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MOLECULAR DISSECTION OF NEUROFIBROMATOSIS TYPE 1 TUMORIGENESIS

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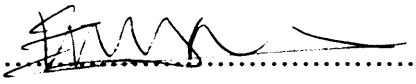
Signed.....  .....(candidate)

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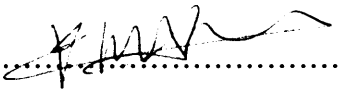
Signed.....  .....(candidate)

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CONTENTS

CHAPTER 6: Germline and somatic mutation detection in NF1-associated tumours

SUMMARY

Neurofibromatosis type 1 (NF1) is an autosomal dominant disease that affects approximately 1/3500 individuals. Although its cardinal features are neurofibromas, Lisch nodules, café-au-lait spots and freckling, the condition is characterised by considerable inter- and intra-familial variability in terms of the clinical phenotype. NF1 patients also present a higher lifetime risk of malignancy, in particular for malignant peripheral nerve sheath tumours (MPNSTs). Located at 17q11, the *NF1* gene comprises 60 exons and spans 350kb of genomic DNA. The detection of germline mutations in the *NF1* gene has long been hindered by the large size of the gene, the relative absence of dramatic mutational hotspots, and the presence of homologous pseudogene sequences. The detection of somatic mutations has faced the additional obstacle of the cellular heterogeneity manifested by neurofibromas.

One of the aims of this investigation was to determine the nature of the germline and/or somatic mutations in a panel of 75 NF1-related tumours, 5 neurofibroma-derived Schwann cell lines and 57 NF1 patients, using complementary mutation detection techniques, and to compare the two mutational spectra. A germline mutation was identified in approximately 65% of the patients, whereas a somatic mutation was found in 37% of the NF1 tumours and derived cells. Schwann cell culture was also found to greatly improve somatic mutation detection. The present study also addressed the possible correlation between *NF1* germline mutation and the likelihood of the patient developing gliomas or MPNSTs.

A growing body of evidence has indicated a potential relationship between the *NF1* gene and the function of the mismatch repair (MMR) genes. Defects in the MMR pathway are characterized by instability of microsatellite repeats. One of the aims of this study was to determine the extent of MSI in large panel of 151 NF1-related tumours. A statistically significant occurrence of MSI in NF1 MPNSTs, as compared to benign NF1 tumours, was observed in this study.

The *NF1* gene is considered to be a tumour suppressor gene and the only currently known function of its protein product, neurofibromin, is to regulate Ras, thereby negatively influencing cell growth. Although it is now accepted that inactivation of the *NF1* gene is necessary and sufficient for neurofibroma formation, little is still known about NF1 tumour development and the evolution to malignancy. The present study investigated the possible involvement of nine candidate genes in NF1 tumours by assessing loss of heterozygosity, promoter hypermethylation, and expression in NF1-related tumours. The *CDKN2A/p16^{INK4a}*, *RB1*, *TP53* and *MGMT* genes have previously been found to be altered in NF1 tumours, and this was confirmed in this study. However, new candidate genes were also found to be involved in NF1 MPNSTs and rare malignancies (*RARB*, *MLH1* and *RASSF1A*). This is the first study which demonstrates that *RASSF1A* may be involved in NF1 malignancy. Identification of genes that are aberrantly methylated in NF1 tumour may provide therapeutic targets for NF1.

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To my late granddad, whose wisdom I miss everyday.

Abbreviations

ACSA	automated comparative sequence analysis
AK3	adenylate kinase 3
ATP	adenosine triphosphate
BLBP	brain lipid binding protein
CALS	café-au-lait spots
cAMP	cyclic adenosine monophosphate
CDK	cyclin-dependant kinase
CC	colon cancer
CGH	comparative genomic hybridisation
CIN	chromosomal instability
CRE	cAMP response element
CREB	CRE binding protein
CTD	C-terminal domain
DHPLC	denaturing high performance liquid chromatography
DNMT	DNA-methyltransferase
EGFR	epidermal growth factor receptor
ESE	exonic splicing enhancer
FISH	fluorescence *in situ* hybridisation
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GRD	GAP (GTPase activating protein) related domain
HA	heteroduplex analysis
HD	homozygous deletion
HDAC	histone deacetylases
HNPCC	hereditary non-polyposis colon cancer
LOH	loss of heterozygosity
MDBP	methyl-CpG binding proteins
MPNSTs	malignant peripheral nerve sheath tumour
MMR	mismatch repair
MR	mitotic recombination
MSI	microsatellite instability
MS-PCR	methylation-specific PCR
MTC	medullary thyroid carcinoma
NF1	Neurofibromatosis type 1
NF2	Neurofibromatosis type 2
NFNS	Neurofibromatosis-Noonan
NMD	nonsense-mediated mRNA decay
NRG	neuregulin
OMgp	oligodendrocyte-myelin glycoprotein
OPG	optic pathway gliomas
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PTT	Protein truncation test
RAR	retinoic acid receptors
RFLP	restriction fragment length polymorphism
SC	Schwann cells
SCF	stem cell factor
SNP	single nucleotide polymorphism
SSCP	single strand conformation polymorphism
STS	soft tissue sarcoma
TS	tumour suppressor

CHAPTER 1: GENERAL INTRODUCTION

1.1 Neurofibromatosis type 1

Neurofibromatosis type 1 (NF1) is one of two inherited conditions characterised by multiple skin tumours arising from the nerves. Of predominant incidence, NF1 manifests itself by variable phenotypes in affected individuals both within the same family and between affected families, and represents a model for multifactorial tumour formation.

1.1.1/ History of neurofibromatoses

Although a description of patients with features of NF1 dates back to the 13th Century, it was not until 1882 that the disorder was characterized by von Recklinghausen, who observed that the tumours originated from the tissue surrounding the small nerves and termed them "neurofibromas"; NF1 is sometimes still referred as "von Recklinghausen Neurofibromatosis". In the 1900s, NF1 was identified as an automosomal dominant condition (Preiser and Davenport, 1918), and a hallmark feature of the condition, the iris nodule, was described by the ophthalmologist Lisch. From 1970, the different types of neurofibromatosis were recorded as separate, as it became obvious that the conditions were quite distinct, both clinically and genetically. In 1987, the National Institutes of Health adopted a classification for the main types of NF, which also acknowledged the less frequent ones: von Recklinghausen neurofibromatosis was renamed NF type 1 (NF1), whereas the condition presenting schwannomas and intracranial tumours was termed NF type 2 (NF2). A few years later, the *NF1* gene was mapped to chromosomal location 17q11.2 (Barker *et al.*, 1987; Viskochil *et al.*, 1990 Wallace *et al.*, 1990; Gutmann *et al.*, 1991), and the *NF2* gene, to 22q12 (Rouleau *et al.*, 1987; Trofatter *et al.*, 1993).

1.1.2/ Clinical aspect of NF1

With a prevalence of 1/3500 (Huson *et al.*, 1989), NF1 represents almost 90% of all NF cases. The cardinal features of the condition are neurofibromas, café-au-lait spots (CALS) and axillary freckling.

1.1.2.1/ NF1-related tumours

1.1.2.1.1/ Neurofibromas

Neurofibromas are a major feature of the disease and appear in two distinct forms, peripheral (dermal or cutaneous, and nodular or subcutaneous) and plexiform neurofibromas.

Most represented in the condition, dermal neurofibromas are soft, fleshy tumours, often associated with pigmentation of the skin (Huson *et al.*, 1988). These discrete lesions of the nerve sheath rarely present before seven years of age, appearing mostly at adolescence (Riccardi, 1992; Huson *et al.*, 1988) and can vary greatly in size (up to several centimetres) and in number (Korf *et al.*, 1997). Moreover, the number and size of neurofibromas appear to increase during pregnancy in women with the condition, and diminish after giving birth. These observations suggest a role for hormonal involvement in the growth of neurofibromas (Riccardi, 1992; Dugoff and Sujanski, 1996). Neurofibromas are composed predominantly of Schwann cells (~80%), but also contain fibroblasts, axons, mast cells, perineurial cells and extracellular matrix (Menon *et al.*, 1990). Although these lesions seldom become malignant, they are a source of great distress to the patients (Riccardi, 1992; Huson *et al.*, 1988).

Less common then dermal neurofibromas and found in deeper parts of the body, nodular neurofibromas arise from major peripheral nerve trunks. These firmer lesions are a source of pain, although they rarely evolve to malignancy. Neurological symptoms can be observed due to the location of these tumours (Huson *et al.*, 1988; Korf *et al.*, 1997).

Plexiform neurofibromas arise in approximately 10% of patients affected with NF1. These deep lesions are composed of the same cellular types as dermal neurofibromas, but present large areas of growth, along the length of a nerve, which can spread from a few

centimetres to whole body segments. Unlike discrete neurofibromas, plexiform neurofibromas are usually congenital and can evolve to malignancy with a lifetime risk for developing a malignant peripheral nerve sheath tumour (MPNSTs) in NF1 patients is of the order of 8%-13% (Ruggieri and Huson, 1999; Evans et al, 2002; Ferner and Gutmann, 2002).

1.1.2.1.2/ Malignant peripheral nerve sheath tumours (MPNSTs)

Malignant peripheral nerve sheath tumours (MPNSTs) occur at a higher frequency (from 2% up to 13% in the most recent estimate) in NF1 patients than in the general population (0.001%) (Ruggieri and Huson, 1999; Evans *et al.*, 2002). The malignant tumours predominantly arise from existing plexiform neurofibromas (King *et al*, 2000; Ferner and Gutmann, 2002), which make them difficult to diagnose early in NF1 patients. A recent statistical analysis on the influence of the burden of neurofibromas on malignancy showed that patients with internal plexiform neurofibromas are 20 times more likely to develop an MPNST (Tucker *et al.*, 2005). The indicator of malignancy, such as pain associated with a growing lump, can be overlooked by a patient suffering from many lumps. The prognosis is indeed poorer in NF1 patients than in the general population and the tumours can metastasize to many tissues (Ferner and Gutmann, 2002; Evans *et al.*, 2002). NF1 sufferers also develop MPNSTs at a younger age, 20-30 years younger then the general population, and have a higher lifetime risk (King *et al.*, 2000; Evans *et al.*, 2002). MPNSTs respond poorly to chemotherapy or radiotherapy, and complete surgical excision is the most effective for patient survival (Wong *et al.*, 1998)

1.1.2.1.3/ Optic gliomas

Gliomas can be of different types: optic gliomas, astrocytomas, brain stem gliomas and spinal cord tumours. NF1-associated optic pathway gliomas (OPG) are classified by the World Health Organization as grade I astrocytic neoplasms, or pilocytic astrocytoma (Kleihues *et al.*, 2002). OPGs are the most common central nervous system tumour associated with children affected with NF1 and can affect the optic nerves, optic chiasm and hypothalamus (Huson *et al.*, 1988). Approximately one quarter of NF1 patients are

diagnosed with optic gliomas (Listernick *et al.*, 1999). Compared with non-NF1 optic gliomas, tumours associated with NF1 present different initial signs and location on the optic nerve, and can stay latent over a long period of time, but can however be the source of significant visual impairment (Listernick *et al.*, 1999; Ruggieri *et al.*, 2004).

Allelic loss and absent *NF1* gene expression has been reported in NF1-related gliomas (Gutmann *et al.*, 2000; Kluwe *et al.*, 2001). By contrast, LOH of the *NF1* gene is uncommon in sporadic pilocytic astrocytomas (von Deimling *et al.*, 1993; Jensen *et al.*, 1995; Kluwe *et al.*, 2001) and expression of neurofibromin has been demonstrated in these tumours (Platten *et al.*, 1996; Peters *et al.*, 1999). Recently, it has been suggested that *NF1* gene loss in astrocytes leads to the preferential activation of K-Ras for glioma formation (Dasgupta *et al.*, 2005b). In astrocytes, neurofibromin has also been associated with cAMP regulation and its loss has been correlated with attenuated Rap1 activation, which contributes to cell proliferation (Dasgupta *et al.*, 2003). Additionally, experiments on a mouse model of Nf1-associated OPG supported the hypothesis that the tumours which arise early form hyperproliferating $Nf1^{-/-}$ astroglial cells and stressed the role of heterozygous $Nf1^{+/-}$ microglia in OPG tumorigenesis (Bajenaru *et al.*, 2005).

1.1.2.1.4/ Other tumours associated with NF1

Apart from neurofibromas and MPNSTs, patients with NF1 manifest an increased incidence of a number of tumours such as phaechromocytomas (Riccardi, 1992; Walther *et al.*, 1999) and leukaemias (Shannon *et al.*, 1994; Side *et al.*, 1997). Rhabdomyosarcomas are a rare type of soft tissue sarcoma (STS) of the primitive muscle cells, but are found at increased incidence in NF1 patients (Yang *et al.*, 1995) and particularly in affected children (Reich *et al.*, 1999; Lampe *et al.*, 2002; Hadjustilianou *et al.*, 2002).

1.1.2.2/ Café-au-lait spots

Present in virtually all NF1 patients, café-au-lait spots (CALS) often appear at birth or during the first years of life, and may increase during childhood. The number and size of these flat, evenly pigmented macules vary in number between individual NF1 patients.

However, the presence of 6 or more CALS of greater size than 1.5 cm is usually considered as a diagnostic criterion for NF1 (Ruggieri and Huson, 1999).

1.1.2.3/ Skin fold freckling

Observed early in childhood, these small hyperpigmented macules are found in more than half of patients, and can appear in the axillae, groin, and base of the neck and under the breast in women (Huson *et al.*, 1988). The unusual locations of these lesions may denote the importance of their immediate environment.

1.1.2.4/ Lisch nodules

These harmless, dome-shaped, pigmented hamartomas were first identified in 1918, and are considered an important diagnostic criterion for NF1 (Waardenberg, 1918; Lisch, 1937). These lesions of the iris appear during childhood, usually between the appearance of CALS and neurofibromas and are found in over 90% of patients (Ruggieri and Huson, 1999).

1.1.2.5/ Other NF1-related features

In addition to the hallmarks of NF1 mentioned above, there are a number of "minor" features, found in a significant proportion of NF1 patients, but not used as part of the diagnostic criteria. These include macrocephaly (~50% of patients), short stature (~30% of patients), multiple juvenile xanthogranulomas (1-2% of children), angiomas, hypertelorism and thoracic abnormalities (Ruggieri and Huson, 1999).

Finally NF1 patients can also manifest a wide-range of complications, which are not specific to NF1, but occur at an increased frequency in NF1 patients. These can be neurological (cognitive deficits, seizure, epilepsy, multiple sclerosis), ophthalmological (neurofibromas involving the eyelid, glaucoma) or orthopaedic (scoliosis) and include general medical complications such as hypertension and gastrointestinal problems (Ruggieri and Huson, 1999).

1.1.2.6/ Diagnostic criteria

The diagnostic criteria for NF1 was determined at the NIH conference of 1987, and later outlined by Stumpf *et al.* (1988) as follows:

For an individual to present with NF1, two or more of the following should be identified:

1/ In a pre-pubertal individual, at least 5 CALS over 5mm in diameter; 6 CALS over 15mm in diameter post-puberty.

2/ Inguinal or axillary freckling.

3/ Two or more neurofibromas (of any kind), or one plexiform neurofibroma.

4/ Two or more Lisch nodules

5/ Optic glioma

6/ Osseous lesions, with or without pseudoarthrosis.

7/ A first-degree relative exhibiting two or more of the above criteria.

By contrast, NF2 (Section 1.3.1) should be diagnosed if a patient presents with one of the three followings:

1/ Bilateral vestibular schwannomas.

2/ A first-degree relative with NF2, and exhibit either plexiform neurofibroma, or a unilateral internal auditory canal mass consistent with an acoustic neuroma.

3/ A first-degree relative with NF2 and exhibiting two of the followings: neurofibroma, glioma, meningioma and intracranial or spinal cord tumour.

1.1.3/ NF1-related conditions

1.1.3.1/ Segmental NF1

Patients with segmental NF1 present with typical features of NF1, but these are limited to a particular area of the body. The incidence of the disease is estimated at 1/70,000-80,000 (Ruggieri and Huson, 1999). Although the first case was reported in 1931, the condition has recently been shown to result from somatic mosaicism for *NF1* gene mutations (Ruggieri and Huson, 1999; Tinschert *et al.*, 2000).

1.1.3.2/ Watson syndrome

Individuals affected with Watson syndrome present CALS, Lisch nodules and neurofibromas, at a lower frequency than NF1 patients. Features such as pulmonary stenosis, short stature and cognitive impairment are characteristic of the condition (Ruggiery and Huson, 1999). Alterations in the *NF1* gene have been shown to segregate with the condition (Upadhyaya *et al.*, 1992; Tassabehji *et al.*, 1993).

1.1.3.3/ NF1 Noonan syndrome

Noonan syndrome (NS) affects 1/2000 individual and is characterized by distinct facial feature, muscle weakness, learning difficulties and small stature (Noonan, 1994). Some NF1 patients have been found to present mild NS features and *NF1* gene alterations have been found in these patients (Colley *et al.*, 1991; Leppig *et al.*, 1994; Bahuau *et al.*, 1998; Baralle *et al.*, 2003). However, the presence of identical mutations in individuals with classical NF1 suggested that the Neurofibromatosis-Noonan (NFNS) phenotype was additive (Bahuau *et al.*, 1998; Baralle *et al.*, 2003). The gene responsible for NS has recently been identified as the *PTPN11* gene located a12q24.1 (Tartaglia *et al.*, 2001). More recently, an individual with NFNS was shown to have both an inherited mutation in the *PTPN11* gene and a *de novo* mutation in the *NF1* gene (Bertola *et al.*, 2005).

1.1.3.4/ Spinal neurofibromatosis (SNF)

The condition is characterised by a predominance of spinal cord tumours and multiple CALs. This rare form of NF1 was first identified in three families: in two families, the condition segregated with markers in the *NF1* gene (Pulst *et al.*, 1991; Poyhonen *et al.*, 1997) and a truncating mutation in exon 46 of the *NF1* gene was identified in the third (Ars *et al.*, 1998). More recently, an investigation of *NF1* mutations in patients with spinal neurofibromas identified eight patients from six families who satisfied the criteria for SNF (Kluwe *et al.*, 2003a). In total, seven *NF1* mutations associated with SNF have been found: three missense, two nonsense and two splicing mutations in exons 46 and 47. These are considered milder *NF1* mutations (as they are located at the end of the gene) and may account for the reduced NF1 clinical spectrum in these patients. It has

been proposed that SNF may represent a subgroup of NF1 patient, or a distinct disorder involving another locus (Kluwe *et al.*, 2003a).

1.3.3.5/ Autosomal dominant multiple café-au-lait spots

This familial condition is characterised by the presence of CALS, but no other features of NF1. Two studies excluded linkage of this trait to the *NF1* locus, suggesting a dominant gene genetically separate from NF1 (Charrow et al., 1993; Brunner et al., 1993). By contrast, Abeliovich et al.(1995) identified close linkage between familial CALS and the *NF1* locus, and concluded that the three generations of the family had a trait allelic to *NF1*.

1.1.4/ Neurofibromatosis type 2 (NF2)

NF2 is the other major type of neurofibromatosis, but is less frequent than NF1, with a birth incidence of 1/33000. The condition presents some overlapping features with NF1: CALS (fewer than 1% in NF2) and peripheral nerve tumours. However, the tumours characteristic of NF2 are vestibular schwannomas, also called acoustic neuromas, and not neurofibromas. These lesions occur in over 90% of the patients and develop bilaterally from the eighth cranial nerve. NF2 also presents ophthalmologic features, which are principally asymptomatic cataracts. Tumours such as meningiomas (50% of patients), pilocytic astrocytomas and ependymomas have also been reported (Evans *et al.*, 1992; Parry *et al.*, 1994; Ruggieri and Huson, 1999).

1.1.5/ Genetic aspect of NF1

1.1.5.1/ Prevalence, penetrance and expression of clinical features

One of the most common autosomal dominant disorders, NF1 has an estimated birth incidence of 1/2500, and a disease prevalence of approximately 1/4500, in population-based studies (Ruggieri and Huson, 1999).

The penetrance of the disease is of virtually 100% by age 5 (Ruggieri and Huson, 1999), although the clinical features observed could vary greatly, even in the same family where individuals carry the same germline mutation (Easton *et al.*, 1993). A number of studies have addressed the genotype-phenotype relationship, but no correlation has been found for the common clinical features of NF1 (Easton *et al.*, 1993; Castle *et al.*, 2003; Szudek *et al.*, 2003), apart from the small number of patients who harbour a large deletion of the NF1 and are reported to have more severe features (Kayes *et al.*, 1994; Wu *et al.*, 1995; Cnosson *et al.*, 1997; Leppig *et al.*, 1997; Tonsgard *et al.*, 1997; Upadhyaya *et al.*, 1996, 1998; De Raedt *et al.*, 2003; Section 1.3.1.3). A borderline statistical significance was found between missense mutations and occurrence of Lisch nodules (Castle *et al.*, 2003). "Trends", such as a high proportion of truncating mutations and occurrence of gliomas, and mutations in exon 10b in patients with scoliosis, have also been described (Ars *et al.*, 2003).

A number of theories have been put forward to explain the inter-individual variations in NF1 clinical features.

At the *NF1* gene level, the role of the second somatic hit has been implicated in the variability of these features. However, no relationship has yet been identified. The number of known somatic microlesions is still small, but the germline and somatic microlesion spectra appear similar thus far, suggesting that the genotype/phenotype relationship might be more complex. Somatic mosaicism has also been offered to account for the phenotype variability and shown to underlie segmental NF1 (Riccardi and Lewis, 1988; Section 1.3.2). Variable levels of abnormal transcript in different patients bearing the same mutation altering the correct splicing has also been proposed as an explanation for the phenotypic variability between patients harbouring the same mutation (Ars *et al.*, 2003).

Large deletions (Section 1.3.1.3) are thought to underlie a predisposition to malignancy, as well as other distinctive features, such as an excessive burden of neurofibromas.(Dorschner *et al.*, 2000; De Raedt *et al.*, 2003). Because such large deletions also involve flanking DNA and the three embedded genes, these observations add also to the idea that genes contiguous to the *NF1* gene might take part in the

phenotypic variability (Kayes *et al.*, 1992). However, apart from the three embedded genes, no other genes close to or at the *NF1* locus have been implicated. One of the embedded genes (Section 1.2.3), *OMGP*, is thought to be a growth suppressor gene and its heterozygosity in NF1 patients with large deletions could contribute to the excessive tumour burden observed in these patients (Habib *et al.*, 1998; Vourc'h and Andres, 2004). It is noteworthy that a number of patients harbouring large deletions involving the *NF1* gene did not exhibit the same distinctive phenotype (Rasmussen *et al.*, 1998; Upadhyaya *et al.*, 1998; Mantripragada *et al.*, 2005), suggesting a role for additional events.

A role for modifier genes (Easton *et al.*, 1993; Bahuau et al, 2001; Szudek *et al.*, 2000) was first put forward on the observation that monozygotic twins shared certain NF1 traits (e.g. CALS, neurofibromas, head circumference) with high similarity. By contrast, distant relatives exhibited more variable clinical phenotypes (Easton *et al.*, 1993). Genes influencing mitotic recombination, and thus the rate of LOH as a second hit, have also been proposed as modifier genes to account for the variation in neurofibromas number (Serra *et al.*, 2001b). Recently, Wiest *et al.* (2003) suggested that functional variants of genes involved in mismatch repair might modulate the *NF1* mutation rate in a given patient. This hypothesis served to explain an excessive burden of neurofibromas in a patient where the identified somatic mutations were predominantly microlesions. Thus, in that patient, the mismatch repair gene variant would have a lower efficiency, and allow the more frequent occurrence of a microlesion as a second hit.

Finally, environmental and stochastic factors have also been proposed to explain inter-individual clinical phenotypic variation in NF1 (Riccardi, 1993).

1.1.5.2/ Origin and rate of mutations

The *NF1* gene presents one of the highest mutation rates reported in any human disorder, approximately 1/10,000 gametes per generation and with 50% of the patient being *de novo* cases (Huson *et al.*, 1989; Li *et al.*, 1995). New mutations exhibit a bias towards paternal origin (Jadayel *et al.*, 1990; Stephens *et al.*, 1992; Upadhyaya *et al.*, 1994),

whereas large gene deletions are of maternal origin (Lazaro *et al.*, 1996; Ainsworth *et al.*, 1997; Upadhyaya *et al.*, 1998).

The large size of the *NF1* gene alone cannot however account for the high mutation rate (Upadhyaya *et al.*, 1994; Rodenhiser *et al.*, 1997a) and a number of explanations have been offered. The presence of numerous pseudogene sequences have been implicated as acting as a mutation "reservoir" for the functional *NF1* gene (Marchuk *et al.*, 1992), but this postulate has not yet been validated (Luijten *et al.*, 2001a). Another explanation would be the post-zygotic occurrence of disease-causing somatic mutations in patients who are clinically unaffected (Zlotogora, 1993).

1.2 The *NF1* gene and its expression

2.1/ Identifying the *NF1* gene

The search for the *NF1* gene was first based on the autosomal dominant character of the condition. Linkage data from NF1 families were gathered internationally and used in conjunction with a marker segregating with the disease to identify a preliminary position of the *NF1* gene. In 1987, the *NF1* gene location was narrowed down, using genetic markers, to the pericentromeric region of chromosome 17 (Seizinger *et al.*, 1987a,b,c; Barker *et al.*, 1987; Diehl *et al.*, 1987). By 1989, the putative *NF1* gene was thought to be located on the long arm of chromosome 17, close to the centromere (Fain *et al.*, 1989; Goldgar *et al.*, 1989). The discovery of two germline translocations (Schmidt *et al.*, 1987; Ledbetter *et al.*, 1989), in that region, in two unrelated individuals, provided a breakthrough for the isolation of the gene. These two mutations were balanced translocations between chromosomes 1 and 17 (Schmidt *et al.*, 1987), and chromosomes 17 and 22 (Ledbetter *et al.*, 1989). Since no chromosomal material was lost during the translocation, the breakpoints were probably located internally to the putative *NF1* gene and the translocations served to disrupt its function. The same year, a 600kb DNA fragment was identified by pulsed field gel electrophoresis (PFGE). An *Nru*I restriction enzyme fragment harboured the two translocation breakpoints and was thought to contain the *NF1* gene (Fountain *et al.*, 1989a, 1989b; O'Connell *et al.*, 1989a, 1989b). The two breakpoints were found to be separated by 55kb of DNA (Collins *et al.*, 1989). Further

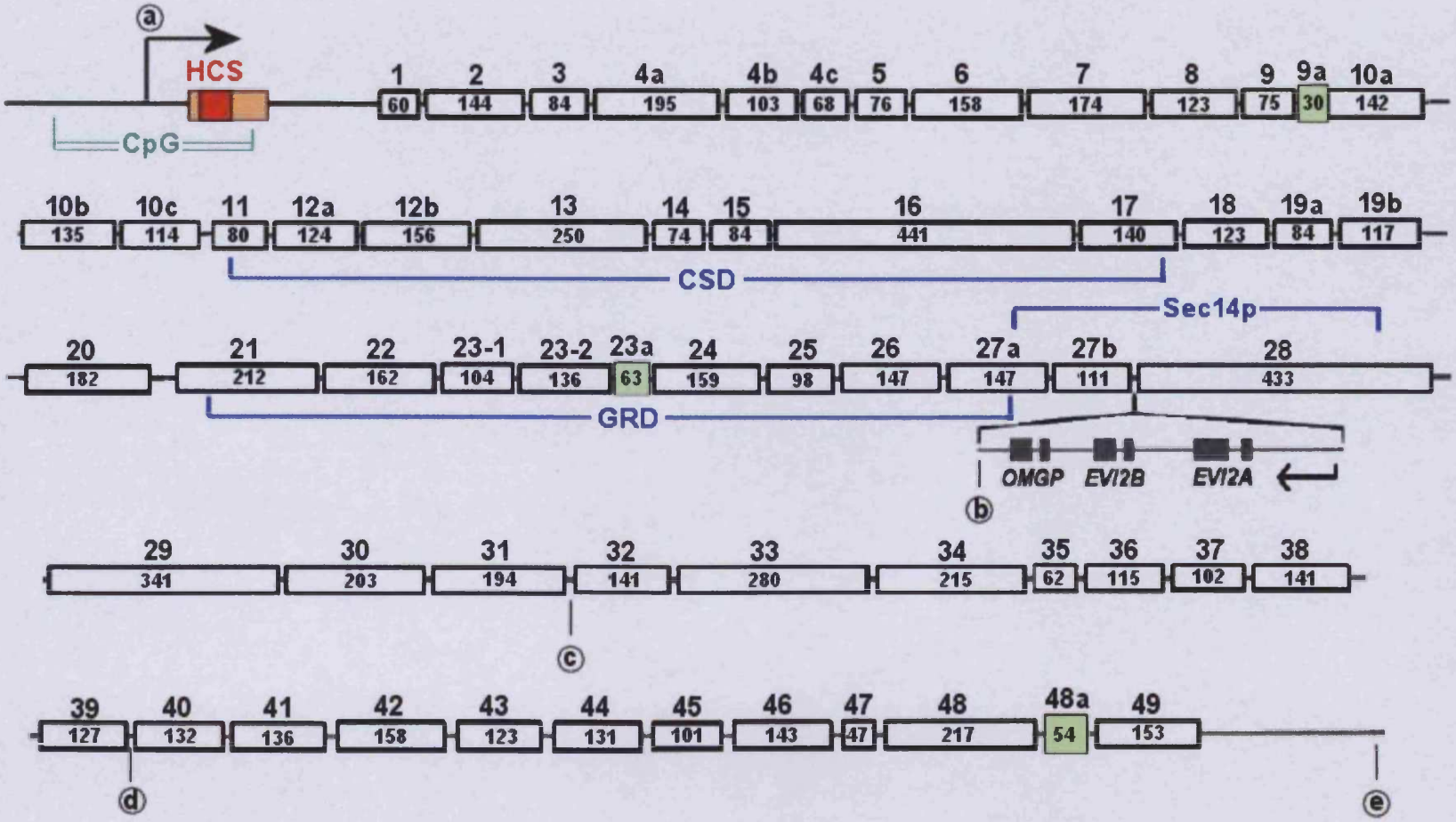

Figure 1.1 : Organisation of the *NF1* gene (derived from Viskochil, 1999).
Exons are represented by boxes and the boxed numbers represent the exon size in base-pairs. Alternatively spliced exons are indicated by green boxes. The orange box represent the core promoter (-341bp to -261bp), with the NF1-HCS (putative core promoter element/transcriptional activator) indicated by a red box (Lee and Friedman, 2005). *CpG*, CpG island (-731bp to –261bp; Lee and Friedman, 2005); *CSD*, cysteine/serine-rich domain (Fahsold *et al.*, 2000); *GRD*, GAP-related domain; *Sec14p*, Sec14p-like domain (Aravind *et al.*, 1999). The three embedded genes are shown in bold and arrow indicate the sense of transcription. a: transcription start site, b: approximate location of the t(1;17) balanced translocation breakpoint; c: site of the t(17;22) translocation breakpoint; d: adenylate kinase 3 (*AK3*) pseudogene; e: polyadenylation site representing the 3' end of the *NF1* gene.

study by positional cloning identified four candidate genes in the region: *EVI2A* (Cawthon *et al.*, 1990), *EVI2B* (Cawthon *et al.*, 1991), *OMGP* (Viskochil *et al.*, 1991) and TBR (Viskochil *et al.*, 1991). Upon mutation screening in the 4 genes, only TBR was found to exhibit disease-causing mutations, and was therefore identified as the *NF1* gene (Cawthorn *et al.*, 1990, Viskochil *et al.*, 1990, Wallace *et al.*, 1990).

1.2.2/ *NF1* gene organisation

The *NF1* gene comprises 60 exons, 3 of which are alternatively spliced. It spans over 350kb of genomic DNA and encodes an mRNA of approximately 12kb (Li *et al.*, 1992; Danglot *et al.*, 1995; Shen *et al.*, 1996). The 8457 bp reading-frame encodes a 327 kDa protein of 2818 amino-acids (Viskochil *et al.*, 1990; Wallace *et al.*, 1990; Marchuk *et al.*, 1991; Viskochil *et al.*, 1993).

The *NF1* GAP-related domain (*NF1*-GRD) is encoded by exons 21 to 27a (Buchberg *et al.*, 1990, Xu *et al.*, 1990a,b). This GRD is functionally homologous to the catalytic domains of mammalian p120 GTPase activating protein (GAP), yeast proteins IRA1 and IRA2, and *Drosophila* GAP (Xu *et al.*, 1990a; Martin *et al.*, 1990; Ballester *et al.*, 1990; Buchberg *et al.*, 1990). Moreover, the *NF1* homologs in human, mouse, rat and *Fugu rubripes* share over 90% identity at the nucleotide sequence level (Bernards *et al.*, 1993; Suzuki *et al.*, 1996; Kehrer-Sawatzki *et al.*, 1998), whereas human and *Drosophila* are 60% identical (The *et al.*, 1997).

The *NF1* promoter region contains a 5' untranslated region (5'UTR) of 484bp upstream from the initiation codon and several DNA binding motifs conserved through evolution, suggesting an important role in gene transcription regulation (Hajra *et al.*, 1994). Additionally, a CpG island lies at the 5' end of the *NF1* gene and encompasses the promoter region. More recently, several potential transcription factor binding sites have been identified in the 5' upstream region and around the transcription start site, and a 24bp segment, highly identical in mouse, rat and *Fugu*, may contain the core promoter element for NF1 transcription (Lee and Friedman, 2005).

The stop codon is located in exon 49, which also contains a 3' UTR, of approximately 3.5kb in length, and a number of potential polyadenylation signals (Li *et al.*, 1995).

Furthermore, the 3' UTR is highly conserved between human and mouse (over 75%; Bernards *et al.*, 1993), suggesting a functional importance for the sequence.

1.2.3/ Embedded genes

Hybridization of probes and sequence comparison studies have also identified three genes entirely contained within the 60kb of intron 27b and transcribed in reverse orientation to the *NF1* gene (Cawthon *et al.*, 1990; Viskochil *et al.*, 1991). The function of these genes and their relation to NF1 is sill unclear.

The *OMGP* gene encodes the oligodendrocyte-myelin glycoprotein (OMgp), which is expressed in the oligodendrocytes of the central nervous system and may function as a cell adhesion protein (Mikol *et al.*, 1990). Moreover, in murine models, OMgp was shown to act as a tumour suppressor and downregulate mitogenic signalling pathways in the same way of *NF1* (Habib *et al.*, 1998). The OMgp protein is also a member of the NgR ligand family, proteins involved in growth inhibition after brain injury in the adult nervous system, and was found to inhibit neurite growth in vitro (Wang *et al.*, 2002; Vourc'h and Andres, 2004).

The *EVI2A* (Cawthon *et al.*, 1990) and *EVI2B* (Cawthon *et al.*, 1991) genes encode transcripts of 1.6kb and 2.1kb, respectively, but their function has yet to be fully characterised. Both transcripts are found in bone marrow and peripheral blood. However, *EVI2A* is expressed in brain tissues, whereas *EVI2B* transcript is found in fibroblasts. Both proteins may interact with other proteins through a leucine-zipper domain, have a trans-membrane domain and appear to be glycosylated. Furthermore, *EVI2B* is the only one of the three embedded genes to possess a similar sequence in intron 27b of the *Fugu NF1* gene (Kehrer-Sawatzki *et al.*, 1998).

Finally, intron 37 of the *NF1* gene encompasses a pseudogene, *AK3* (adenylate kinase 3), which is transcribed in the same orientation as *NF1* (Xu *et al.*, 1992).

1.2.4/ Alternative splicing

Alternative splicing is an important mechanism in achieving proteosome diversity and in regulating gene expression at different developmental stages and tissue types

(Vandenbroucke *et al.*, 2002). Alternative splicing can also be deregulated in tumorigenesis (Bracco *et al.*, 2003).
In the *NF1* gene, there are three exons, all small in-frame coding segments, alternatively spliced in or out of the mature mRNA. The functional significance of each variant form has yet to be determined. The presence of the exon potentially modifies the structure and/or function of the protein, with a possible role in tumour growth or in certain tissues. Mutations have yet to be found in these exons.

First identified, the exon 48a (Cawthon *et al.*, 1990) splice variant is specifically expressed in cardiac and skeletal tissue (Gutmann *et al.*, 1993, 1995). The variant has been termed "type III RNA" and the protein encoded presents no homology to any known sequence. Incidentally, "knock-out" *Nf1* mice have been observed to die in gestation following severe heart defects, suggesting a role for *Nf1* for normal growth control and muscle development (Jacks *et al.*, 1994; Brannan *et al.*, 1994).

The exon 9a variant is expressed exclusively in the brain (Danglot *et al.*, 1995, Geist and Gutmann, 1996), and, although its functional significance is still unknown, its conserved expression in mouse may indicate a role in nervous system differentiation and development.

The exon 23a (or "type II RNA") variant is ubiquitously expressed and more abundant than the *NF1* RNA variant excluding exon 23a (or "type I RNA"; Suzuki *et al.*, 1991; Bernards *et al.*, 1992). Due to the specific position of exon 23a, which encodes 21 amino acids in the centre of the *NF1*-GRD (Bernards *et al.*, 1992; Andersen *et al.*, 1993), the type II isoform has different Ras-GTP binding and catalytic activities from that of isoform I (Zhu and Parada, 2001). Splice variant II exhibits reduced GAP activity *in vitro* with enhanced affinity for H-ras (Andersen *et al.*, 1993). It also presents some homology to a nuclear localization sequence (Suzuki *et al.*, 1991). The two isoforms are expressed in varying ratios in different cell types (Nishi *et al.*, 1991; Gutmann *et al.*, 1993). Recently, induction of the type I variant, and thus up-regulation of the *NF1*-GRD, has been shown to result from NGF-induced Ras activation in neuronal cells; *NF1*-GRD

(type I) control the active state of Ras by feedback regulation, leading to neurite extension (differentiation; Yunoue *et al.*, 2003). Finally, differential expression of type I, II and III have been implicated in tumorigenesis progression (Nishi *et al.*, 1991; Mochizuki *et al.*, 1992; Uchida *et al.*, 1992; Iyengar *et al.*, 1999; Wimmer *et al.*, 2002; Cacev *et al.*, 2005).

1.2.5/ Pseudogenes

Fluorescence *in situ* hybridization (FISH) analysis using *NF1* cDNA sequences probes identified a number of pseudogenes on chromosomes 2q12-13, 12q11, 14p11-q11, 15q11.2, 18p11.2, 21p11-q11 and 22p11-q11 (Marchuk *et al.*, 1992; Legius *et al.*, 1992; Gasparini *et al.*, 1993; Suzuki *et al.*, 1994; Purandare *et al.*, 1995; Cummings *et al.*, 1993; Hulsebos *et al.*, 1996. Kehrer-Sawatzki *et al.*, 1997; Barber *et al.*, 1998; Luijten *et al.*, 2000a, Fantes *et al.*, 2002). The pseudogenes display at least 90% homology to the *NF1* sequence, and also contain nucleotide substitutions, insertions and deletions. Chromosomes 15 (Legius *et al.*, 1992) and 22 (Gasparini *et al.*, 1993) exhibit more than one *NF1* homologous sequence. Luijten *et al.* (2001a) further demonstrated that some pseudogenes most likely arose from sequential inter-chromosomal transposition events. In that manner, a fragment of 640kb was duplicated and transposed from 2q11 to 14q11. The same mechanism, this time employing 14q11 as a template, gave rise to an *NF1* pseudogene at locus 22q11 (Luijten *et al.*, 2001a).

Although none of the partial *NF1* sequences encode a functional domain, it has been suggested that the pseudogenes could act as a mutational reservoir, thereby serving to increase the *NF1* mutation rate by inter-chromosomal gene conversion (Marchuk *et al.*, 1992; Section 1.1.5.1). However, very few disease-causing mutations characterised in the *NF1* gene appear to have equivalents in a pseudogene, suggesting that inter-chromosomal gene conversion has made only a very limited contribution to the *NF1* mutation rate (Luitjen *et al.*, 2001b).

In 2002, Gervasini *et al.* (2002) claimed to have identified a direct tandem repeat duplication of the *NF1* gene at locus 17q11.2 by high-resolution FISH on stretched chromosomes and DNA fibres. If validated, this *NF1*-related sequence would be the first to be located on chromosome 17 and the most complete copy of the *NF1* gene to date, missing only the exon 22 region. This putative new pseudogene, resulting from intrachromosomal duplication, has been claimed to shed new light on the contribution of gene conversion to the mutation rate of the *NF1* gene. However, other groups have so far failed to reproduce such findings (Kehrer-Sawatzki *et al.*, 2002, 2003; De Raedt *et al.*, 2004). If confirmed, however, this finding would be of the utmost importance for future mutation detection, because PCR primers should be designed to amplify only the *NF1* gene. Similarly, it might warrant the re-examination of all mutations identified thus far. The use of *NF1* mRNA might circumvent the problem created by pseudogenes, as these are not expressed. Whether or not the alleged pseudogene on chromosome 17 is transcribed remains to be determined.

1.2.6/ Neurofibromin

1.2.6.1/ Distribution of neurofibromin

Neurofibromin is present in almost all tissue types and organ systems, with the highest expression levels being noted in the brain (Wallace *et al.*, 1990; Daston *et al.*, 1992). Through the use of immunohistochemical staining using neurofibromin-specific antibodies, the protein has been found in the cytoplasm of cells from various tissues (Daston *et al.*, 1992; Gutmann *et al.*, 1992) and shown to colocalise with microtubules (Gregory *et al.*, 1993; Section 1.2.6.3). Complex patterns of expression depending on cell type, alternative splice variants (Section 1.2.4) and subcellular localisation have also been noted.

1.2.6.2/ Neurofibromin and the Ras pathway

The only known functional domain of the *NF1* gene is encoded by exons 21 to 27a and is termed the *NF1* GAP-related domain (*NF1*-GRD), as substantiated by its high homology

to the catalytic domain of GAP (GTPase activating proteins) related genes, including the mammalian p120GAP (Xu *et al.*, 1990a,b; Martin *et al.*, 1990; Ballester *et al.*, 1990; Buchberg et *al.*, 1990). Additionally, the human neurofibromin and its homologues in mouse, *Drosophila* and yeast present a similar structure and a catalytic region (or GRD; Tanaka *et al.*, 1991; Shen *et al.*, 1996; The *et al.*, 1997).

As with other GTPase activating proteins, neurofibromin, through its *NF1*-GRD, is thought to stimulate the conversion of active RasGTP to inactive RasGDP, thereby downregulating Ras and preventing uncontrolled cell proliferation. The *NF1*-GRD has been estimated to stimulate the GTPase activity of Ras more than 1000-fold (Xu *et al.*, 1990b; Bollag and McCormick, 1991; Ahmadian *et al.*, 1997). It has been found to interact with and downregulate GTP-bound p21ras and was shown to stimulate the intrinsic GTPase activity of the Ras proteins in both yeast Ras2 and human H-Ras (Ballester *et al.*, 1990; Martin *et al.*, 1990; 63:843; Xu *et al.*, 1990b; Buchberg *et al.*, 1990).

Activation of Ras is achieved through GTP-binding and subsequent conformational change of the protein. The activating conversion from the GDP-bound to the GTP-bound form is controlled by guanine nucleotide exchange factors. The opposite, inactivating GTP to GDP conversion occurs through the stimulation of the Ras GTPase activity by GTPase activating proteins (GAPs). The Ras proteins are involved in complex signal transduction pathways, and moderate cellular response to different factors, including mitogens and differentiation factors. The three main Ras effectors are PI3-K, RAL-GEFs and Raf kinase. Perhaps the best described signal transduction pathway, Raf activates a series of kinases in cascade in the MAPK kinase pathway leading to cell proliferation or differentiation (Macaluso *et al.*, 2002).

Thus, inactivation of neurofibromin might be predicted to lead to increased Ras signalling and cell proliferation. The *NF1* gene is therefore considered to be a tumour suppressor gene (Harber and Harlow, 1997). In keeping with this postulate, studies in NF1-related MPNSTs have shown that lack of (or reduced) neurofibromin correlated with increased levels of activated Ras-GTP (Basu *et al.*, 1992; deClue *et al.*, 1992; Guha *et al.*, 1996). Introduction of *Nf1*-GRD in $Nf1^{-/-}$ cells has also been shown to be sufficient

to restore normal cell growth *in vitro* and *in vivo*, through direct interaction with Ras (Hiatt *et al.*, 2001).

More recently, excessive Ras activity has been associated with NF1-related learning deficiencies, and suspected to lead to impairment in long-term potentiation caused by increased GABA-mediated inhibition (Costa *et al.*, 2002).

1.2.6.3/ Other roles of neurofibromin

Neurofibromin has an N-terminal 80 amino-acids region of the NF1-GRD has been found to interact with microtubules. Neurofibromin has been shown to co-precipitate with both α and β forms of tubulin. The interaction resulted in the inhibition of the GAP activity of the GRD, which could be restored by the introduction of colchicines (a microtubule depolymerising agent) and competitive inhibition of the neurofibromin-tubulin interaction (Bollag *et al.*, 1993). By contrast, mammalian p120GAP did not interact with tubulin. Additionally, neurofibromin has been found to co-elute with MAP2, a microtubule-associated protein (Gregory *et al.*, 1993). Mutations at conserved residues of the N-terminal region have been shown to cause a loss of interaction between NF1-GRD and microtubules (Xu and Gutmann, 1997). NF1 has also been reported to bind to all members of the syndecan family, surface molecules that can induce cytoskeletal rearrangements (Hsueh *et al.*, 2001). NF1 has also been found to be involved with other cytoskeletal structures, including the kinesin-1 containing complex (Hakimi *et al.*, 2002), and the F-actin cytoskeleton (Li *et al.*, 2001).

Outside of the NF1-GRD and its effect on cell growth control, the *NF1* gene may have other yet-unidentified functional domains and be involved in other cellular pathways and processes. For example, despite the initial bias of the earliest mutational studies, the *NF1*-GRD does not exclusively harbour the mutations identified in the gene. A putative second functional domain has been identified upstream of the GRD, when a possible mutational hotspot was found in exons 11-17 (Fahsold *et al.*, 2000). The region encodes a cysteine/serine-rich domain, with three potential cAMP-dependant protein kinase recognition sites, subject to phosphorylation by protein kinase, and three cysteine pairs suggestive of ATP binding (Izawa *et al.*, 1996). Additionally, mutations in the

Drosophila neurofibromin have been shown to inhibit the cAMP (cyclic adenosine monophosphate) and protein kinase-signalling pathway, through regulation of adenylyl cyclase (AC), a process potentially involved in learning deficits (Guo *et al.*, 1997, 2000; The *et al.*, 1997). In mammalian neurones, neurofibromin has been shown to positively regulate intracellular cAMP levels (Tong *et al.*, 2002), which are involved in modulating cell growth and differentiation in the brain. Finally, neurofibromin has been implicated in the regulation of cAMP generation in astrocytes (Dasgupta *et al.*, 2003). Furthermore, in Nf1-deficient melanoma cell lines, loss of *NF1* expression was not correlated with elevated levels of Ras-GTP and induced expression of *NF1* has been shown to inhibit growth and differentiation without altering Ras-GTP levels (Johnson *et al.*, 1993). In melanocytes from NF1 patients, reduced *NF1* expression was not found to alter regulation of RasGTP (Griesser *et al.*, 1995). Schwann cells from *Nf1*-deficient mice, when treated with a farnesyl-transferase inhibitor, were shown to exhibit a diminished growth rate, but retained their invasive properties, suggesting that Nf1 may act as more than a regulator of Ras (Kim *et al.*, 1997a). In Ras-transformed fibroblasts, NF1 was shown to cooperate with Ras in the anchorage-dependent growth capacity of the cells, and NF1 overexpression was shown to induce an increase in focal adhesion kinase (FAK) expression, and modify the level of expression of MAPKs (Corral *et al.*, 2003). Taken together, these results would suggest that the Ras-independent NF1 function exerts itself through cytoskeletal rearrangements (Corral *et al.*, 2003).

Amino acid residues which lie N-terminal to the GRD domain are required for the neurofibromin ubiquitin-mediated proteolysis (Cichowski *et al*, 2003). These authors have shown that neurofibromin is dynamically regulated by the ubiquitin-proteasome pathway and that this is an important mechanism of controlling both the amplitude and duration of Ras-mediated signalling.

Recently it has been shown that the mTOR pathway is tightly regulated by neurofibromin. In the absence of growth factors, mTOR is activated in both NF1 tumours and in *NF1*-deficient primary cells and astrocytes (Dasgupta *et al*, 2005a; Johannessen *et al*, 2005).This mTOR pathway hyperactivation was reflected by high levels of ribosomal S6 activation, and expression of proteins involved in promoting ribosome biogenesis was increased in the absence of neurofibromin (Dasgupta *et al*, 2005a)

1.3 The *NF1* gene mutational spectrum

The search for *NF1* gene mutations has been hindered by several factors: the large size of the gene, the lack of mutational hotspots and the presence of pseudogenes. The *NF1* gene also exhibits a high mutation rate, with 30-50% of NF1 patients representing new mutations (Huson *et al.*, 1989). Below is an overview of the known *NF1* gene mutational spectrum, based on the Appendix table incorporating all the *NF1* microlesions identified to date.

1.3.1/ *NF1* germline mutations

The *NF1* germline spectrum has been thoroughly investigated in large studies (Fashold *et al.*, 2000; Messiaen *et al.*, 2000; Han *et al.*, 2001; Ars *et al.*, 2003), and displays a wide range of mutations. To date, over 730 mutations have been identified (Human Gene Mutation Database; http://www.hgmd.org; De Luca et *al.*, 2004; Mattocks *et al.*, 2004; Upadhyaya et *al.*, 2004; Kluwe *et al.*, 2004).

1.3.1.1/ Chromosomal rearrangements

A number of chromosome 17 deletions (Andersen *et al.*, 1990; Upadhyaya *et al.*, 1996a; Riva *et al.*, 1996, Perry et al., 2002), inversions (Asamoah *et al.*, 1995) and translocations (Schmidt *et al.*, 1987; Ledbetter *et al.*, 1989; Fahsold *et al.*, 1995; Kehrer-Sawatzki *et al.*, 1997) have been reported. Historically, the t(1; 17) (Schmidt *et al.*, 1987) and t(17; 22) (Ledbetter *et al.*, 1989) translocations were key to the discovery of the *NF1* gene locus.

1.3.1.2/ Gross rearrangements

A number of gross deletions (Lazaro *et al.*, 1994a, 1995; Heim *et al.*, 1995a; Xu *et al.*, 1992; Osborn *et al.*, 1999; Frahm *et al.*, 2004a) and gross insertions (Wallace *et al.*, 1991; Upadhyaya *et al.*, 1992, Fahsold et al., 2000) have been reported. The lesions can involve one or more exons, and generally lead to a truncated protein. It is estimated that 5-10% of

NF1 patients harbour large 17q11 deletions in the germline, encompassing the *NF1* gene and several neighbouring genes, some of which may be transcribed (Cnossen *et al.*, 1997; Lopez-Correa *et al.*, 2001; De Raedt *et al.*, 2004; Kluwe *et al.*, 2004).

Patients have been diagnosed with a more severe phenotype, termed the "microdeletion syndrome", that include early onset of an excessive number of neurofibromas, an increased risk of MPNST development, mental retardation, dysmorphism and cardiovascular malformations (Kayes *et al.*, 1994; Wu *et al.*, 1995; Cnossen *et al.*, 1997; Leppig *et al.*, 1997; Tonsgard *et al.*, 1997; Riva *et al.*, 2000; De Raedt *et al.*, 2003; Kluwe *et al.*, 2003b; Venturin *et al.*, 2004b; Kehrer-Sawazki *et al.*, 2005). However, not all patients have this phenotype (Rasmussen *et al.*, 1998; Upadhyaya *et al.*, 1998). About 80% of *de novo NF1* large deletions are estimated to occur on maternally derived chromosomes (Upadhyaya *et al.*, 1998; Lopez-Correa *et al.*, 2001).

The most common type of deletion spans approximately 1.5Mb, mostly occurs at paralogous sequences (termed NF1REPs, for repetitive elements) through interchromosomal recombination and, in half of the cases, at a 2kb recombination hotspot within these NF1REPs (Lopez-Correa *et al.*, 1999; Dorschner *et al.*, 2000; Lopez-Correa *et al.*, 2000; Jenne *et al.*, 2001; Lopez-Correa *et al.*, 2001; Jenne *et al.*, 2003; Forbes *et al.*, 2004; Kehrer-Sawazki *et al.*, 2004).

Other large deletions (ranging from 700kb to over 7Mb) have also been reported, including a 1.2Mb deletion mediated by intrachromosomal recombination between the *JJAZ1* gene and pseudogene elements (Kayes *et al.*, 1992, 1994; Wu *et al.*, 1995,1997; Cnossen *et al.*, 1997; Leppig *et al.*, 1997; Tonsgard *et al.*, 1997; Upadhyaya *et al.*, 1998; Dorschner *et al.*, 2000; Riva *et al.*, 2000; Jenne *et al.*, 2003; Kehrer-Sawazki *et al.*, 2003; Petek *et al.*, 2003; Kehrer-Sawazki *et al.*, 2004; Venturin *et al.*, 2004a, 2004b; Gervasini *et al.*, 2005; Kehrer-Sawazki *et al.*, 2005). Recently, maps of the *NF1* large deletion (or "microdeletion", because these are too small to be seen by FISH) region have been published, offering detailed information on the deletion breakpoints and additional genes involved (Jenne *et al.*, 2003; De Raedt *et al.*, 2004).

Using a newly designed array-CGH, Mantripragada *et al.* (2005) identified 39 heterozygous deletions in 161 NF1 samples; 26 cases (including 1 somatic deletion in an MPNST) were 1.44-1.58 Mb deletions mediated by the NF1-REPS, whereas 13

(including 2 somatic deletions in MPNSTs) were atypical deletions, generally ranging from 6kb to 1.6Mb. Additionally, new deletion breakpoints were identified, including an intron 1 (*NF1* gene) breakpoint, which may represent a new deletion hotspot.

Gross insertions and duplications are rare and five have been reported to date, ranging from 23bp to 10kb (Wallace *et al.*, 1991; Upadhyaya *et al.*, 1992; Tassabehji *et al.*, 1993; Fahsold *et al.*, 2000; De Luca *et al.*, 2004).

1.3.1.3/ Micro-insertions and micro-deletions

This type of lesions account for approximately 49% of the microlesions identified so far (Appendix). The majority of the deletions usually involve 1 to 4 base-pairs and the largest reported were 23bp (Heim *et al.*, 1995; Mattocks *et al.*, 2004). Less represented than deletions, insertions generally involve 1 or 2 base-pairs, with the largest one reported of 8bp (Ars *et al.*, 2000a). The majority of these mutations alter the open reading frame of the gene, and are expected to result in a truncated protein. Additionally, a number of in-frame deletions have been reported (Shen *et al.*, 1993; Abernathy *et al.*, 1997; Wu *et al.*, 1999; Serra *et al.*, 2001a; Ars *et al.*, 2003; De Luca *et al.*, 2003; Mattocks *et al.*, 2004). Indels, which are mutations that combine a deletion and an insertion, are much rarer (approximately 1% of all microlesions).

1.3.1.4/ Base-pair substitutions

These lesions can either be missense mutations (amino-acid substitutions), nonsense mutations (introduction of a stop codon) and mutation at or close to a conserved splice site. As discussed in this section, the former two may also alter splicing, so that these mutations "categories" are not as strict as would first appear. Nucleotide substitutions in the 5'UTR and promoter region of *NF1* have scarcely been assessed and a handful have been described, although these did not appear to be of functional significance (Osborn *et al.*, 2000; Horan *et al.*, 2004).

Taken together, nucleotide substitutions account for the other 50% of all microlesions. Transitions are only marginally more represented (60%) than transversions (40%).

Additionally, 18% of all transitions occur at a CpG dinucleotide or, more rarely, at a CpNpG trinucleotide. Following methylation at its 5 position, the cytosine of a CpG dinucleotide is prone to undergo spontaneous deamination to thymine and this mechanism, coupled with less than completely efficient DNA repair, is thought to underlie the C>T transition (Cooper and Youssoufian, 1988).

Although the pathogenicity of an amino-acid substitution is not always clear, the variant protein resulting from such a change can potentially be unstable or alternatively exhibit an altered function, especially if the substitution is located in the GRD. A missense mutation at the GRD conserved residue 1423 (Lys) was shown to segregate with the disease in an NF1 family and was also identified as a somatic mutation in non-NF1 sporadic tumours. While not affecting the binding affinity of the mutant protein to Ras-GTP, the mutation was shown to reduce the GAP activity to 200-400 folds lower than that of the wild-type neurofibromin (Li *et al.*, 1992). Similarly, Upadhyaya *et al.* (1997b) reported a variant neurofibromin resulting from a R1391S mutation; the GAP activity of the Ser1391 variant protein was 300 times lower than that of the wild-type neurofibromin.

The donor and acceptor splice sites of exons feature the highly conserved GT or AG dinucleotide motifs. Most of the *NF1* mutations affecting mRNA splicing are located in these obligate bases, although some mutations have been reported further into the intron.

Nonsense mutations result in the appearance of a stop codon in the sequence, and may therefore result in a truncated protein. However, this rigid definition has been revised in the light of functional assays. Nonsense mutations can also have a role in aberrant splicing by disrupting exonic splicing enhancer (ESE) elements, as demonstrated by Zatkova *et al.* (2004). Nonsense, missense and transcriptionally silent mutations can induce mutation-associated exon skipping, thereby modifying the transcript (Cartegni *et al.*, 2002). This new type of splicing mutation is gaining recognition in a number of genes, although the underlying mechanism is not completely understood (Cartegni *et al.*, 2002). One such mechanism involves the disruption or creation of ESE or exonic splicing

silencer (ESS) elements that serve as recognition motifs for diverse proteins required in splicing (Blencowe, 2000).

The Zatkova study re-examined reported NF1 mutations for their effect on ESEs using two prediction programs and *in vitro* experiments. The ESE-finder program (http://rulai.cshl.edu/tools/ESE/; Cartegni *et al.*, 2003) scores candidate ESE motifs for SR proteins. These serine/arginine-rich (SR) proteins bind to purine-rich ESE sequences present in constitutively and alternatively spliced exons (Blencowe, 2000). The RESCUE-ESE program (http://genes.mit.edu/burgelab/rescue-ese/) predicts sequences with ESE activity. The mutation-induced exon skipping effect and reduction in splicing enhancement was also evaluated using separate minigene assays. The combined *in silico* and *in vitro* approach demonstrated that one missense and six nonsense mutations disrupted functional ESE. These results emphasized the need to consider a mutation not only at the genomic level, but also at its functional level.

Lastly, a number of studies have reported environmentally-induced splicing defects (Messiaen *et al.*, 1999; Park *et al.*, 1998; Wallace *et al.*, 1998; Ars *et al.*, 2000b; Wimmer *et al.*, 2000; Thomson and Wallace, 2002). New NF1 isoforms produced by such events as a 31bp insertion in an intron (Wallace *et al.*, 1998) or exon-skipping (Thomson and Wallace, 2002) were identified by RT-PCR of RNA extracted from cooled or aged blood. Such isoforms are found indifferently in NF1 patients and unaffected individuals. It has been proposed that these alterations might be present, albeit at a lower level, *in vivo* (Thomson and Wallace, 2002).

1.3.1.5/ Recurrent mutations

Although the most recent studies on *NF1* mutations still report a high proportion of new mutations (55-74%; Ars *et al.*, 2003; Mattocks *et al.*, 2004, De Luca *et al.*, 2004), recurrent microlesions have begun to emerge from the collected data, with over 25 mutations represented in more than 6 patients. The most represented is a missense mutation in exon 31 (C5839T, R1947X) identified in 21 patients. Furthermore, there

appear to be regions of clustered mutations, such as nucleotides 6789-92 in exon 37, which harbour 6 different mutations, and are mutated in 47 patients (Section 3.2.3.4).
To obtain a clearer picture of the current distribution of mutations on the *NF1* gene, the entirety of the microlesions identified to date (Appendix) has been plotted in Figure 1.2. In order to reflect true commonly mutated exons, the distribution of the mutations was weighted, as published in Fahsold *et al.* (2000). Of note, the *NF1*-GRD does not seem to harbour more mutations than the rest of the gene, despite the bias of earlier studies. It also becomes clear that exons 37, 4b and, to a slightly lesser extent, 7 represent mutational "warm spots" in the *NF1* gene. This distribution is very close to that seen by Fahsold and co-workers (2000) in one of the largest studies of its kind.
These results, while stressing the absence of a true mutational hotspot for *NF1*, are however valuable for future studies, in defining a number of exons and recurrent mutations that can be prioritized in terms of screening order.

1.3.2/ *NF1* gene somatic mutations

1.3.2.1/ Loss of heterozygosity (LOH)

The first studies to assay *NF1* allelic loss focused on malignant tumours (Skuse *et al.*, 1989; Menon *et al.*, 1990; Glover *et al.*, 1991; Xu *et al.*, 1992) and identified the loss of all or part of chromosome 17, including the *NF1* locus. Homozygous inactivation of the *NF1* gene by LOH has been observed in NF1-related malignant tumours such as phaeochromocytomas (Xu *et al.*, 1992) and neurofibrosarcoma (Legius *et al.*, 1993) and in the bone marrow of children with NF1-related haematological malignancies (Shannon *et al.*, 1994; Miles *et al.*, 1996). More recent studies have also noted the frequent incidence of LOH in MPNSTs (up to 50% of tumours; Frahm *et al.*, 2004a; Upadhyaya *et al.*, 2004), although it has yet to be determined to be of significance)
However, early studies failed to demonstrate LOH of chromosome 17 in benign tumours (Menon *et al.*, 1990; Skuse *et al.*, 1991; Lothe *et al.*, 1995). The cellular heterogeneity of neurofibromas may account for such results, as LOH may be masked by the presence of normal cellular DNA (Peltonen *et al.*, 1988; Menon *et al.*, 1990).

In 1995, Colman *et al.* reported for the first time the loss by LOH of the remaining wild-type *NF1* allele in 36% of neurofibromas using intragenic *NF1* RFLP markers (Colman *et al.*, 1995). Moreover, the group showed that in the same patient, two neurofibromas presented LOH in two distinct locations, and therefore arose from two independent somatic events.

Since then, a number of studies on NF1 benign tumours have reported LOH as the second somatic hit, ranging in frequency 3% to 57% (Coleman *et al.*, 1995; Daschner *et al.*, 1997; Serra *et al.*, 1997; Kluwe *et al.*, 1999a,b; Eisenbarth *et al.*, 2000; John *et al.*, 2000; Rasmussen *et al.*, 2000; Serra *et al.*, 2001a,b; Upadhyaya *et al.*, 2004). The number of extragenic and intragenic markers used to assess LOH, limited sensitivity of the gel/silver staining technique, as well as the cellular heterogeneity of neurofibromas are likely to account for the variation between studies.

The common 1.5Mb large deletion (Section 1.3.1.3) has also been recently reported in an MPNST (Mantripragada *et al.*, 2005).

1.3.2.2/ Microlesions

The first somatic microlesion identified was a 4bp deletion in exon 4b in a benign neurofibroma (Sawada *et al.*, 1996). In 2000, John *et al.* reported the first case of a plexiform neurofibroma with both germline and somatic mutations. The germline mutation was located at the donor splice-site of exon 3 and consisted of a G to A substitution. The somatic mutation, seen only in the DNA derived from the tumour, was a truncating nonsense mutation (R816X).

To date, 38 somatic mutations have been reported (Sawada *et al.*, 1996; Eisenbarth *et al.*, 2000; John *et al.*, 2000; Serra *et al.*, 2000, 2001a; Wiest *et al.*, 2003; Upadhyaya *et al.*, 2004; Appendix). The somatic mutations include micro-insertions, micro-deletions, nonsense, missense and splice-related mutations. The distribution (Figure 1.3) and proportions are similar to those of the germline microlesions. Of note, CpG dinucleotides account for 46% of all somatic transitions. Approximately 26% (10/38) of the somatic mutations have also been reported in the germlines of other patients.

1.3.3/ *NF1* as a tumour suppressor gene

Several lines of evidence support the role of *NF1* as a tumour suppressor gene. Firstly, clinical data shows that NF1 patients are prone to multiple tumours, both benign and malignant (Ruggieri and Huson, 1999; Section 1.1.2.1). In mouse models, *Nf1* heterozygous mice also showed a predisposition to phaeochromocytomas and myeloid leukaemia, and LOH was found in half of these mice (Jacks *et al.*, 1994).

Secondly, NF1 tumorigenesis has been shown to follow the "two hits" model proposed by Knudson (1971), where an inactivating mutation (first hit) is inherited and a second inactivating event must occur for tumour formation.

In keeping with this model, homozygous inactivation of the *NF1* gene was observed first by LOH, then by microlesions in NF1-related malignant and benign tumours (Sections 1.3.2.1 and 1.3.2.2). These findings served to emphasize the heterogeneous nature of the *NF1* somatic mutational spectrum, and showed that each tumour, even in the same patient, results from an independent somatic event (Colman *et al.*, 1995).

Thirdly, *NF1* inactivation has also been reported in non-NF1 sporadic malignancies including melanomas (Andersen *et al.*, 1993), neuroblastomas (The *et al.*, 1993) adenocarcinoma (Li *et al.*, 1992) and breast cancer cells (Ogata *et al.*, 2001). More recently, LOH of the *NF1* gene has been described in approximately 20% of sporadic colorectal tumours, and differential expression of isoforms II and I were observed between normal and tumour tissue (Cacev *et al.*, 2005). In a similar fashion, a shift from predominant type II expression towards type I expression has been related to ovarian tumour development (Iyengar *et al.*, 1999). LOH of the *NF1* gene has also been reported in breast cancer tumours in a family diagnosed with both breast cancer and NF1 (Güran and Safali, 2005).

Finally, the presence of a GAP-related domain in the *NF1* gene, and the biochemical evidence linking this domain to Ras downregulation (section 1.2.6.2), underlies a role for the *NF1* gene in cell growth regulation.

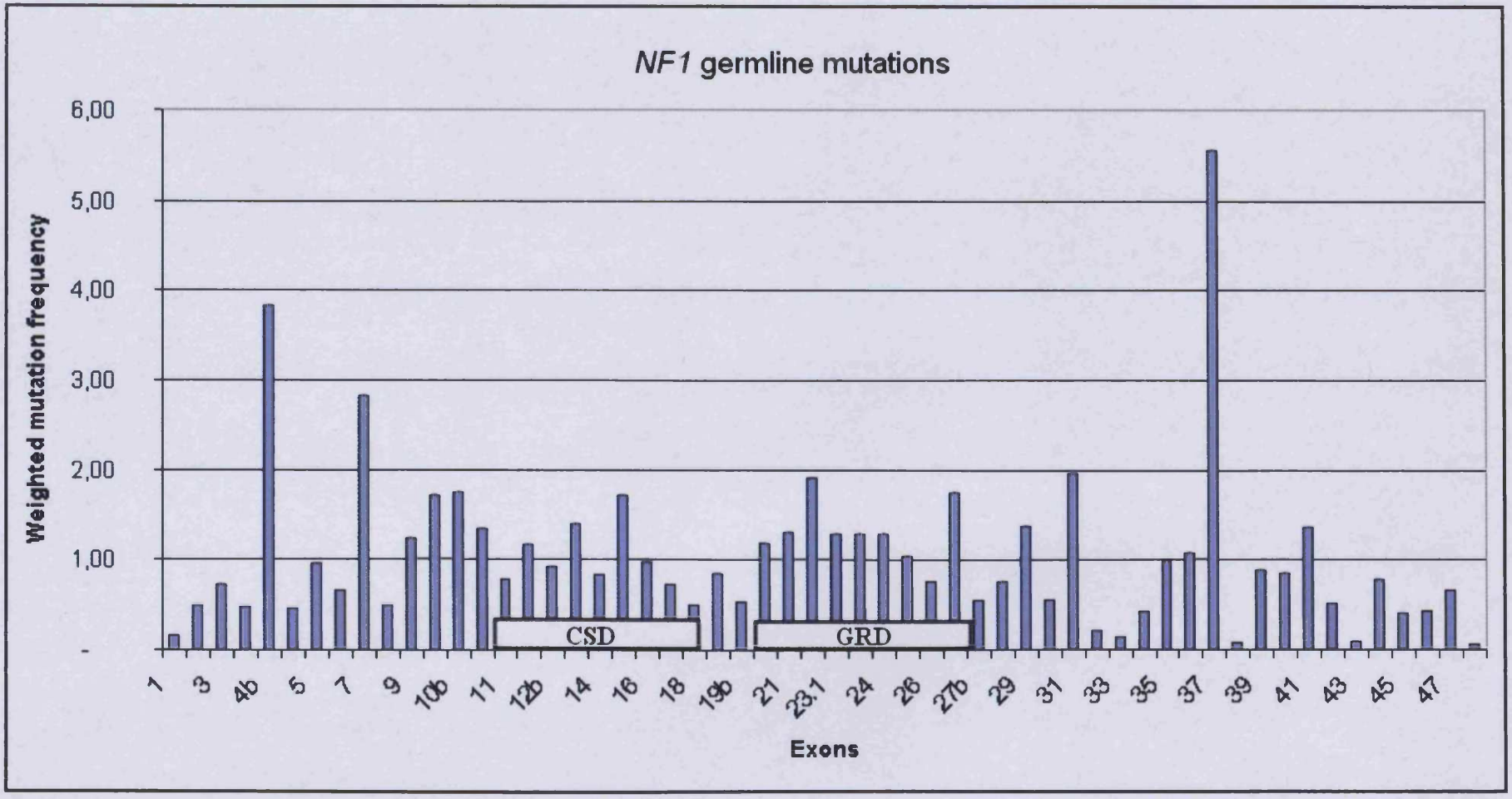

Figure 1.2 : Weighted disribution of NF1 germline mutations (microlesions) reported to date.
The distribution has been weighted as described by Fahsold *et al.* (2000). Intronic/splicing mutations have been allocated to the nearest exon. For each exon, the number of mutation was divided by the number of base pairs per exon + 10bp (to account for the close intronic sequences). The values shown are the ratio between each exon density, and the average mutation density for the whole gene. This distribution accounts for recurrent mutations, each represented as many times as it was found in patients

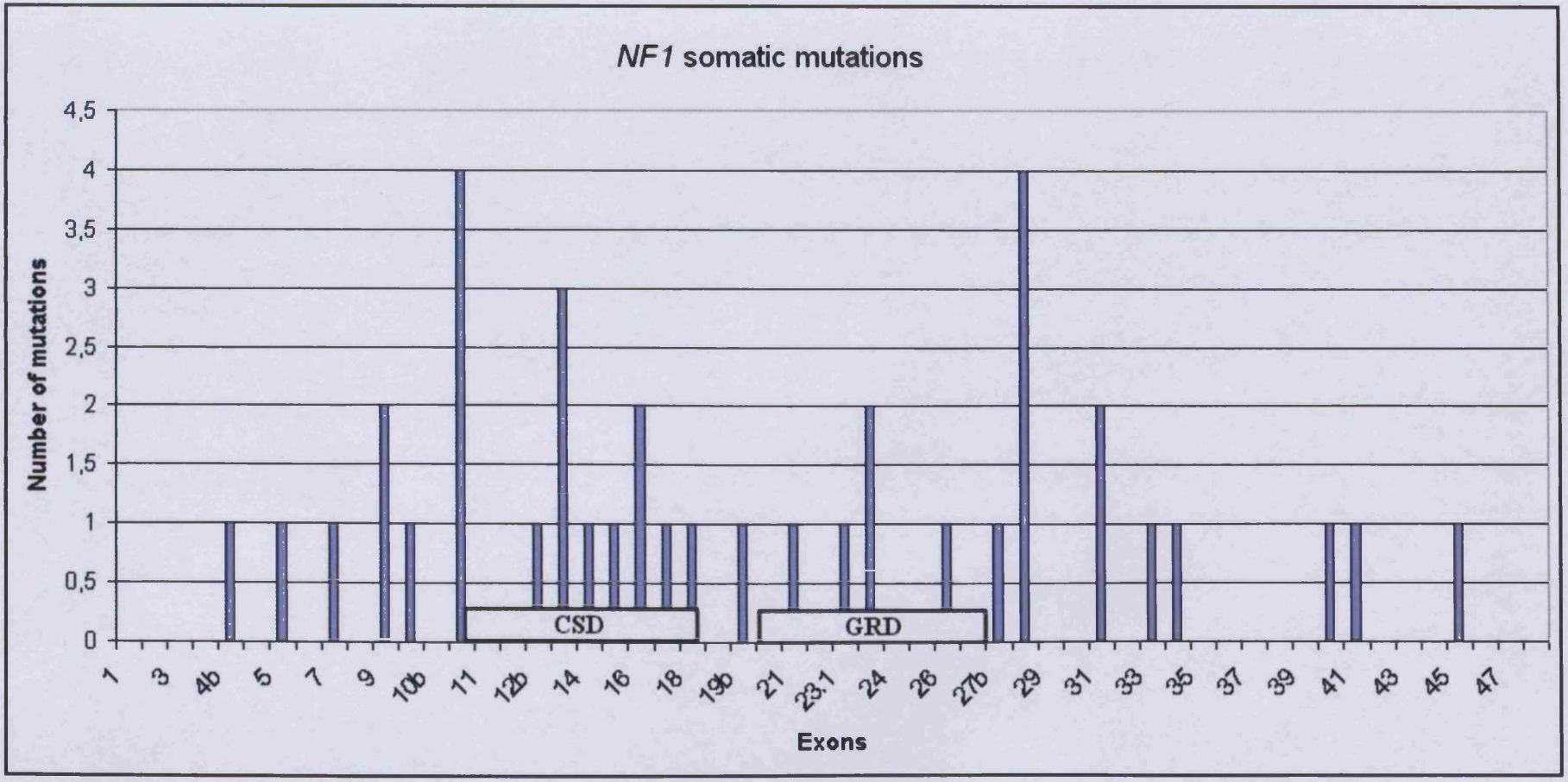

Figure 1.3: Disribution of NF1 somatic mutations (microlesions) reported to date.
Due to the small number of mutations, the distribution has not been weighted. Intronic/splicing mutations have been allocated to the nearest exon.

1.3.4/ Evolution to malignancy

NF1 patients have an increased risk of developing a malignancy as compared to the rest of the population (Ferner and Gutmann, 2002). Approximately 6% of plexiform neurofibromas undergo malignant transformation, and an elevated risk for certain malignant complications has also been reported (Gutmann and Collins, 1995; Ruggieri and Huson, 1999; Section 1.1.2.1.4).

Although loss of neurofibromin is necessary for neurofibroma formation (Eisenbarth *et al.*, 2000; Serra *et al.*, 2001a), it is not sufficient for the progression of the tumour to malignancy and additional mutations are required for malignant progression (Carroll and Stonecypher, 2004). The mechanisms underlying MPNST development are not completely understood; however, there have been several investigations into candidate genes involved in NF1 malignancy.

Using fluorescent *in situ* hybridisation (FISH), and more recently comparative genomic hybridisation (CGH), a number of studies have assessed the karyotypic differences between MPNSTs and benign tumours (Jhanwar *et al.*, 1994; Mertens *et al.*, 1995; Lothe *et al.*, 1996; McComb *et al.*, 1996; Mechtersheimer *et al.*, 1999; Plaat *et al.*, 1999; Mertens *et al.*, 2000; Schmidt *et al.*, 2000; Perry *et al.*, 2002; Frank *et al.*, 2003; Mawrin *et al.*, 2002; Gil *et al.*, 2003; Bridge *et al.*, 2004; Frahm *et al.*, 2004a). MPNSTs were found to have complex karyotypes and carry cytogenetic abnormalities, both structural (gain/loss) and numerical (tetrasomy, pentasomy,...). Virtually every chromosome has been found to be affected, and whether chromosomal loss (or tumour suppressor gene inactivation) is more frequent than gain (or oncogene activation) is arguable between studies. Although the results can vary greatly between studies, a number of recurrent breakpoints and chromosomal regions involved in MPNSTs have emerged, in particular 1p, 7p22, 11q13-23, 17q, 20q13 and 22q11-13. These aberrations could point to genes involved in malignant progression, such as *ERK* (extracellular signal regulated kinase, in the Ras pathway) on 1p36.1, *ETV1* (involved in sarcomas) and *PMS2* (MMR gene) on 7p22, and *NF2* on 22q12.2 (Bridge *et al.*, 2004).

To a lesser extent, plexiform neurofibromas have also been reported to have complex anomalies at various chromosomes (Wallace *et al.*, 2000; Koga *et al.*, 2002; Perry *et al.*,

2002). However, karyotypic alterations in neurofibromas are rare (Riccardi and Elder, 1986; Glover *et al.*, 1991; Mertens *et al.*, 2000); most notably, one study that assessed specifically neurofibroma-derived Schwann cells failed to identify karyotypic aberration (Wallace *et al.*, 2000).

In a large study of 489 genes by real-time PCR, Lévy and co-workers (2004) identified 28 genes that were significantly up- or down-regulated in MPNSTs compared to plexiform neurofibromas. The genes were mainly involved in cell proliferation, senescence, apoptosis and extracellular matrix remodelling, or in the Ras or Hedgehog (Hh)-Gli signalling pathways. Some down-regulated genes were also Schwann cell or mast cell specific. The considerable amount of data reported in this study points to pathways and cell-specific alterations in NF1 tumour malignant transformation, and should warrant additional investigation in the altered genes identified. Of note, a recent study has shown expression of transcription factor Gli1, which mediates Hh signalling, in plexiform neurofibromas, but not in control samples (Endo *et al.*, 2004).

A number of genes have also been the objects of extended investigation in NF1 tumours, and the findings are reported in the following sections.

1.3.4.1/The *TP53* gene and p53 pathway

The tumour suppressor *TP53* plays a major role in maintaining genome integrity in response to various cellular stresses, by inducing cell cycle arrest or apoptosis (Levine 1997; Vousden, 2000; Vogelstein *et al.*, 2000). Post-transcriptional stabilization of p53 is induced by events such as hypoxia, oncogene activation and DNA damage (Kastan *et al.*, 1991; Vousden and Lu, 2002). The p53 protein triggers cell cycle arrest by transactivating key downstream effector genes and allowing time to repair damaged DNA (Deng *et al.*, 1995). Additionally, increased levels of p53 can activate apoptotic pathways through both transcription-dependent and -independent mechanisms (Fridman and Lowe, 2003). Therefore, loss of p53 function through mutation can impair apoptosis in a cell with compromised DNA damage repair, leading to the accumulation of mutations in that

cell. *TP53* is the most frequently mutated tumour suppressor gene in human cancer, with mutations found in more than half human tumours (Olivier *et al.*, 2002; Beroud and Soussi, 2003). Furthermore, the *TP53* DNA damage response pathway can be indirectly altered by upregulation of MDM2 (Prives, 1998), and *TP53* apoptosis pathway can be interrupted by loss of $p14^{ARF}$ (Lowe and Sherr, 2003).

In NF1 tumours, over-expression and accumulation of p53 have been observed in MPNSTs whereas low (or absent) expression is found in neurofibromas and *TP53* alterations (deletions, mutations) have been reported in up to 100% MPNSTs, but not neurofibromas (Nigro *et al.*, 1989; Menon *et al.*, 1990; Glover *et al.*, 1991; Xu *et al.*, 1992; Jhanwar *et al.*, 1994; Legius *et al.*, 1994; Kindblom *et al.*, 1995; Lothe *et al.*, 1995; Halling *et al.*, 1996; Luria *et al.*, 1997; Kluwe *et al.*, 1999a; Kourea *et al.* 1999a; Mertens *et al.*, 2000; Rasmussen *et al.*, 2000; Schmidt *et al.*, 2000; Birindelli *et al.*, 2001; Leroy *et al.*, 2001; Koga *et al.*, 2002; Mawrin *et al.*, 2002; Zhou *et al.*, 2003; Frahm *et al.*, 2004), although bi-allelic inactivation seems rare (Lothe *et al.*, 2001)

In mouse models, *p53* mutations in conjunction with *Nf1* mutations (alleles in *cis*) have been shown to accelerate tumorigenesis and contribute to the apparition of MPNSTs (Vogel *et al.*, 1999). Cell lines derived from MPNSTs harvested from these mice also aberrantly expressed epidermal growth factor receptor (EGFR, Li *et al.*, 2002,), suggesting that EGFR expression in MPNSTs may provide an additional growth advantage.

A gene in the p53 pathway, $p14^{ARF}$, has also been found inactivated in MPNSTs (Perrone *et al.*, 2003), following promoter methylation. Protein $p14^{ARF}$, encoded by the *CDKN2A* locus through a separate promoter and alternate reading frame (ARF), is a tumour suppressor that inhibits HDM2 (Quelle *et al.*, 1995; Sherr and McCormick, 2002). In mouse models, $Nf1^{+/-}/p14^{ARF+/-}$ mice have been shown to die earlier than *p14* null mice and present tumour types typical to *p14* null mice. However, in contrast to *p14* null mice, $Nf1^{+/-}/p14^{ARF+/-}$ mice also presented a multiple tumour phenotype and metastatic tumours, probably following activation of Ras. These results suggest that *NF1* and $p14^{ARF}$ may cooperate in conferring a multiple tumours phenotype (King *et al.*, 2002).

Finally, it has been suggested that both the p53 and Rb pathways may be co-inactivated in a large proportion of MPNSTs, resulting in modifications in growth control and apoptosis in these tumours (Perrone *et al.*, 2003).

1.3.4.2/ The Rb pathway

The regulation of the cell cycle is mediated by a cascade of phosphorylation / dephosphorylation of key proteins. In phase G1, such reactions are under the control of the Rb and p53 pathways. The Rb pathway is responsible for keeping the cell in G1 phase or allowing the G1 to S transition (Czajkowski *et al.*, 2002). Tumour suppressor protein Rb prevent cell growth by regulating the progression from G1 to S phase and blocking transcription factors, like E2Fs, needed to activate genes involved in DNA replication. The Rb protein is itself tightly regulated by cyclin-D- and E-dependant kinases, which can inactivate Rb through phorphorylation. Protein $p16^{INK4a}$, one of the two tumour suppressors encoded by the *CDKN2A* locus, inhibits cyclin-D-dependant kinases, CDK4 and CDK6, thereby maintaining Rb in its active state (Sherr, 2001).

1.3.4.2.1/ The *CDKN2A/p16^{INK4a}* gene

An increasing body of evidence suggests that the *CDKN2A* (or *INK4a/ARF*) locus frequency of involvement in human cancers may be second only to that of *TP53*. Inactivation of the *CDKN2A* locus by hypermethylation and/or deletion is seen in many cancers although subtle mutations are rare (Kamb *et al.*, 1994; Cairn *et al.*, 1995; Williamson *et al.*, 1995; Chin *et al.*, 1998; Foulkes *et al.*, 1997; Cachia *et al.*, 2000; Esteller *et al.*, 2000; Sherr, 2001; Esteller *et al.*, 2002; Berggren *et al.*, 2003).

In NF1 tumours, lack of *CDKN2A/p16^{INK4a}* expression was found by *in situ* expression studies in MPNSTs, but not in dermal and plexiform neurofibromas (Nielsen *et al.*, 1999; Birindelli *et al.*, 2001; Zhou *et al.*, 2003). Gross alteration of the gene, including homozygous deletions, and 9p deletion are the most common and correlated with loss of *CDKN2A/p16^{INK4a}* expression in up to 87% of MPNSTs (Lothe *et al.*, 1996; Berner *et al.*, 1999; Kourea *et al.*, 1999b; Nielsen *et al.*, 1999; Mertens *et al.*, 2000; Schmidt *et al.*, 2000; Birindelli *et al.*, 2001; Perry *et al.*, 2002; Perrone *et al.*, 2003; Zhou *et al.*, 2003;

Bridge *et al.*, 2004; Frahm *et al.*, 2004; Ågesen *et al.*, 2005). Promoter methylation of *CDKN2A/p16*^{INK4a}, has also been reported in NF1 and sporadic MPNSTs, but does not appear to be a major mechanism of *CDKN2A/p16*^{INK4a} inactivation in MPNSTs (Perrone *et al.*, 2003; Gonzalez-Gomez *et al.*, 2003a).
A splice-related mutation, affecting both *CDKN2A* tumour suppressors (TS) p16^{INK4a} and p14^{ARF}, was found in a family presenting with breast cancer and multiple other tumours, including neurofibromas (Prowse *et al.*, 2003).

Located at 9p21 and contiguous to *CDKN2A*, the *CDKN2B* locus encodes the tumour suppressor p15^{INK4b}, also involved in the Rb pathway. Although both belong to the INK4 family of cyclin-dependant kinase inhibitors and inhibit cyclins CKD4 and CDK6, thereby leading to cell cycle arrest in G1, p15^{INK4b} responds to extracellular signals whereas p16^{INK4a} is triggered by intracellular signals (Kamb, 1995). All three TS genes at 9p21 have been shown to be deleted in the majority of sporadic and NF1 MPNSTs and the involvement of one or more of these TS genes in NF1 MPNSTs was estimated to be 78% (Kourea *et al.*, 1999a,b; Perrone *et al.*, 2003).

1.3.4.2.2/ The *RB1* gene

Loss of expression of Rb can be achieved through mutations, aberrant methylation of the promoter and disruption of the genes regulating Rb phosphorylation status, and is generally mutually exclusive with other alterations in the pathway (Weinberg, 1995; Sherr and McCormick, 2002; Konishi *et al.*, 2002; Gonzalez-Gomez *et al.*, 2003b; Kishi *et al.*, 2005). Deletion at the *RB1* locus was detected in both sporadic and NF1 MPNSTs, and correlated with loss of protein expression (Berner *et al.*, 1999; Mawrin *et al.*, 2002). Others have reported a generally maintained expression in both MPNSTs and neurofibromas, but tumour recurrence occurred from MPNSTs that were Rb-negative (Kourea *et al.*, 1999b). It has also been suggested that expressed *RB1* in MPNST may still harbour a heterozygous deletion (Ågesen *et al.*, 2005). Finally, aberrant promoter methylation has recently been reported in 11% benign NF1 tumours and 33% MPNSTs (Gonzalez-Gomez *et al.*, 2003a).

1.3.4.2.3/ Other genes

Cyclin D1 has been found to be weakly expressed in MPNSTs, although this could be due to their labile nature (Ågesen *et al.*, 2005). By contrast, overexpression of cyclin E has been observed in MPNSTs (Kourea *et al.*, 1999a).

Ågesen *et al.* (2005) have reported CDK2 and CDK4 to be expressed in all MPNSTs whereas Birindelli *et al.* (2001) detected CDK4 in only 1/26 MPNSTs. A CDK inhibitor, p21cip, has also been found to be of aberrant size in one MPNST (Ågesen *et al.*, 2005). p27kip1 has been found to accumulate in the cytoplasm of MPNST cells, and it has been suggested that epigenetic modification, rather than mutation, may account for this alteration (Kourea *et al.*, 1999a).

1.3.4.3/ EGFR

EGFR (epidermal growth factor receptor) is a membrane receptor tyrosine kinase with oncogenic properties when mutated (Conti *et al.*, 2005). Wild-type Schwann cells do not express EGFR (DeClue *et al.*, 2000), but a number of observations implicate EGFR in NF1 malignant progression. Low-level amplification of the *EGFR* gene and gain of chromosome 7 have been observed in both sporadic and NF1 MPNSTs (Perry *et al.*, 2002; Bridge *et al.*, 2004), in particular a subset of high-grade MPNSTs with homozygous *p16* deletions (Perry *et al.*, 2002). In these tumours, gene amplification may provide a means for overexpression. In cell lines derived from NF1 and sporadic MPNSTs, cells were found to respond to EGF with increased EGFR and MAP kinase phosphorylation (DeClue *et al.*, 2000). EGFR overexpression was also seen in murine MPNST (DeClue *et al.*, 2000; Watson *et al.*, 2004) and, in a mouse model with inactivating mutations in both the *Nf1* and *p53* genes, MPNSTs were shown to aberrantly express EGFR (Li *et al.*, 2002). These results have suggested that aberrant expression of EGFR might confer an additional growth advantage.

More recently, transgenic mice were engineered to express human EGFR in Schwann cells, via the control of a promoter specific to these cells (Ling *et al.*, 2005). Interestingly, the mice developed nerve hyperplasia and occasional neurofibroma formation, a similar

nerve pathology to that seen in nerves from mice and human where NF1 function is lost (Cichowski *et al.*, 1999; Vogel *et al.*, 1999; Zhu *et al.*, 2002). This suggest a model where *Nf1* loss predisposes Schwann cells to up-regulate EGFR, thereby leading to neurofibroma formation.

Additionally, studies by Miller *et al.* (2003) identified elevated levels of brain lipid binding protein (BLBP) in mouse and human MPNSTs, and showed that elevated expression of BLBP was induced in normal Schwann cells following introduction of EGFR, but not activated Ras. This would suggest a role for EGFR and BLBP in the disruption of the Schwann cell - peripheral nerve interaction in NF1 tumours.

Finally, elevated CD44 expression has recently been shown to contribute to the invasive behaviour of MPNSTs. CD44 is a transmembrane glycoprotein that has been implicated in Schwann cell invasion and metastasis. The high expression of CD44 is also thought to be dependent on Src kinase activity, which itself may be linked to over-expression of EGFR (Su *et al.*, 2003).

Other growth factors that may be involved in MPNST tumorigenesis have been thoroughly reviewed by Carroll and Stonecypher (2005). These include the hepatocyte growth factor (HGF) and receptor c-Met, both expressed in neurofibromas and, at greater levels in MPNSTs, stem cell factor (SCF) and receptor c-Kit, PDGF, midkine and transforming growth factor- β (TGF-β).

1.4 NF1 and Schwann cells

1.4.1/ Schwann cells in the peripheral nervous system

Schwann cells (SC) are a type of neural-crest derived glial cells of the peripheral nervous system that help separate and insulate neurons, by myelinating the axons (Jessen and Mirsky, 2002). Furthermore, SC are a regenerative cell type; when a nerve injury occurs, mature SC can dedifferentiate and proliferate, in a process called Wallerian degeneration, and redifferentiate following axon signals (Fawcett and Keynes, 1990). Normal, non-

myelinating Schwann cells express neurofibromin (Daston *et al.*, 1992), at higher levels in developing nerve (Daston and Rainer, 1992).

The aspects of SC life, from differentiation to survival, are under the influence of their close relationship with the neurons. *In vivo*, a family of neuronaly-expressed mitogens, the neuregulins (NRG) is though to play a critical role in SC proliferation, differentiation, survival and migration (Zorick and Lemke, 1996; Aldkofer and Lai, 2000; Meintanis *et al.*, 2001). All NRG are transmembrane proteins with an extracellular EGF-like domain and phosphorylate a tyrosine-kinase receptor erbB from the EGFR receptor family, leading to signal transduction through the MAPkinase pathway (Falls, 2003). Moreover, when a lesion occurs, SC can secrete NRG1 and 2 in an autocrine manner to dedifferentiate and repair the nerve (Aldkofer and Lai, 2000).

The complexity of mechanisms underlying SC growth is only now beginning to be unravelled. *In vitro*, SC are particularly difficult to culture, and do not respond to standard culture conditions, which resulted in continued attempts to refine SC-specific isolation and to develop SC culture techniques (Brockes *et al.*, 1979; Carey and Stahl, 1990; Cheng *et al.*, 1998; Muir *et al.*, 2001; Meintanis *et al.*, 2001; Mauritz *et al.*, 2004).

In vitro studies have led to the discovery of two main mechanisms underlying SC growth. Firstly, Rahmatullah and co-workers reported that heregulin (a member of the NRG1 family) and forskolin (an activator of cAMP) work synergistically to promote SC proliferation (Rahmatullah, 1998). In the cells, forskolin increases the level of cAMP, an essential co-mitogen for a range of SC growth factors (Jessen and Mirsky, 1992). Heregulin and forskolin appear to elevate cyclin D3 levels, through up-regulation of transcription factor C/EBPβ (Fuentealba *et al.*, 2004), thereby leading to the inactivation by phosphorylation of Rb. This cascade of events results in DNA synthesis and proliferation. Forskolin may also potentiate the heregulin/erbB2:erbB3/MAPkinase pathway by promoting the recycling and/or preventing degradation of the erbB receptors, suggesting that the synergistic interaction may also derive, at least in part, from post-transcriptional mechanism (Fregien *et al.*, 2005). Secondly, Kim *et al.* (2000; 2001) proposed that heregulin was a complete mitogen of SC and alone promoted the elevation of cyclin D1 and hence cell growth. Although these two mechanisms, and seemingly

contradictory results, could be attributed to discrepancies between laboratories and conditions used, they may also reflect the complexity of signals in a particular cell type. A recent study using microarray analysis of genes up- and down-regulated in SC following treatment with heregulin and/or forskolin, underlined the great number of genes involved. The cyclin D1 (*CCND1*) gene was amongst the genes that could be upregulated by heregulin alone, whereas the cyclin D3 (*CCND3*) gene was in the group of genes promoted by synergistic action of heregulin and forskolin (Schworer *et al.*, 2003).

Understanding the signals involved in SC proliferation and survival is of importance, not only in the nerve damage repair arena, but also in the context of NF1, where tumours are thought to arise from SC.

1.4.2/ Schwann cell in NF1

The organisation found in healthy peripheral nerve is lost in neurofibromas, and the tumours are composed primarily of 40-80% Schwann cells and fibroblasts, as well as other cell types, but to a lower proportion (Peltonen *et al.*, 1988; Tucker *et al.*, 2002).

Several findings support the idea that SC is the primary defective type of cells in neurofibroma. Neurofibroma-derived SC have been shown to harbour the somatic loss of the *NF1* gene (Kluwe *et al.*, 1999b; Serra *et al.*, 2000; Frahm *et al.*, 2004a) and cytogenetic abnormalities (Wallace *et al.*, 2000), and lack neurofibromin expression compared to fibroblasts (Rutkowski *et al.*, 2000). *In vivo* FISH studies on MPNSTs and plexiform neurofibromas showed that *NF1* deletions were restricted to S100-positive SC (Perry *et al.*, 2001).

Importantly, altered Ras activity has been reported in neurofibromas-derived SC but not fibroblasts (Sheela *et al.*, 1990; Kim *et al.*, 1995; Kim *et al.*, 1997; Sherman *et al.*, 2000). Neurofibromin acts as a Ras-GAP and downregulates Ras in normal tissues (Xu et *al.*, 1990b), and decreased levels of neurofibromin have been associated with elevated levels of Ras in NF1 tumours (Basu *et al.*, 1992; DeClue *et al.*, 1992). Interestingly, increased levels of Ras signalling, through the Raf/ERK pathway, are able not only to block the induction of SC differentiation, but also sufficient to cause de-differentiation in SC

(Harrisingh *et al.*, 2004). Although sustained Ras signalling alone can induce cell cycle arrest in cultured SC (Ridley *et al.*, 1988; Kim *et al.*, 1995), its synergistic interaction with the activated cAMP pathway can also facilitate the proliferation of SC in culture (Kim *et al.*, 1997b). Harrisingh and Lloyd (2004) proposed that, in the context of the heterogeneous background of *NF1+/-* cells, *NF1-/-* SC could be less differentiated due to neurofibromin loss, and could respond to neighbouring signals by unregulated proliferation. In this model, elevated levels of Ras may also promote defects in SC re-differentiation, keeping the cells in an immature state.

Neurofibroma-derived SC, but not fibroblasts, have been also shown to exhibit angiogenic and invasive properties (Sheela *et al.*, 1990; Muir, 1995). Neurofibroma-derived SC do not form colonies in soft agar, or induce tumours when grafted subcutaneously in immunodeficient (nude) mice. However, and in contrast to normal SC, neurofibroma-derived SC, when grafted into the sciatic nerve of nude mice, not only survive but also demonstrate infiltrative growth (Muir *et al.*, 2001). Moreover, a recent study demonstrated the increased chemotactic and chemokinetic migration properties of Schwann cells derived from *Nf1* null mice (Huang *et al.*, 2004). In the mouse model, biallelic inactivation of *NF1* was shown to be required for neurofibroma formation (Jacks *et al.*, 1994; Cichowski *et al.*, 1999; Vogel *et al.*, 1999).

Through the use of a conditional (cre/lox) allele, Zhu *et al.* (2002) demonstrated that selected ablation of the *Nf1* function in SC in mouse model, was sufficient to induce formation of neurofibroma, and emphasized the role of an haploinsufficient environment for tumorigenesis.

1.4.3/ Culture of NF1 tumour-derived Schwann cells

Culture of SC has always been hindered by low-proliferation, early senescence and contamination by fibroblasts (Serra *et al.*, 2000). In the case of SC obtained from NF1 tumours, one must make a distinction between SC from neurofibromas (dermal or plexiform) and Schwann cells from MPNSTs. The latter do not respond to Schwann cells-specific conditions and can grow under standard culture conditions. These MPNST-derived cells do not exhibit the spindle shape characteristic of SC and show an increased

proliferative capacity as compared to neurofibroma-isolated SC (Frahm *et al.*, 2004a). Moreover, more than half of MPNSTs-derived SC were shown to be S100-negative (Weiss *et al*, 1983; Woodruff *et al.*, 2000; Frahm *et al.*, 2004a), and, while *NF1* allele loss is restricted to S100-positive cells in neurofibromas, such deletions have also been characterised in S100- negative MPNST-derived SC (Perry *et al.*, 2001; Frahm *et al.*, 2004a). It is likely that these SC, by becoming highly neoplastic, have dedifferentiated and lost Schwann cells markers such as the S100 protein, while still retaining the *NF1* mutation acquired during the tumour initiation stage (Perry *et al.*, 2001).

A established culture protocol for neurofibroma-derived SC (Rosenbaum *et al.*, 2000) allowed interesting insights into the composition of the SC population in neurofibromas. Serra and co-workers reported the existence of a mixed SC population in benign tumours, of which only a proportion of the cells sustain mutations in both alleles (Serra *et al.*, 2000).

$NF1^{-/-}$ SC growth seems to be at a disadvantage when forskolin, an agent that elevates cAMP in the cells, is added to the medium (Serra *et al.*, 2000). In mouse models, $Nf1^{-/-}$ SC have been reported to have increased Ras and cAMP levels and it has been suggested that one role of neurofibromin may be to antagonise the accumulation of cAMP in the cell (Kim *et al.*, 2001). Alternatively, treatment by heregulin and forskolin appear to be favourable to $NF1^{+/-}$ Schwann cells (Serra *et al*, 2000). In the light of recent publications on the synergistic interaction of heregulin and forskolin on normal Schwann cells growth, one can ponder whether the same conditions benefit heterozygous Schwann cells due to the remaining presence of neurofibromin (half that of the normal cell). While $NF1^{-/-}$ SC exhibit an altered morphology with an elongated shape, $NF1^{+/-}$ SC demonstrate an intermediate phenotype to $NF1^{-/-}$ and normal SC, which may be in part attributed to the expression of the remaining *NF1* allele (Kim *et al.*, 1995).

1.5 Methylation

1.5.1/ Epigenetics in the normal cell

DNA methylation is the major epigenetic modification in mammals, and of key importance in the regulation of gene expression (Mukai and Sekiguchi, 2002). The major modified base, 5' methylcytosine, was first reported in mammalian DNA in 1948 (Hotchkiss *et al.*, 1948) and pairs with guanine in the DNA double helix. In mammals, 4-8% of cytosine residues are methylated in normal human DNA and most of the methylation occurs in CpG dinucleotides, of which 70-90% are methylated (Gruenbaum *et al.*, 1981; Esteller and Herman, 2004). CpG islands are stretches of GC-rich DNA that are located at the 5'-end (encompassing the regulatory regions and promoter up to the first exon) of ~60% of all genes (McClelland and Ivarie, 1982). There are approximately 45000 CpG islands in the human genome.

In mammals, genes are usually unmethylated. There are exceptions: X-chromosome inactivation by methylation (Riggs and Pfeifer, 1992; Mukai and Sekiguchi, 2002) and gene imprinting with methylation of one parental allele (Li *et al.*, 1993; Mukai and Sekiguchi, 2002). DNA methylation has also been implicated in developmental regulation and normal DNA methylation was proven to be essential for embryo development in the Dnmt KO mouse model (Li *et al.*, 1992).

The normal state of CpG island hypomethylation in the genome is tightly regulated by a number of processes, not all of which are completely understood. Firstly, DNA-methyltransferases (DNMTs) are involved in the process of methylating the CpG dinucleotide. DNMTs are thought to be recruited by as yet unknown DNA-binding protein, to methylate a CpG site (Mukai and Sekiguchi, 2002). Three major proteins, coded by different genes, have so far been identified: DNMT1, thought to be involved in maintaining established methylation, and DNMT 3a and 3b, thought to have a role in establishing new methylation patterns. Interestingly, *DKO* (*DNMT1 -/- DNMT3b -/-*) cells show a massive release of epigenetic silencing, with restoration of previously lost gene function. Taking into account that *DNMT3-/-* or *DNMT1-/-* cells failed to exhibit any change in methylation status, these results suggest a cooperation of DNMTs in

methylation (Rhee *et al.*, 2002; Paz *et al.*, 2003). Moreover, DNMTs have been found to be associated with histone deacetylases (HDAC; Mukai and Sekiguchi, 2002).

Secondly, chromatin is a dynamic structure and seems to play an important role in both normal and abnormal methylation. The elementary unit of chromatin is the nucleosome: 147bp of DNA wound almost twice around an octamer of histones, two core dimers of H3 and H4 histones and two dimers of H2A and H2B histones. Nucleosomes are linked by 10-60bp of linker DNA (Peterson and Laniel, 2004). Histones are subjected to many post-translational modifications, including methylation and acetylation. These modifications are heritable; this has been coined the "histone code", although this term may actually be a misnomer as the same modification can have different, and even opposite effects (Peterson and Laniel, 2004). The modification of conformation in chromatin is under the influence of ATP-dependent chromatin modification complexes, a large family of proteins involved in different processes requiring flexible states of chromatin (Cho *et al.*, 2004). In brief, "open" chromatin is characterized by irregularly and widely spaced nucleosomes, composed of acetylated histones. This configuration leaves the DNA open to, for example, transcription factors. "Closed" chromatin is made up of tightly and regularly spaced nucleosomes with deacetylated histones. This configuration has been found in methylated CpG islands (Tazi and Bird, 1990; Stirzaker *et al.*, 2004). Moreover, the methylation status of histones seems to interact with the methylation status of DNA, although the mechanism is not well understood (Stirzaker *et al.*, 2004).

1.5.2/ Aberrant methylation in cancer

In cancer cells, a number of processes are observed: a general hypomethylation of the genome, hypermethylation of genes in key pathways and disregulation of DNMTs (Herman, 2004; Esteller and Herman, 2004). While hypomethylation can result in the activation of oncogenes, hypermethylation of the promoter can silence a gene, thus causing a loss in expression.

Allelic inactivation by methylation in a tumour suppressor gene already sustaining a genetic mutation in one allele would satisfy Knudson's "two hit" hypothesis for

tumorigenesis (Knudson, 1983). Tumour-associated methylation was first demonstrated in the calcitonin gene in lung cancer and lymphoma (Baylin *et al.*, 1986). An epigenetic second hit was also found to be frequent in the remaining, non-mutant allele of genes involved in hereditary colorectal and breast cancer (Esteller *et al.*, 2001a).

Gene silencing by hypermethylation is thought to arise either by direct interference with the binding of the transcription factor machinery (Clark *et al.*, 1997), or by steric blocking through proteins called methyl-CpG binding proteins. The methyl-binding domains (MBDs) of these proteins bind methyl-cytosines, but these proteins also possess a transcription repression domain, which recruits protein complexes, including an HDAC (Mukai and Sekiguchi, 2002; Stirzaker *et al.*, 2004).

The heterochromatin/ euchromatin model explains how a normally hypomethylated CpG region can become hypermethylated (Baylin, 2002). The heterochromatin, densely methylated, encompasses the unexpressed region of the genome, such as repeat sequences. The euchromatin, or actively transcribed genes, is hypomethylated. It is protected from the densely methylated heterochromatin and maintained in an active state by boundary elements. *De novo* methylation of a promoter CpG island would occur when such boundary elements are lost, allowing the heterochromatin to "leak" into the euchromatin. Methylation would spread from the edge of the island toward the most protected, central parts. This model would explain the heterogeneity of methylation found in tumour cell populations (Yan *et al.*, 2003), as the hypermethylation of CpG in the island can occur differently in different cells, and the time-dependency of some cancers (the boundary elements would gradually become inactivated with age). A number of elements have been identified that support this theory. Clusters of Sp1 elements have been found both upstream and downstream of the transcriptional start site of some genes (Graff *et al.*, 1997) and their disruption has been reported to facilitate *de novo* methylation (Brandeis *et al.*, 1995; Mummaneni *et al.*, 1995). Hypermethylation may also involve elements acting as "centre of initiation" for *de novo* methylation, such as *Alu* repeats located upstream of a CpG island in the heterochromatin (Turker *et al.*, 1999; Graff *et al.*, 1997).

Aberrant methylation is thought to be involved in the early stages of tumorigenesis, as well as in tumour progression and metastasis. Rather than being mutually exclusive,

genetics and epigenetics seem to act synergistically in cancer (Baylin *et al.*, 2001; Feinberg, 2004). A model of integrated epigenetics supported the age-dependency of cancer, whereby accumulation of methylation due to age acts as a permissive background for a mutation to occur, or provide a wider array of cells to be targeted by mutations. Epigenetic alterations can be observed early in tumorigenesis, and, in some cases, in pre-neoplastic tissues (Baylin *et al.*, 2001). The presence of aberrant methylation may also be the event tipping the scales towards tumorigenesis instead of apoptosis. Moreover, accumulation of epigenetic changes has been correlated with an increase in tumour aggressiveness (Herman, 2004) and methylation-associated activation / silencing of genes involved in tumour invasion and metastasis has been reported (Feinberg, 2004)

Although a lot remains to be uncovered in the mechanism of aberrant methylation, the reversibility of this epigenetic change makes it an attractive target for therapy. Many studies have focused on defining epigenetic markers for each cancer types, and identifying genes, new and old, affected by hypermethylation.

1.5.3/ Genes and pathways hypermethylated in cancer

A recent study by Esteller and co-workers mapped a profile of cancer-related genes across 600 tumours representing 15 major tumour types and showed a pattern of methylation specific to each cancer type. Some gene promoter methylation is shared between cancers and others are specific to a tumour type. However these results helped outline the molecular pathways disrupted in each human cancer (Esteller *et al.*, 2001b). Moreover, the group also observed that different tumour types might have simultaneous inactivation of different pathways e.g. disruption of cell cycle and DNA repair, through inactivation of different genes in the same pathway. Thus, two different tumour types may exhibit different gene methylation profiles, but the epigenetic changes still result in the disruption of the same pathway.

Recently, many groups have attempted to map multigene methylation in one tumour type by looking at the epigenetic inactivation of candidate genes contributing to tumour progression (Morris *et al.*, 2003; Kuroki *et al.*, 2003a; Mehrotra *et al.*, 2004; Ueki *et al.*, 2000).

Table 1.1 : List of genes methylated in human cancers.
ESCC, oesophageal squamous cell carcinoma ; *NSCLC*, non-small cell lung cancer; *SCLC*, small cell lung cancer; *MSI-H*, high microsatellite instability; *SGC*, salivary gland carcinoma; *MSP*, methylation-specific PCR.

Gene	Methylation pattern	Method used	Reference
RASSF1A	17% lymphoma, 5%leukemia	Bisulfate/MSP	Takahashi *et al.*, 2004.
	31.7-100% glioma primary tumours/cell lines	Bisulfate/MSP	Gao *et al.*, 2004;Hesson *et al.*, 2004 Horiguchi *et al.*, 2003
	40% primary lung tumours	Bisulfate/seq	Dammann *et al.*, 2000
	49-65% primary breast tumours/cell lines	Bisulfate/MSP	Burbee *et al.*, 2001; Dulaimi *et al.*, 2004
	80% breast cancer primary tumours/cell lines	MSP/ COBRA	Yan *et al.*, 2003
	67-100% breast cancer metastasis	Bisulfate/MSP	Mehrotra *et al.*, 2004
	51-73% ESCC tumours/cell lines	Bisulfate/MSP	Kuroki *et al.*, 2003
	62-80% thyroid carcinomas	Bisulfate/MSP	Schagdarsurengin *et al.*, 2002
	63% NSCLC	Bisulfate/MSP	Burbee *et al.*, 2001
	64% primary adenocarcinomas	Bisulfate/MSP	Dammann *et al.*, 2003
	67% pediatric tumours	Bisulfate/MSP	Wong *et al.*, 2004
	83% pancreatic endocrine tumours	Bisulfate/MSP	Dammann *et al.*, 2003
	100% SCLC	Bisulfate/MSP	Burbee *et al.*, 2001
NORE1A	10% SCLC tumour cell lines	COBRA assay	Hesson *et al.*, 2003
	15% Wilm's tumour	Bisulfate/MSP	Morris *et al.*, 2003
	16.6% colorectal tumour cell lines	COBRA assay	Hesson *et al.*, 2003
	17.6% NSCLC tumour cell lines	COBRA assay	Hesson *et al.*, 2003
	19% renal cell carcinoma	Bisulfate/MSP	Morris *et al.*, 2003
	24% NSCLC primary tumour	MSP/ COBRA	Hesson *et al.*, 2003
	33.3% kidney tumour cell lines	COBRA assay	Hesson *et al.*, 2003
	40% breast tumour cell lines	COBRA assay	Hesson *et al.*, 2003
RARB	20% pancreatic carcinomas	sequencing/MSP	Ueki *et al.*, 2000
	24% sporadic, 6-36% BRCA breast tumour	Bisulfate/MSP	Esteller *et al.*, 2001b; Mehrotra *et al.*, 2004
	78-90% breast cancer metastasis	Bisulfate/MSP	Mehrotra *et al.*, 2004
	30% lymphoma and leukemia	Bisulfate/MSP	Takahashi *et al.*, 2004.
	55-70% ESCC primary tumours	Bisulfate/MSP	Kuroki *et al.*, 2003; Wang *et al.*, 2003
	73% MSI-H HNPCC	Bisulfate/MSP	Yamamoto *et al.*, 2002
	73% ESCC cell lines	Bisulfate/MSP	Kuroki *et al.*, 2003
	97.5% prostate carcinomas	Bisulfate/MSP	Jerónimo *et al.*, 2004
RB1	**14% NF1-associated tumours**	Bisulfate/MSP	Gonzalez-Gomez *et al.*, 2003
	6.25% prostate carcinomas	Bisulfate/MSP	Konishi *et al.*, 2002
	15% schwannomas	Bisulfate/MSP	Gonzalez-Gomez *et al.*, 2003b
	19% malignant nervous system tumours	Bisulfate/MSP	Gonzalez-Gomez *et al.*, 2003c
	47.1% SGC	Bisulfate/MSP	Kishi *et al.*, 2005
CDKN2A	**19% NF1-associated tumours**	Bisulfate/MSP	Gonzalez-Gomez *et al.*, 2003
	1-15% leukemia primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001; Takahashi *et al.*, 2004
	7.3% gliomas	Bisulfate/MSP	Gao *et al.*, 2004
	9% bladder cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	10% Wilm's tumour	Bisulfate/MSP	Morris *et al.*, 2003
	15% liver cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	15% schwannomas	Bisulfate/MSP	Gonzalez-Gomez *et al.*, 2003b
	17% breast cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	17% meningiomas	Bisulfate/MSP	Bello *et al.*, 2004
	18% pancreatic carcinomas	Bisulfate/MSP	Ueki *et al.*, 2000

Table 1.1 continued

Gene	Methylation pattern	Method used	Reference
CDKN2A	18% ovary cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	20% uterus cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	23% kidney cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	27% head-neck cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	30% cervical cancer	Bisulfate/MSP	Yang *et al.*, 2004
	30% brain cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	31% lung cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	33% esophagus cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	36% stomach cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	37% colon cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	37.5% laryngeal cancer	Bisulfate/MSP	Sasiadek *et al.*, 2004
	39% pancreas cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	25-48% lymphomas	Bisulfate/MSP	Esteller *et al.*, 2001; Takahashi *et al.*, 2004
MLH1	44% colon cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	84% MSI+ sporadic colorectal cancers	Bisulfate/MSP	Herman *et al.* 1998
	43% uterus cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	6% leukemia primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	32% stomach cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	5% liver cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	55.8% non-small cell lung cancer	Bisulfate/MSP	Wang *et al.*, 2003
	22.6% in laryngeal cancer	Bisulfate/MSP	Sasiadek *et al.*, 2004
	4% pancreatic carcinomas	sequencing/MSP	Ueki *et al.*, 2000
MSH2	93% non-small cell lung cancer	Bisulfate/MSP	Wang *et al.*, 2003
MGMT	**43% NF1-associated tumours**	Bisulfate/MSP	Gonzalez-Gomez *et al.*, 2003
	4% bladder cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 1999; Esteller *et al.*, 2001
	6% leukemia primary tumours	Bisulfate/MSP	Esteller *et al.*, 1999; Esteller *et al.*, 2001
	8% kidney cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	8% cervical cancer	Bisulfate/MSP	Yang *et al.*, 2004
	11% melanoma primary tumours	Bisulfate/MSP	Esteller *et al.*, 1999
	11% pancreas cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 1999; Esteller *et al.*, 2001
	16% stomach cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	16% meningiomas	Bisulfate/MSP	Bello *et al.*, 2004
	20% esophagus cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	20% schwannomas	Bisulfate/MSP	Gonzalez-Gomez *et al.*, 2003b
	21-24% lung cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 1999; Esteller *et al.*, 2001
	22.7% gastric carcinomas	Bisulfate/MSP	Park *et al.*, 2001
	28-32% head-neck cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 1999; Esteller *et al.*, 2001
	25% lymphoma cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 1999; Esteller *et al.*, 2001
	30% Wilm's tumour	Bisulfate/MSP	Morris *et al.*, 2003
	34% brain cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 2001
	38% gliomas	Bisulfate/MSP	Esteller *et al.*, 1999
	38-39% colon cancer primary tumours	Bisulfate/MSP	Esteller *et al.*, 1999; Esteller *et al.*, 2001
	88% oligodendroglial tumours	sequencing/MSP	Möllemann *et al.*, 2005

The following outlines a number of pathways and genes commonly found to be disrupted in cancer through aberrant methylation. A non-exhaustive list of cancers harbouring hypermethylation of these genes' promoters can be found in Table 1.1.

1.5.3.1/ The Rb pathway

As mentioned in Section 1.3.4.2, disruption of the Rb pathway is one of the cornerstones of human cancer, and feature importantly in NF1. In cancer, control of the cyclinD/pRb pathway is lost in virtually all tumour types, via disruption of *CDKN2A/p16*^{INK4} or *RB1* function, but not both at the same time (Sherr *et al.*, 1995; Baylin and Herman, 2000). The mechanism of disruption also varies for each tumour type: in colon cancer, only hypermethylation of *CDKN2A/p16*^{INK4} is observed (Sherr and McCormick, 2002). Hypermethylation of the *RB1* gene promoter has been reported in nervous system tumours (Gonzalez-Gomez *et al.*, 2003b; Ichimura *et al.*, 1996; Tsuzuki *et al.*, 1996; Ueki *et al.*, 1996), bladder and renal cancer (Ishikawa *et al.*, 1991) and salivary gland cancer (Kishi *et al.*, 2005).

Loss of *CDKN2A/p16*^{INK4} expression by deletions, or rarely, by subtle mutation has been reported in both cell lines and primary tumours in many cancers (Kamb, 1994; Section 1.3.4.2.1). The cell-cycle regulator is also silenced by hypermethylation in many tumours types including colorectal, lung, bladder, cervical, and breast cancer, melanomas, and gliomas (Esteller *et al.*, 2001b; Yang *et al.*, 2004; Gao *et al.*, 2004). In colorectal tumours, silencing by hypermethylation of *CDKN2A/p16*^{INK4} is independent of that of *p14*^{ARF}, the other tumour suppressor encoded by the *CDKN2A* locus (Quelle *et al.*, 1995; Esteller *et al.*, 2000a,).

1.5.3.2/ The Ras pathway to apoptosis

Ras proteins are involved in many cellular pathways and oncogenic Ras (through mutation or deregulation of regulator such as neurofibromin) has been implicated in the deregulation of cell cycle and uncontrolled cell proliferation (Bos, 1989).

However, Ras has also recently been found to mediate apoptotic signals. The seemingly contradictory effects of Ras signalling may in fact coexist as part of a complex balance of

signals from Ras' many effectors with the outcome differing depending on the cell type and context. For example, in a normal cell, high-levels of Ras may protect the cell by determining cell death, whereas, in a neoplastic cell, aberrantly activated Ras may promote cell growth. There are three major pathways that modulate Ras-mediated apoptosis: protection from apoptosis via PI3-K/NF-κB, promotion via RASSF1/NORE1/Mst1, and the Raf/MEK/ERK pathway straddling the two states (Cox and Der, 2003).

1.5.3.2.1/ The *RASSF1* gene

Located at 3p21.3, in a 120kb region that often shows LOH in lung and breast cancer (Sekido *et al.*, 1998, Wistuba *et al.*, 2000), the tumour suppressor gene *RASSF1* (Ras association domain family 1) was identified as a new human Ras effector homologue (Damman *et al.*, 2000), and became the founding member of family that includes NORE1.

The tumour suppressor gene *RASSF1* encodes seven transcripts, A-G, derived from alternative splicing and separate promoter use (Dammann *et al.*, 2000; Agathanggelou *et al.*, 2005). Isoforms A and C are ubiquitously expressed, whereas isoforms B, D and E are expressed in haematopoietic, cardiac and pancreatic cells, respectively. A Ras association or RalGDS/AF-6 domain, similar in structure to the RasGTP binding domain of Ras effector Raf1, is encoded by exons 4-5 and located at the COOH terminus of isoforms A-E. The C-terminus region (where the RAS association domain is) also has a high sequence homology for the mouse Ras effector protein Nore1 (Dammann *et al.*, 2000). Isoform RASSF1A (and D-G) also possesses a NH_2-terminal protein kinase C (PKC) conserved region 1 (C1) domain encoded by exons 1α and 1β. In its COOH terminus, RASSF1A (and B-E) possess a SARAH (Sav/RASSF/Hpo) domain that mediates interactions with several proteins, including Mst1 (Agathanggelou *et al.*, 2005). Additionally, RASSF1A is known to bind directly to Ras-GTP (Vos *et al.*, 2000), and form homodimers and heterodimers with NORE1, both of which can complex with Mst-1 and regulate its kinase activity (Ortiz-Vega *et al.*, 2002). Mst1 is a serine/threonine kinase that initiates apoptosis when overexpressed (Graves *et al.*, 1998). Additionally, the

scaffold protein CNK1 has been shown to bind to RASSF1A but not NORE1. CNK1 can also induce apoptosis, which can be abrogated by dominant-negative inhibitors of Mst1 (Rabizadeh *et al.*, 2004). RASSF1 exogenous expression is also thought to promote cell cycle arrest and apoptosis, by inhibiting cyclin D1 accumulation (Khokhlatchev *et al.*, 2002; Vos *et al.*, 2000; Shivakumar *et al.*, 2002).

It has also been suggested that RASSF1A may be involved in a mechanism distinct from a Ras-mediated pathway, at least in some cell types (Kim *et al.*, 2003). A consensus phosphorylation site for the ATM kinase has been identified, suggesting that RASSF1A function may be phosphorylation dependent and that RASSF1A could take part in the ATM pathway leading to cell cycle arrest and apoptosis (Shivakumar *et al.*, 2002; Vos *et al.*, 2004).

Finally, the RASSF1A isoform was found to associate with and stabilise polymerised microtubules, and that this interaction is necessary for RASSF1A function to block Ras-induced genomic instability (Liu *et al.*, 2003; Vos *et al.*, 2004). Additionally, RASSF1A is thought to contribute to the cell cycle regulation, through modulation of APC/C (anaphase promoting complex/cyclosome) activity (Agathanggelou *et al.*, 2005).

The *RASSF1A* (and D-G) promoter CpG island spans a 650bp region (85 CpGs, 71.5% GC) encompassing the entire promoter region and almost all of the first exon of the gene (Yan *et al.*, 2003; Agathanggelou *et al.*, 2005). Transcript A is expressed in all normal tissues but is absent from several cancer cell lines, which correlates with its hypermethylated promoter status (Dammann *et al.*, 2000; Burbee *et al.*, 2001). Methylation of *RASSF1A* was first observed in lung cancer (Dammann *et al.*, 2000; Burbee *et al.*, 2001), but has since been reported in at least 37 tumour types (Agathanggelou *et al.*, 2005), including pancreatic (Dammann *et al.*, 2003), brain (Horiguchi *et al.*, 2003) and breast tumours (Burbee *et al.*, 2001). Epigenetic inactivation of *RASSF1A* was also observed in a high percentage of primary gliomas and derived cell lines (Hesson *et al.*, 2004; Gao *et al.*, 2004). A cDNA microarray and RT-PCR approach also uncovered genes from many different cellular processes to be targeted by RASSF1A in lung and cancer cell lines (Agathanggelou *et al.*, 2003). The *RASSF1A* promoter was found to be hypermethylated to different degrees in breast tumours and correlated with

alteration of the chromatin configuration in this region, both epigenetic modifications potentially leading to a stable silencing of the gene in these tumours (Yan *et al.*, 2003). Finally, hypermethylation of *RASSF1A* has also been detected in pre-cancerous tissues (Zochbauer-Muller *et al.*, 2003; Honorio *et al.*, 2003), and it has been suggested that *RASSF1A* could be used as a marker for early detection and monitoring (Agathanggelou *et al.*, 2005).

Taken together, these observations strongly support the tumour suppressor gene status of *RASSF1A* and suggest that the gene plays a crucial role in cell processes of apoptosis, cell cycle progression and regulation of the microtubule network.

1.5.3.2.2/ The *NORE1* (*RASSF5*) gene

In 2002, Dammann and co-workers identified the human homologue to the mouse Nore1, previously characterised in 1998, as a potential Ras effector (Vavvas *et al.*, 1998). Located at 1q32.1, *NORE1*, also termed *RASSF5*, encodes at least two isoforms differing in their 5' termini. Both isoforms share highly conserved Ras association domain encoded by exons 3-6; however, NORE1A contains 2 additional 5' exons, 1α and 2α (Tommasi *et al.*, 2002). Human NORE1A is a 418 amino acid protein and also contains a zinc finger, RA (Ras/Rap association) domain and a carboxyterminal tail, the latter two being conserved in the family. The carboxyterminal tail binds selectively to the proapoptotic protein kinase MST1 and NORE1 has been found to induce apoptosis (Khokhlatchev *et al.*, 2002). It has also been proposed that NORE1 constitutively form a complex with MST1, maintaining the later in an inactive form, until association with Ras is induced following stimulation (Praskova *et al.*, 2004). However, the mechanism underlying NORE1 function is still unclear. Whereas Vos and co-workers (Vos *et al.* 2002) found evidence that, like RASSF1, NORE1-mediated apoptosis is Ras-dependent, Aoyama *et al.* (2004) reported that NORE1A inhibits tumour cell growth by delaying cell cycle progression, independently of its capacity to bind Ras or the Mst1 kinases.

NORE1A possess a CpG island at its 5' end, different from that of *NORE1B* (Hesson *et al.*, 2003). No aberrant methylation has been observed in *NORE1B*, but *NORE1A* hypermethylation was observed in a number of cell lines and primary tumours, including

lung cancer, and correlated with down-regulation of *NORE1A* expression (Hesson *et al.*, 2003). Additionally, *NORE1A* was observed to display aberrant hypermethylation in glioma cell lines, but not in primary tumour, suggesting a possible role in late gliomagenesis (Hesson *et al.*, 2004). No correlation has been observed between *RASSF1A* and *NORE1A* hypermethylation (Morris *et al.*, 2003).

1.5.3.3/ DNA repair

1.5.3.3.1/Mismatch repair genes: *MLH1* and *MSH2*

DNA mismatch repair (MMR) is a pathway that is conserved from bacteria to humans. The MMR pathway targets base-base mismatches and insertion/deletion loops that can occur during replication and homologous recombination, or after DNA damage, and can give rise to frameshifts (Schofield and Hsieh, 2003). The majority of HNPCC cases are linked to the two main MMR genes, *MLH1* and *MSH2*, inactivated by point mutation and promoter hypermethylation (Kinzler and Vogelstein, 1996; Bocker *et al.*, 1999). Inactivation of *MLH1* by hypermethylation has been linked to non-familial cases and thought to be the major cause of MSI in some cancers, such as colorectal cancer (Herman *et al.*, 1998), pancreatic carcinomas (Ueki *et al.*, 2000), endometrial and gastric tumours (Esteller, 2000b). However, aberrant hypermethylation of the *MLH1* promoter was not correlated to MSI in oesophageal squamous cell carcinoma (Hayashi *et al.*, 2003). Aberrant hypermethylation of the *MLH1* promoter has also been found in pre-cancerous tissues, suggesting an early epigenetic inactivation in some cancer (Mukai and Sekiguchi, 2002). The *MSH2* promoter is not prone to hypermethylation in MSI+ tumours (Herman *et al.*, 1998; Cunningham *et al.*, 1998), and appears to be rare in cancer. Hypermethylation of the *MSH2* promoter has however been reported in 93% of non-small cell lung cancer (Wang *et al.*, 2003).

Finally, *MLH1* and *MGMT* were reported to be inactivated by hypermethylation at a high frequency in colorectal cancer (Esteller, 2001c). Observations on $Mgmt^{-/-}/Mlh1^{-/-}$ mice suggested that *Mlh1* might have a critical role in steering the cell fate towards mutation induction or apoptosis, when *Mgmt* repair is lost (Kawate *et al.*, 2000).

1.5.3.3.2/ The *MGMT* gene

Located at 10q26, the O6-methylguanine DNA methyltransferase (*MGMT*) gene encodes a protein that repairs DNA by removing the mutagenic and cytotoxic alkyl-adducts from the O6-position of guanine. O6-methylguanine can pair with a thymine during replication thus leading to the conversion of a GC pair to an AT pair. Moreover, the O6-alkylguanine-DNA adduct can block DNA replication. MGMT repairs these lesions by transferring the alkyl group to an active cysteine within its sequence, inactivating one MGMT molecule for each repair in the process (reviewed in Esteller *et al.*, 1999).

The *MGMT* gene harbours a CpG island in its 5' region, encompassing the promoter and first exon (Harris *et al.*, 1991). Aberrant promoter methylation has frequently been associated with loss of *MGMT* expression in cell lines and primary tumours (Esteller *et al.*, 1999; Danam *et al.*, 1999). The *MGMT* promoter has been found to be hypermethylated in a wide range of cancers, including gliomas (Möllemann *et al.*, 2005; Esteller and Herman, 2004) and has been associated with sporadic tumours with low MSI (Whitehall *et al.*, 2001). Moreover, MGMT showed hypermethylation in precancerous tissues in colorectal adenomas, suggesting that the epimutation may occur as an early event in some cancers (Esteller *et al.*, 2000b).

It has been suggested that silencing of *MGMT* triggers a mutator pathway, possibly by overloading the mismatch repair system (Whitehall *et al.*, 2001; Esteller and Herman, 2004). *MGMT* inactivation has also been strongly linked to the appearance of oncogenic G to A mutations in K-Ras (Esteller *et al.*, 2001c; Whitehall *et al.*, 2001; Park *et al.*, 2001) and to inactivating G to A mutations, particularly in non-CpG dinucleotides, in p53 (Esteller *et al.*, 2001c; Nakamura *et al.*, 2001).

1.5.3.4/ Retinoids and the *RARB* gene

Retinoids, a group of natural and synthetic analogues of vitamin A, have important effects on cell growth, differentiation and apoptosis (Sun and Lotan, 2002). Retinoid signal transduction occurs through two different types of receptors, the retinoic acid receptors (RAR) and the retinoid X receptors (RXR), each of which consist of three subtypes (α, β, and γ; Chambon, 1996; Sun and Lotan, 2002).

Located at 3p24, the retinoic acid receptor β (*RARB*) gene is thought to play a central role in the growth regulation of epithelial cells and in tumorigenesis (Roman *et al.*, 1992; Swisshelm *et al.*, 1994). The RARβ-dependent pathway is also thought to prevent cell-cycle progression via degradation of cyclinD1 (Dragnev *et al.*, 2001).

The *RARB* gene generates several RARβ isoforms through separate promoter usage and alternative splicing: RARβ1 is under the control of promoter P1, whereas promoter P2 directs RARβ2 and RARβ4. It is thought that the diversity in structure and pattern of expression allow for the modulations in retinoic cid effects (Virmani *et al.*, 2000).

RARβ2 expression is frequently found to be down-regulated or lost in breast cancer through the epigenetic inactivation of promoter P2 and deletion (Yang *et al.*, 2001; Yang *et al.*, 2002). Silencing of RARβ2 by hypermethylation has also been reported in many cancers, including colon (Cotes *et al.*, 1998), oesophageal squamous cell (Kuroki *et al.*, 2003a; Wang *et al.*, 2003), head and neck squamous cell (Youssef *et al.*, 2004), prostate (Jerónimo *et al.*, 2004) and lung (Virmani *et al.*, 2000) cancers. RARβ2 is also thought to play an important role in the early steps of tumour progression in some cancers (Kuroki *et al.*, 2003a; Wang *et al.*, 2003). These results add to the growing evidence of RARβ2 as a tumour suppressor.

1.5.4/ Hypermethylation in the context of NF1

1.5.4.1/ The *NF1* gene promoter

The *NF1* promoter lies within a CpG island, thus making it possible for an aberrant methylation event to suppress the *NF1* gene expression and induce tumour formation (Mancini *et al*, 1999). The CpG island is thought to be 471bp long and start 731bp (-731bp to –261bp) upstream of the translation initiation site (Lee and Friedman, 2005). Recently, conserved regions, suspected to affect *NF1* transcriptional regulation, have been identified; of note, a core promoter element, termed NF1HCS, has been identified and found to overlap much of the CpG island (Lee and Friedman, 2005).

Furthermore, several transcription factors binding sites have been identified in the CpG island, including an AP2 and Sp1 binding motifs, a cAMP response element (CRE) and a

putative repressor element located 3kb upstream of the transcription start site (Hajra *et al.*, 1994; Purandare *et al.*, 1996). Mancini and co-workers established a methylation map of the promoter region and demonstrated that normal DNA is unmethylated around the *NF1* transcriptional start site. Furthermore, they showed that the Sp1 and CRE binding sites were functionally sensitive to site-specific methylation, where methylation inhibits binding of Sp1 and CREB (CRE binding protein) *in vitro* (Mancini *et al*, 1999). Methylation at the two cytosines in the CpCpG nucleotides in the Sp1 binding site has been reported to inhibit binding by 95% *in vitro*, and endogenous mCpmCpG methylation has also been found in the hypermethylated *RB1* promoter in retinoblastoma tumours (Clark *et al.*, 1997). Sp1 has also been proposed to play an important role in maintaining the CpG islands in an unmethylated state (Graff *et al.*, 1997; Baylin, 2002), suggesting that blocking Sp1 binding by methylation of CpCpG could play a role in initiating *de novo* CpG methylation (Clark *et al.*, 1997).

However, Mancini and co-workers, as well as other groups, failed to identify tumour-related methylation of the *NF1* promoter, either at the transcription factors binding-sites (Luijten *et al.*, 2000b; Harder *et al.*, 2004) or at other CpG sites (Horan et *al.*, 2000). More recently, the methylation status of the NF1 promoter was reassessed in a larger panel of NF1 neurofibromas and neurofibroma-derived Schwann cells. Although the degree of methylation was greater than that previously reported, its low level did not greatly differ from the one observed in normal Schwann cells. Interestingly, the majority of methylated CpGs appeared to cluster within the putative transcription factor binding sites (Fishbein *et al.*, 2005). Taken together, these results suggest that methylation of the *NF1* promoter is unlikely to play a major role in tumour formation.

1.5.4.2/ Hypermethylation in NF1 tumours

Although the methylation status of tumour related genes have been assessed in different cancers, it is only recently that such a study has been performed in NF1-related tumours. Gonzalez-Gomez *et al.* determined the methylation status of 11 genes (*RB1*, *p14ARF*, *CDKN2A/p16INK4a*, *TP73*, *TIMP-3*, *MGMT*, *DAPK*, THBS1, *CASP8*, *TP53* and *GSTP1*) in NF1-associated and sporadic tumours: 17 dermal neurofibromas, 1 plexiform

neurofibroma and 3 neurofibrosarcomas, (Gonzalez-Gomez *et al.*, 2003a). Aberrant methylation ranging from 5% to 52% was identified in most genes; however no methylation was identified in *DAPK*, *CASP8*, *TP53* and *GSTP1*. These findings suggest that methylation-mediated silencing of genes, previously found to be inactivated in other cancers, may contribute to neurofibroma progression.

1.6 Microsatellite instability (MSI)

1.6.1/ MSI in cancer

Microsatellites (MS) are short tandem repeats of 1-5bp units, and are represented in large numbers over the human genome (Weber and May, 1989). In the human genome, ~50,000 to 100,000 (CA)n repeats are scattered throughout the genome and MS are highly polymorphic in the human population. The mutation rate at repeat sequences is higher than at other genomic sites, although still quite low, $5x10^{-4}$ to $5x10^{-5}$ (Hearne *et al.*, 1992). The alleles are generally stably inherited from generation to generation and are usually used for linkage analysis (Weissenbach, 1992).

In 1993, collaborative studies on HNPCC uncovered the instability of microsatellite sequences in somatic cells of patients suffering from cancers belonging to the HNPCC spectrum (Thibodeau *et al.*, 1993; Aaltonen *et al.*, 1993; Peltomaki *et al.*, 1993). The mechanism was referred to as both microsatellite instability (MSI; Thibodeau *et al.*, 1993) and replication error phenotype (RER, Peltomaki *et al.*, 1993). The latter can however be a misnomer, as MSI is not a symptom of increased replication error, but rather the outcome of defects in the repair process, and manifest itself as a global instability phenomenon affecting primarily microsatellite sequences (Boland, 1998).

In a panel of 90 colorectal tumours analysed with four microsatellite (MS) markers, Thibodeau and co-workers identified length alterations (from 2 bp to much larger) in 28% of the tumours and observed an inverse correlation between large length modification and LOH at 3 marker loci. Aaltonen *et al.* (1993) linked the process to a locus later identified as the first MMR gene in HNPCC. Using MS markers, the same group discovered MSI in 43 to 71% of the HNPCC tumours and at lower incidence in sporadic colon cancer tumours.

Mutations in 5 genes functioning in mismatch repair, and most frequently *MLH1* and *MSH2*, were subsequently found to be segregated with the HNPCC phenotype (Toft and Arends, 1998; Fishel *et al.*, 1993; Leach *et al.*, 1993). Mismatch repair proteins correct base-pair mismatches, which arise during DNA replication and can lead to spontaneous mutations if uncorrected. DNA polymerases tend to be more prone to replication slippage on repetitive sequences such as mononucleotide runs or microsatellites, which results in deletions or insertions of repeat units, altering the length of the repeat. In DNA MMR-deficient cells, misaligned repetitive sequences caused by polymerase slippage remain unrepaired.

To standardize the investigation for MSI in HNPCC tumours, guidelines and a reference panel of 5 markers, with the possibility of additional markers, was proposed. A classification of MSI-High (MSI-H), MSI-Low (MSI-L) and MSS (microsatellite stable) was also implemented (Boland *et al.*, 1998).

The observation of MSI is not, however, limited to tumours from the HNPCC spectrum and sporadic colorectal cancer. Arzimanoglou *et al.* (1998) reviewed the issue of MSI in human solid tumours: MSI has been reported in many cancers, including brain, gastric, breast, lung, prostate and gynaecological tumours. Of note, MSI in these cancers is rarely related to mutations (inherited or somatic) in the MMR genes most commonly involved in HNPCC (Arzimanoglou *et al.*, 1998).

MMR genes are considered to be "mutator genes": they do not cause cancer directly by altering cell growth, but greatly increase the probability of secondary mutations arising that then contribute toward the development of a malignant phenotype (Hanahan and Weinberg, 2000). Genes containing repeat sequences are particularly prone to this type of genetic alteration; thus, MSI reflects increased genomic mutability and the increased accumulation of mutations in oncogenes, tumour suppressor genes and/or other mutator genes which accelerate neoplasia (Fearon, 1992). The list of potential target genes that may be involved in carcinogenesis is constantly growing and includes genes involved in apoptosis (Bax, Caspase-5), regulation of cell growth (IGFIIR), transcription factors and other MMR genes (Boland *et al.*, 1998; Duval and Hamelin, 2002). These genes have

been reported to be mutated in colorectal and other MSI tumours at varying frequencies. The importance of MSI in non-coding regions has also been addressed. Reference markers, such as BAT25 and BAT26, were found to have varying allelic length depending on the degree of advancement of the tumour (the larger the deletion, the more advanced the tumour), suggesting a role as "molecular clock" in tumour progression (Pedroni *et al.*, 2001; Duval *et al.*, 2001).

1.6.2/ MSI in NF1 tumours

As discussed in Section 4.3.2, there has been a limited report of MSI in NF1 tumours. A number of groups reported the rare contribution of MSI in MPNSTs, and a general absence of instability in benign tumours (Serra *et al.*, 1997; Berner *et al.*, 1999; Luijten *et al.*, 2000b; Birindelli *et al.*, 2001). By contrast, Ottini *et al.* (1995) reported MSI in 50% of neurofibromas. As with MSI investigations in other cancers, the differences in markers used may in part account for the discrepancies observed (Arzimanoglou *et al.*, 1998).

1.6.3/ MMR genes and NF1

An interesting link between NF1 and MMR genes emerged with the reports of individuals, homozygous for MMR genes defects and presenting characteristics of NF1.
In 1999, Ricciardone *et al.* reported three individuals from consanguinous families who were homozygous for germline mutations in *MLH1* and exhibited hallmark features of NF1 (Ricciardone *et al.*, 1999). All three patients suffered from acute haematological malignancies, which can also occur in the setting of NF1.
Additional cases with homozygous *MLH1* mutations have been described with clinical features of NF1 (CALS, multiple axillary freckling, neurofibromas) and non-colonic cancers, including gliomas and gastrointestinal tumours (Wang *et al.*, 1999; Vilkki *et al.*, 2001; Gallinger *et al.*, 2004). A homozygous *MLH1* mutation, which produces a functional but instable protein, was found in a patient who had mild neurofibromatosis (CALS, one neurofibroma), but no haematological cancer (Raevaara et *al.*, 2004). This case differs from the previous ones in that the mutation is not expected to result in the complete lack of the MMR protein, which may account for the milder phenotype.

Cases with homozygous inactivation of other MMR genes have also been described. Patients with inactivated *MSH2* (Whiteside *et al.*, 2002; Bougeard *et al.*, 2003), *PMS2* (Trimbath *et al.*, 2001; De Vos *et al.*, 2004) and MSH6 (Hegde *et al.*, 2005; Menko *et al.*, 2004) have been reported to develop NF1 features, haematological malignancies and additional cancers (brain tumours, colorectal neoplasia, oligodendroglioma).

Thus it would appear that loss of activity of the MMR complex, rather than that of a particular gene, is at the origin of the phenotype of childhood malignancy involving neurofibromatosis features and haematological cancers (Whiteside *et al.*, 2002). These findings also suggest that MMR deficiency may lead to mutations during embryogenesis in preferential target genes, such as *NF1* and genes involved in haematopoiesis (Raevaara et *al.*, 2004). *NF1* might not only be one of these target genes but also a key step in tumour progression.

In addition, the *NF1* gene has recently been found to be a mutational target gene in MMR-deficient cells, with its inactivation representing an early and important even in malignant progression (Wang *et al.*, 2003; Gutmann *et al.*, 2003). By contrast, no constitutional deficiency in *MLH1* or *MSH2* was observed in *de novo* NF1 cases, suggesting that MMR gene alteration is not frequently involved in these cases (Wang *et al.*, 2003).

In the mouse model, MMR deficiency has been found to predispose to haematological malignancies, such as lymphomas (Baker *et al.*, 1995; De Wind *et al.*, 1995; Edelmann *et al.*, 1996; Prolla *et al.*, 1998), whereas $Nf1^{+/-}$ mice are prone to the development of myeloid leukaemia and other tumours (Jacks *et al.*, 1994). $Nf1^{+/-}/Mlh1^{-/-}$ mice, however, develop myeloid leukaemia and die earlier than either $Nf1^{+/-}$ or $Mlh1^{-/-}$ mice, suggesting that *Mlh1* loss accelerates *Nf1* inactivation and tumorigenesis, and high MSI was also observed in the tumours (Gutmann *et al.*, 2003).

1.7 Mutation detection techniques

The number of identified *NF1* germline (and more recently somatic) mutations is increasing as mutation detection techniques become more reliable and sensitive. Examples of techniques commonly used in previous NF1 studies, as well as in this

Table 1.2: Examples of mutation detection techniques used in NF1.
MED, multi-exons deletion; *LR*, large rearrangements. Of note, LOH assays feature a different of primers in the different studies, which may partly explain the discrepancies in LOH identified.

	Germline mutation detection			
Technique	Detection rate	Detects	Does not detect	Reference
FISH	1% (1/67)	large deletion	microlesions	Messiaen *et al.*, 2000
	31% (4/13)	large deletion		Wu *et al.*, 1995
	1% (1/67)	balanced translocation		Messiaen *et al.*, 2000
DHPLC	66% (68/103)	microlesions	MED, LR	De Luca *et al.*, 2004
DHPLC/cDNA-DHPLC	38% (12/31)			Upadhyaya *et al.*, 2004
PTT	39% (7/18)	truncating mutations	missence mutations	De Luca *et al.*, 2004
	53% (21/40)			Osborn *et al.*, 1999
	83% (56/67)			Messiaen *et al.*, 2000
cDNA SSCP/HD	40% (142/189)	microlesions	MED, LR some single nt mutations	Ars *et al.*, 2003
HA	9% (6/67)	point mutations	MED, LR	Messiaen *et al.*, 2000
SSCP/HA	80% (16/20)	point mutations	MED, LR	Upadhyaya *et al.*, 1997
Direct sequencing	75% (18/24)	microlesions	MED, LR	Kluwe *et al.*, 2003
ACSA	85.6% (89/104)	microlesions	MED, LR	Mattock *et al.*, 2004
CMC	36% (9/25)	point mutations	MED, LR	Purandare *et al.*, 1994
	16% (4/25)	truncating mutations		Purandare *et al.*, 1994
DS	86% (6/7)	point mutations	MED, LR	Wu *et al.*, 2000
Combined techniques				
cDNA SSCP/HA/DS	55% (44/80)			Ars *et al.*, 2000
DS/TGGE/PTT	58% (301/524)			Fahsold *et al.*, 2000
PTT/FISH/HA/SB	95 (64/67)			Messiaen *et al.*, 2000

Table 1.2 continued

	Somatic mutation detection			
Technique	Detection rate	Detects	Does not detect	Reference
cDNA SSCP/HD	15% (13/84)	microlesions	MED, LR some single nt mutations	Serra *et al.*, 2001
LOH	18% (17/91)			Upadhyaya *et al.*, 2004
	27% (32/116)	deletions	microlesions	Serra *et al.*, 2001
DHPLC/cDNA-DHPLC	23% (12/53)	microlesions	MED, LR	Upadhyaya *et al.*, 2004
PTT	43% (3/7)	truncating mutations	missence mutations	Eisenbarth *et al.*, 2000
Combined techniques				
DHPLC/LOH	32% (29/91)			Upadhyaya *et al.*, 2004
LOH/PTT/DS	71% (5/7)			Eisenbarth *et al.*, 2000
cDNA SSCP/HD/LOH	39% (45/116)			Serra *et al.*, 2001

project, will be briefly described in the following sections. Examples of the detection rate of these techniques (or combination thereof) in NF1 are available in Table 1.2.

1.7.1/ Identifying chromosomal aberrations

Fluorescence *in situ* hybridisation (FISH) relies on the hybridisation of a labelled DNA or cDNA probe to intact metaphase and anaphase chromosomes fixed on a slide (Baurmann *et al.*, 1993). After removal of any unbound probe, visualisation of the probe distribution by fluorescence microscopy allows detection of large chromosome alteration, such as deletions and translocations.

Another molecular cytogenetic approach, serial karyotypic analysis (SKY) has recently been used on MPNSTs, in conjunction to FISH, to obtain more precise identification of the chromosomal rearrangement (Bridge *et al.*, 2004).

1.7.2/ Screening large deletions/insertions

Large deletions or insertions can be identified by Southern blotting (Southern, 1975). This technique relies on the electrophoretic separation of genomic DNA fragments digested by restriction enzymes. The DNA is then transferred to a nylon membrane by blotting, and hybridised with radioactive probes, which detect missing or abnormally size fragments. This technique can detect fragments of up to 30kb in size. However, a modification of this technique, pulsed field gel electrophoresis (PFGE), has been used to detect large alterations in the *NF1* gene (Upadhyaya *et al.*, 1990). PFGE can resolve DNA fragments of up to 10Mb, by periodically alternating the direction of the electric field applied to the gel (Carle *et al.*, 1986).

A simple PCR assay has been designed to identify a common 1.5Mb deletion encompassing the entire *NF1* gene, 1 pseudogene and 17 contiguous genes (Dorschner *et al.*, 2000; Lopez-Correa *et al.*, 2001; De Raedt *et al.*, 2004). The deletion is undetectable by FISH, which earned it the name of "microdeletion". The recombination mechanism responsible for the deletion is mediated by regions of high identity, the NF1-REPS, which include a 12kb recombination hotspot. Recombination creates a chimeric 3.4kb deletion junction fragment, which can be amplified using primers designed within the paralogous NF1-REPS (Lopez-Correa *et al.*, 2001).

Comparative genomic hybridisation (CGH) is a recently developed technique that relies on the differences in fluorescent signals between a sample DNA and a control DNA, simultaneously hybridised to a normal chromosome spread or array, and allows genome-wide detection of chromosomal gain and losses (Kallioniemi *et al.*, 1992; Pinkel and Albertson, 2005). CGH has been used on NF1 tumours to determine karyotypic aberrations (Lothe *et al.*, 1996; Mechtersheimer *et al.*, 1999; Schmidt *et al.*, 2000; Koga *et al.*, 2002). More recently, an array-CGH has been devised to detect deletions ranging from approximately 1 exon (from *NF1*) to 2.24Mb (entire region covered by the array) on 17q11 (Mantripradagada *et al.*, 2005).

1.7.3/ LOH assay

Detection of LOH (Loss of heterozygosity) is a key technique in identifying tumour suppressor genes. The term of "LOH" has been used broadly in the literature, and can encapsulate different meanings. LOH has been used to describe a functional effect in the two-hit model, whereby a tumour suppressor is absent in cells, thus leading to tumour growth. LOH then represents the loss of the normal, remaining functional allele through any of several mechanism, including micro-lesion, epigenetic inactivation, or chromosomal event (e.g. deletion, mitotic recombination; Tischfield, 1997).

In the present case, LOH refers to the loss of heterozygosity of *NF1* gene markers (microsatellite or RFLP) in NF1 tumour compared to a corresponding blood sample. The definition of LOH is thus restricted to the somatic occurrence of a reduction to homozygosity (through mitotic recombination, for example), or hemizygosity (through loss of the remaining allele). Occasionally, apparent LOH has been reported for a RFLP marker in a coding region, and found to actually result from a pathogenic mutation that also modified the enzyme restriction site.

LOH has been shown to represent between 25-40% of the *NF1* somatic mutational spectrum (Section 1.3.2.1). The loss of part or the entire chromosome can be detected in the affected tumour cell by apparent homozygosity for a genetic marker shown to be heterozygous in blood DNA. Various types of markers, including microsatellite or restriction site polymorphisms, have been used in numerous NF1 studies (Colman *et al.*, 1995; Daschner *et al.*, 1997; Serra *et al.*, 1997; Kluwe *et al.*, 1999a,b; Eisenbarth *et al.*, 2000; John *et al.*, 2000; Rasmussen *et al.*, 2000; Serra *et al.*, 2001a; Upadhyaya *et al.*, 2004) and shown to be very informative for NF1 tumours.

Short tandem repeat polymorphisms, or microsatellite markers, consist of short sequences of a few nucleotides tandemly repeated several times. They are characterised by many alleles that can be typed by PCR, run on a denaturing gel and visualised by silver staining. More recently, the sensitivity of the technique has been improved by using automated analysis of fluorescent signals (through modified primer sets) instead of silver staining (Dietmaier *et al.*, 1999; Faulkner *et al.*, 2004; Cai *et al.*, 2004). Restriction site polymorphism analysis relies on the presence or absence of a specific restriction enzyme recognition site. Primers flanking this region are used for PCR. The product is then

incubated with the enzyme and run on a non-denaturing, high-resolution agarose gel. If there is a mutation at the restriction site, the enzyme will not be able to cut and only one band will appear on the gel.

LOH can be observed for contiguous markers, which will roughly define the size of the deletion. In theory, LOH would be observed on a gel as one band in the tumour DNA (the allele carrying the constitutional deletion) compared with two bands in the corresponding blood lymphocyte DNA (the allele carrying the constitutional deletion and the remaining wild-type allele). However, owing to the cellular heterogeneity of neurofibromas, LOH often appears as a subtle dosage difference, as it is masked by the DNA from other heterozygous cell types.

1.7.4/ Identifying microlesions

Denaturing high performance liquid chromatography (DHPLC) is a relatively new technique presenting the advantage of high throughput and sensitivity. DHPLC relies on the detection of heteroduplexes formed between mismatched in double stranded DNA and can detect nucleotide substitution and small insertions/deletions (Underhill *et al.*, 1997). In effect, the technique used ion-pair reverse-phase HPLC at partially denaturing conditions, and detects DNA homoduplexes and heteroduplexes. The PCR products are denatured by heat and allowed to form homoduplexes and heteroduplexes (in the presence of a mutation), by slow cooling. The binding of the sample to a DNASep column is mediated by triethylammonium acetate (TEAA). The sample is subsequently eluted from the column using a linear gradient of acetonitrile (ACN) under a partially denaturing temperature (determined for each fragment). Heteroduplexes elute earlier than wild-type homoduplexes, due to their lower melting temperature. The eluted DNA fragments are scanned by a UV detector and visualised as corresponding peaks on a chromatogram. The technique has proved very sensitive for mutation detection in many conditions, with reported detection rate of up to 100% (Gross *et al.*, 1999; Jones *et al.*, 2000; Klein *et al.*, 2001). In NF1, a blind study by O'Donovan *et al.*, showed 100% sensitivity and specificity (O'Donovan *et al.*, 1998) for known point mutations and small insertions/deletions in exon 16 of the *NF1* gene. Others have reported a detection rate of

up to 68% in screening for new mutations in unrelated patients (Han *et al.*, 2001; De Luca *et al.*, 2004). A cDNA-DHPLC technique relying on the amplification and resolution by DHPLC of 24 fragments spanning the *NF1* coding region has recently been devised (Upadhyaya *et al.*, 2004).

Heteroduplex analysis (HA) is a PCR-based mutation detection technique, a precursor to DHPLC. HA relies on the formation of heteroduplexes in PCR, which can then be resolved on a polyacrylamide gel. The heteroduplexes, owing to their altered conformation, move differently in the gel to homoduplexes (Keen *et al.*, 1991).

Single strand conformation polymorphism (SSCP) relies on the secondary structure of single stranded DNA. A single base change, such as a mutation, can cause a drastic change in conformation. As a result, the mutant single stranded DNA has a different migration profile on a polyacrylamide gel compared to a wild-type single stranded DNA (Kanazawa *et al.*, 1986). SSCP can be used as a radioactive method (Orita *et al.*, 1989) with radionucleotides incorporated in the PCR product, or non-radioactively, with standard silver staining. The method can be used to screen fragments ranging from 100-500 base pair, and larger fragments can be digested by restriction enzymes prior to running the gel (Kovar *et al.*, 1991).

Both techniques, SSCP and HA have been used in conjunction in order to increase the detection rate. As re-annealing after denaturation also produces homo- and hetero-duplexes, which can be resolved at the bottom of a non-denaturing gel, whilst the SSCP fragments are resolved at the top. A temperature gradient may also be applied across the gel to improve sensitivity, as a particular mutation may become unstable, compared to the wild-type fragment, at a given temperature, and thus show an altered migration profile (Rubben *et al.*, 1995).

The two techniques, or a combination thereof, have commonly been used for mutation (both germline and somatic) detection in NF1 (Cawthon *et al.*, 1990; Shen *et al.*, 1993; Upadhyaya *et al.*, 1994; John *et al.*, 2000; Messiaen *et al.*, 2000; Serra *et al.*, 2001a; Ars *et al.*, 2003). It is noteworthy that in using an RNA-based assay, mutation detection may be hampered by nonsense-mediated mRNA decay (NMD), which can cause an under-representation of the mutated transcript (Osborn and Upadhyaya, 1999).

More recently, automated comparative sequence analysis (ACSA) has been developed (Mattocks *et al.*, 2000; Mattocks *et al.*, 2004). The technique relies on the analysis of a fluorescently-labelled sequenced fragment, separated in its four component electrophoregrams (A, T, G, C), each aligned to a control. Using ACSA, Mattocks *et al.* (2004) reported the highest mutation detection rate for a single technique in NF1 patients.(89%; germline alterations).

1.7.5/ Direct sequencing

Although direct DNA sequencing has perhaps been used more as a means to check the nature and position of a mutation identified using other techniques, recent technological advances now make direct sequencing a reliable screening method. The automation of the technique and increased rapidity now allows for the processing of a 96-well plate in an hour.

1.7.6/ Protein truncation test (PTT)

Approximately 80% of *NF1* mutations lead to a truncated protein (Shen *et al.*, 1996; Fahsold *et al.*, 2000; Ars *et al.*, 2003). First described by Roest *et al.* (1993), PTT detects mutations, in the coding region of a gene, that result in the premature truncation of the protein. The technique relies on PCR amplification of DNA or RNA, using primers containing a T7 promoter sequence and a eukaryotic translation initiation sequence. The product is then subjected to *in vitro* transcription and translation using labelled ^{35}S methionine, which is incorporated in the newly synthesized protein. The proteins can then be analysed on a SDS-PAGE gel, and truncated proteins are identified by their reduced sized (and altered migrating profile). This method has been applied to the *NF1* gene by different groups (Messiaen *et al.*, 1997 Osborn *et al.*, 1999; Ars *et al.*, 2000a; Fashold *et al.*, 2000; Messiaen *et al.*, 2000; Origone *et al.*, 2002; De Luca *et al.*, 2004). It should be noted that caution must be exercised when using an RNA-based technique, due to the "aged blood" effect reported by several groups (Messiaen *et al.*, 1999; Park *et al.*, 1998; Wallace *et al.*, 1998; Ars *et al.*, 2000b; Wimmer *et al.*, 2000; Thomson and Wallace,

2002). Thus, the interpretation of PTT results should be followed by the full characterisation of the truncating mutation in genomic DNA(Messiaen *et al.*, 2000).

1.8 Animal models of NF1

1.8.1/ Naturally occurring models

In nature, a few animal disease models show NF1-like features. The bicolour damselfish (*Pomacentrus partitus*) develops tumours that resemble plexiform neurofibromas and invasive MPNSTs, and also harbour CAL-like hyperpigmentation (Schmale *et al.*, 1983, 1986). The cause of the disease is unclear, although retroviral infection has been suspected (Schmale *et al.*, 1996).

In mammals, multiple neurofibromas and intraocular lesions have been reported in a subset of Holstein cattle (Sartin *et al.*, 1994). No mutation has however been found in the bovine *Nf1* gene, although a polymorphism near the locus has been found to segregate with the disease.

1.8.2/ Chemically-induced models

The chemical agent and carcinogen ENU (*N*-nitroso-*N*-ethylurea) has been shown to induce the formation of tumours, including MPNSTs in rat and hamsters. In prenatal treatment, ENU induced plexiform neurofibromas in rats, although subcutaneous neurofibromas were rare, and, administered postnatally, ENU triggered a high incidence of MPNSTs (Cardesa *et al.*, 1989). These findings suggest that a mutation may need to occur at a specific stage of the development to trigger tumour formation. In hamsters, postnatal ENU treatment induced the development of subcutaneous neurofibromas (Nakamura *et al.*, 1989, 1991). Activating mutations of heregulin receptor and proto-oncogene *ERBB2* has been implicated in these tumours, suggesting a development of SC origin (Nakamura *et al.*, 1994; Section 1.4.1). In both models, *Nf1* mutations have yet to be identified.

1.8.3/ Transgenic models

Transgenic mice containing the HTLV-1 *tax* gene, under the control of its own promoter, have been generated and found develop MPNSTs at three months of age, as well as medullary tumours (similar to phaechromocytomas) and iris lesions (Hinrichs *et al.*, 1987; Green *et al.*, 1992). The viral tax protein can inhibit *Nf1* expression *in vitro*, through interaction with a regulatory element in the murine *Nf1* promoter (Feigenbaum *et al.*, 1996), and overexpression of tax was also shown to up-regulate growth factors, such as NGF and TGF-β1, detected both in normal and tumour tissue in these mice (Green, 1991; Kim *et al.*, 1991).

Other models with phenotypic similarities to NF1 include mice that overexpress the SV40 large (Lg) T antigen and develop neurofibromas and MPNSTs (Mazarakis *et al.*, 1996). The SV40 Lg T antigen has been shown to bind and inhibit p53 and Rb, although no direct connection to Nf1 has been established (Weinberg, 1991).

1.8.4/ Targeted disruption of the *NF1* gene

In 1994, mice carrying one mutated allele of the *Nf1* gene were generated and found to be predisposed to tumours such as myeloid leukaemia and phaeochromocytomas, both of which are rare complications of NF1. However, the *Nf1*-heterozygous mice failed to exhibit common features of NF1, such as neurofibromas, CALS or Lisch nodules (Brannan *et al.*, 1994; Jacks *et al.*, 1994). However, $Nf1^{+/-}$ mice show abnormal responses to skin wounding (Atit *et al.*, 1999) and $Nf1^{+/-}$ mice with C57BL/6 background (resistant to chemical carcinogens) developed skin abnormalities after being subjected to a skin cancer initiator (Atit *et al.*, 2000). *NF1*-homozygous mice were then engineered, but the animals died *in utero* and presented severe heart defects, emphasizing the importance of the *Nf1* gene in normal growth and development (Brannan *et al.*, 1994; Jacks *et al.*, 1994).

To circumvent the lethality of the $Nf1^{-/-}$ genotype, chimeric mice were produced by injecting $Nf1^{-/-}$ murine embryonic stem cells into normal blastocysts. The chimera obtained was a mosaic animal composed of varying degrees of wild-type cells and $Nf1^{-/-}$ cells, allowing some of the mice to survive gestation (Cichowski *et al.*, 1999). The

chimeric mice developed multiple neurofibromas, similar to human plexiform tumours, as well as progressive neuromotor defects, but failed however to develop dermal tumours. More recently, Nf1flox/-; Krox20-cre mice, harbouring *Nf1* inactivation in Schwann cells only, were found to develop neurofibromas (Zhu *et al.*, 2002). This model simultaneously demonstrated that *Nf1* loss in Schwann cells was sufficient for tumour formation, and emphasized the functional role of the *Nf1* haploinsufficient environment.

Another model of Nf1 mice was engineered to evaluate the role of p53 in the evolution to malignancy in NF1-related tumours. The $Nf1^{+/-}$; $p53^{+/-}$ (in *cis*) mice were found to develop MPNSTs; and the tumours harboured LOH at the remaining two wild-type alleles (Cichowski *et al.*, 1999; Vogel *et al.*, 1999), suggesting an important role for *TP53* in NF1 tumours.

Mice with a complete Nf1 deletion specifically in neurons ($Nf1^{synI}$ knockout mice; Zhu *et al.*, 2001) or in glia ($Nf1^{GFAP}$ conditional knockout mice; Bajenaru *et al.*, 2002) both exhibit astrogliosis.

A mouse model for learning impairment, as found in NF1 patients (Section 1.1.2.5) has also been developed (Costa *et al.*, 2001). The mice lacked only neurofibromin variant type II (Section 1.2.4), through targeted alteration of exon 23a. Type II is expressed in the brain, and, although the mice developed normally, they presented learning difficulties, suggesting a role for this variant in the NF1 learning deficit phenotype.

1.9 Aims of the project

- To enlarge the known somatic mutational spectrum of the *NF1* gene in NF1-related tumours by using a wide range of highly sensitive mutation detection techniques.

- To compare the germline and somatic mutation spectra for NF1.

- To attempt to identify candidate genes in NF1 tumour formation and progression.

CHAPTER 2: MATERIAL AND METHODS

2.1 Materials

2.1.1/ Reagents and chemicals

Reagents and chemicals were purchased from Sigma (MI, USA): urea, EDTA, sodium borohydride, formaldehyde, chloroform, sodium bisulphite, ammonium acetate, bromophenol blue, ammonium persulphate, hydroquinone, sodium borohydride, TRIS and mineral oil. Agarose was purchased from Roche (Basel, Switzerland) and NuSieve agarose from BMA (ME, USA). Xylene cyanole FF was obtained from Biorad (Biorad Laboratories, Richmond, California, USA). Boric acid was obtained from Riedel-de Haen (Seeize, Germany). Were purchased from Fisher Scientific (Loughborough, UK): sodium hydroxyde, sodium carbonate anhydrous, sodium chloride and silver nitrate. Were obtained from National Diagnostics (Hull, UK): sodium dodecyl sulphate (SDS, 20% solution), sequagel 6 (6% sequencing gel solution) and sequagel complete. Low melting-point agarose was purchased from Invitrogen (Paisley, UK). Phenol-chloroform solution was bought from Biogene (Kimbolton, UK). GelSlick was purchased from Flowgen (Nottingham, UK). Ethanol, glacial acetic acid, acetonitrile and isopropanol were purchased from BDH (VWR International, Lutterworth, UK). RNAzol was obtained from Biogenesis (Poole, UK). Ethylenediamine-Tetraacetic acid (TEAA) was purchased from Transgenomics (Crewe, UK).

2.1.2/ Enzymes

Taq DNA polymerase and HotStart *Taq* DNA polymerase were purchased from Qiagen (Crawley, UK). Shrimp alkaline phosphatase and Exonuclease I were obtained from Amersham (Little Chalfont, UK). Obtained from New England Biolabs (Herts, UK)were: Methylase (*Mss*I), restriction enzymes (and buffers) *Taq*I, *Rsa*I, *Msp*I and BSA. SuperScript II reverse transcriptase was purchased from Invitrogen (Paisley, UK). Restriction enzyme *Hind*III and RNAsin ribonuclease inhibitor were obtained from Promega (Southampton, UK). The Expand Long Template PCR system, restriction enzyme *EcoR*I and proteinase K were purchased from Roche (Basel, Switzerland).

2.1.3/ Biochemicals

Were obtained from Applied Biosystems: Big Dye Terminator v3.1 cycle sequencing kit, HiDi formamide, and SybrGreen. Deoxyribonucleotides (dNTPs) were obtained from Promega (Southampton, UK).

2.1.4/ Kits

The QIAquick gel extraction kit and PCR purification kit were obtained from Qiagen (Crawley, UK). Montage SEQ96 sequencing reaction cleanup kit and Montage PCR96 Plates were purchased from Millipore (Watford, UK).

2.1.5/ Cell culture reagents

Cell culture reagents were obtained from Gibco (Invitrogen Ltd, Paisley, UK): DMEM, fetal calf serum (FCS), laminin, DMEM-F12, N2 supplement, versene, trypsine and PBS. Were purchased from Sigma (MI, USA): Penicillin/Streptomycin solution (PenStrep, 100x), forskolin (10mg), collagenase type I, poly-L-lysin, insulin, 3-isobutyl-1-methylxanthine (IBMX), DMSO (Dimethyl sulfoxide C2H6OS). Dispase was obtained from Roche. β-heregulin (HRG- 1) was purchased from R&D systems (Abingdon, UK). Lab-Tek 8-wells slides were provided by Naige-Nunc (Sevenoaks, UK). The Rabbit IgG ABC kit was obtained from Vectastain. Gills' haematoxylin was purchased from BDH (VWR International, Lutterworth, UK).

2.1.6/ Primers

Primers were synthesized by MWG Biotech (Ebersberg, Germany)and are summarized in Tables 2.3, 2.4, 2.5, 2.6 and 2.7.

2.1.7/ Instruments

The thermocyclers used were: Primus 96 Plus from MWG (Ebersberg, Germany), PCR express from Hybaid (Middlesex, UK) and GeneAmp PCR system 2700 from ABI (Applied Biosystems, Warrington, UK). The centrifuges were a Universal 32R from Hettich Zentrifugen (Tuttlingen, Germany) and a GS-6KR centrifuge from Beckman (High Wycombe, UK). The bench-top centrifuge was an Eppendorf 5415C (Hamburg, Germany).

The gel tank apparatus and power sources were from Biorad. The spectrophotometer was a GeneQuant Pro (Cambridge, UK). Purified Big Dye reactions were sequenced using an ABI Prism 3100 Genetic analyser from Applied Biosystems (Warrington, UK).
Micro-well plates were purchased from AB gene (Epsom, UK). Centrifuge tubes (10mL and 50mL) were obtained from Sarstedt (Leicester, UK), and micro-centrifuge (Eppendorf) tubes were purchased from Treff Lab (Luton, UK).

Table 2.1: Clinical informations available for 20 NF1 patients.
CALS, café-au-lait spots; NF, neurofibroma ; PNF, plexiform neurofibroma ; MPNST, malignant peripheral nerve sheath tumour.

Patient	Clinical informations
P23	CALS, facial PNF, neurosarcoma, dermal NFs, small stature, macrocephaly,
P135	Multiple CALS, freckling, macrocephaly, ptosis, Lisch nodules, NF
P137	NF, painful nodule on left palm (palmar NF)
P139	Scalp NF
P140	Van der Woude syndrome, cleft palate, anaemia, multiple NFs 1 CALS on back, no freckling
P145	CALS, MPNST of left psoas muscle, PNF (right lower limb), epilepsy, dermal NF Additional complications
P146	Optic glioma, meningioma on the sphenoid wing
P148	CALS, axillary freckling, dermal NFs, additional complications MPNST arose from PNF (right thigh and left buttock)
P151	Pelvic metastase, right lung metastase, lymphoma
P156	Isolated PNF, underlying CALS
P157	Multiple NFs
P159	Large palm and dorsal forefinger NF
P167	PNF from left 11th intercostal nerve, asymptomatic PNF on left thigh and knee
P168	Optic pathway glioma, precocious puberty, PNF (left foot), multiple PNFs involving the right brachial plexus, thoracic spine and lumbosacral region, cognitive impairment, CAL
P169	Brother to P178
P172	PNF (right ankle)
P176	NFs on right eyelid, right nostril, forehead
P177	Severe NF1, dysmorphic, PNF
P178	Brain tumour, PNF, shakiness, erratic heartbeat.
P184	Atypical NFs (C2, sacral), left lateral popliteal nerve NF, right occipital ganglioglioma grade 1-2, pelvic tumour (low-grade MPNST), no cutaneous manifestation of NF1
P186	Very severe NF manifestations, aqueduct stenosis

Table 2.2: Summary of experiments and number of tumours used.

LOH, LOH assay using 17q *NF1* intragenic and extragenic markers (Chapter 6); *SC*, Schwann cell culture and mutation detection by direct sequencing (Chapter 5); *MSI*, microsatellite instability assay, using 6 genome-wide microsatellite markers (Chapter 4); *MSPCR*, methylation-specific PCRs assay (Chapter 3); *CDKN2A*, LOH assay using 4 21p markers (Chapter 4); *TP53*, LOH assay using 1 *TP53* intragenic marker (Chapter 4); *MLH1*, LOH assay using 1 *MLH1* intragenic marker (Chapter 4); √, experiment done; –, not done in this sample (or sample set).

Patient	Tumours	DHPLC	LOH	cDNA	SC	MSI	MSPCR	*CDKN2A*	*TP53*	*MLH1*
P135	2 NF	√	√	–	–	√	–	–	–	1/2
P136	2 NF	√	√	–	–	√	–	–	–	–
P137	10 NF	√	√	1/10	–	8/10	1/10	–	–	1/10
P139	1 NF	√	√	–	–	√	√	–	–	√
P140	5 NF	√	√	–	–	√	1/5	–	–	–
P141	16 NF	√	√	3/16	–	√	2/16	–	–	1/16
P143	7 NF	√	√	1/7	–	√	1/7	–	–	–
P144	2 NF	√	√	–	–	√	–	–	–	–
P147	1 NF	√	√	–	–	√	–	–	–	–
P149	1 NF	√	√	1/1	–	√	–	–	–	–
P150	6 NF	3/6	√	–	3/6	√	–	–	–	–
P152	2 NF	√	√	–	–	√	–	–	–	√
P153	1 NF	√	√	–	–	√	–	–	–	–
P155	1 NF	√	√	–	–	–	–	–	–	–
P157	2 NF	√	√	–	–	√	1/2	–	–	–
P159	1 NF	√	√	–	–	√	–	–	–	√
P175	1 NF	–	–	–	√	–	–	–	–	–
P176	3 NF	√	–	–	–	√	√	–	–	√
P183	1 NF	–	–	–	√	–	–	–	–	–
P186	1 NF	√	–	–	–	√	√	–	–	√
P23	3 MPNST	–	–	–	–	–	1/3	2/3	2/3	–
P130	1 MPNST	√	√	–	–	√	√	√	√	√
P145	1 MPNST	√	√	–	–	√	√	√	√	√
P148	1 MPNST	√	√	–	–	√	√	√	√	–
P151	2 MPNST	√	√	–	–	√	√	√	√	√
P154	1 MPNST	√	√	–	–	√	–	√	√	–
P168	1 MPNST	–	√	√	–	√	√	√	√	√
P169	1 MPNST	√	√	–	–	√	√	√	√	√
P178	1 MPNST	√	√	–	–	√	–	√	√	√
P184	1 MPNST	–	√	√	–	√	√	√	√	√
P146	1 MN	–	–	–	–	–	√	–	–	–
P160	1 RMS	√	√	–	–	√	√	√	√	√
P185	1 MTC	–	√	√	–	√	√	√	√	√
P156	1 PNF	√	√	1/1	–	√	√	–	–	√
P167	1 PNF	√	√	–	–	√	√	–	–	√
P172	1 PNF	–	–	–	–	–	√	–	–	–
P177	1 PNF	–	–	–	–	–	√	–	–	–

2.2 NF1 Patients

Samples were collected over the years from hospitals across the country and worldwide and through patient donations. Samples were obtained with informed consent from the patients, and under the approval of the local ethics committee. All patients satisfied the NIH criteria for NF1. However, in many cases, no additional clinical data were available. The clinical data available for 20 patients are summarized in Table 2.1. The panel of patients with gliomas was obtained from Prof. Gareth Evans (Manchester, UK), and no additional clinical data were available. A summary of the experiments in this thesis, conducted on the main tumour panel, is available in Table 2.2.

2.3 General techniques

2.3.1/ DNA extraction

2.3.1.1/ Extraction from blood

Blood samples were, almost exclusively, processed and DNA was extracted by the Service Laboratory, Medical Genetics, UHW. For a few samples, DNA was extracted using a protocol provided by the latter, as follows. Buffers were provided by the Service Laboratory.

Blood was transferred to a 50mL tube kept on ice. Ice-cold, sterile water was added up to a volume of 50mL. The tube was centrifuged at 3,000rpm, 4°C, for 10-15 min. The supernatant was discarded and pellet was re-suspended in 25mL ice-cold, sterile, sucrose lysis buffer. The tube was left on ice for 30 min, then centrifuged at 3,000rpm, 4°C, for 10-15 min. The supernatant was discarded and the tube drained briefly, upside down, on paper towels. The pellet was re-suspended in 3mL CVS buffer and 50-100μL proteinase K (10mg/mL), and incubated either at 37°C overnight or at 65°C for 3 - 4 hours. A volume of 1.5-2.0 ml of 6M NaCl was added to the tube, which was then shaken for 20 seconds. The tube was centrifuged at 3,500rpm, 4°C, for 30 min. The supernatant was collected in a 15mlL tube, and ethanol (100%) was added to make up the volume. The solution was mixed by inversion until DNA became visible. The DNA pellet was then transferred to a 1.5mL Eppendorf tube with 70% ethanol, and centrifuged at 13,000rpm for 10 min. The tube was drained of ethanol and the pellet was allowed to air dry. DNA was re-suspended in 200-500μL TE buffer (1x; 10mM HCl, 1mM EDTA, pH=8) and stored at –20°C.

2.3.1.2/ Extraction from whole tumour

Fresh frozen tumour tissue specimens were digested at 37°C overnight in 500µL extraction buffer (0.12g Tris, 0.04g EDTA 5M, 0.87g NaCl, 5mL SDS), 50µL proteinase K and 25µL 10% SDS. DNA was then extracted using the phenol/ chloroform procedure. 500µL phenol chloroform was added to each tube and centrifuged for 15 min at 13.000rpm. The supernatant was collected and added to a solution of 7.5M-ammonium acetate (250µL) and 100% ethanol (1.5mL), to precipitate the DNA strand. A final clean-up step was performed in which the DNA strand is collected and put in 500µL 75% ethanol, and then centrifuged at 13.000rpm for 10 min. The pellet was then allowed to air dry and re-dissolved in 100-500µL TE buffer or water, at room temperature for 2 days.

2.3.1.3/ Extraction from cultured cells

Pelleted cultured cells were digested at 37°C overnight in 500 µL extraction buffer, 50 µL proteinase K and 25 L 10% SDS, and the procedure was performed as described in Section 2.3.1.2.

2.3.2/ RNA extraction

2.3.2.1/ Extraction from whole tumour

Frozen tumours (~10mg) were ground with a mortar and pestle in liquid nitrogen until a homogeneous powder was obtained. A small amount of liquid nitrogen and the powderised tumour were transferred to a 10mL centrifuge tube, and the liquid nitrogen was allowed to evaporate. The tumour sample was however not allowed to thaw. RNAzol was immediately added to the tube and homogenized with the tumour "powder" by pipeting, then transferred to a micro-centrifuge tube and put on ice for 5 min. The samples were then centrifuged for 15min at 14000rpm and 4°C. The aqueous phase was collected, mixed with 400µL of isopropanol and left on ice for 45 min. The samples were centrifugated for 15 min at 14000rpm and 4°C. The pellet was re-suspended in 800 µL of 75% ethanol and centrifuged for 8 min at 7500rpm and 4°C. The pellet was allowed to air dry for 5 min and re-suspended in 50µL water and 0.5 µL RNAsein. The RNA was stored at -70°C.

2.3.2.2/ Extraction from cultured cells

Pellets from cultured cells were homogenized in 800 µL RNAzol and placed on ice for 5 min. The samples were transferred to an eppendorf and 80 µL of chloroform was mixed in. The tubes were left on an ice for 5 min; then the procedure was conducted as described in Section 2.3.2.1.

2.3.3/ Polymerase chain reaction (PCR)

2.3.3.1/ Standard PCR

The standard PCR reaction was performed in 12.5µL or 25µL, depending on the final use of the PCR product. Water was used as a negative control in each reaction.

PCR products used as template for sequencing and DHPLC screening required a 25µL volume. A reaction volume of 20µL was set up in microwell plates and contained 0.4pmol forward and reverse primer, 1x buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 15mM $MgCl_2$, pH=8.7 at 20°C), 250µM of each dNTPs and 0.025 units of *Taq* DNA polymerase, and 5µL of DNA (10ng) were added.

A smaller volume was necessary for restriction enzyme digestions and microsatellite/LOH gels. The reaction volume was reduced to 7.5µL (1x buffer, 0.4pmol of each primers, 250µM of each dNTPs, 0.025U *Taq* DNA polymerase) and 5µL of DNA (10ng). Reactions were also set-up in microwell plates.

The usual PCR cycling parameters were:

94°C x 5min, (94°Cx30secs, AT x 30secs, 72°C x 30secs) x 35 cycles, 72°Cx5min, 4°C hold.

AT is the annealing temperature. The primers used in the DHPLC assay and their annealing temperatures and conditions are listed in Table 2.3. Primers and conditions used for the *NF1* LOH study are summarised in Table 2.4. Microsatellite primers and conditions in Table 2.5. Cycling parameters differed for the cDNA PCR (Section 2.3.3.2) and the methylation-specific PCR (Section 2.6.2).

Following the amplification reaction, PCR products were checked on a 2% agarose gel (Section 2.3.5) to ensure that the PCR had been successful.

Table 2.3: Primers and conditions for PCR in DHPLC screening.

AT, annealing temperature for PCR; *Tm*, melting temperature for fragment analysis, designed using the WaveMaker software. The first primer sets used in the study were published in Han *et al.*,(2001). However, a number of primer sets have been re-designed by Sian Griffiths during the course of the study, and the primers sets currently used are presented in this table.

Exon	Sequence (5' - 3')	Size (bp)	AT	Tm (°C)	Reference
1	CTCCACAGACCCTCTCCTTG	242	60	64 67.5	
	TCCCCTCACCTACTCTGTCC				
2	AAGCTGTTAACGTGTTTTTTTTTTC	228	56	54,5	Han *et al.* (2001)
	AAGAAAAGAAAGCAAATTCCCC				
3	TTTCACTTTTCAGATGTGTGTTG	244	60	54 55.5	Han *et al.* (2001)
	TGGTCCACATCTGTACTTTG				
4a	TTAAATCTAGGTGGTGTGT	517	54	54 58	Han *et al.* (2001)
	AAACTCATTTCTCTGGAG				
4b	GATACCACACCTGTCCCCTAA	227	58	54	
	CATGATACTAGTTTTTGACCCAGTG				
4c	TTTCCTAGCAGACAACTATCGA	308	58	54,5	Han *et al.* (2001)
	AGGATGCTAACAACAGCAAAT				
5	TGTTAGCATCCTGAATCAAAA	259	57	54,5	
	TCGTATCCTTACCAGCCATA				
6	AATGCCAGGGATTTTGTTCC	294	59	54.5 56.5 58	
	AAGCCTAAAGTAATACACACCTTGA				
7	GCTACATCTGGAATAGAAGAAACTTCA	399	58	56.5 59	
	CCATTTAGGCTGATGAACACA				
8	CATGTTAGTAAAGAAATACTGCATGG	249	54	56 58.5	
	TTTTGTTTATAAAGGATAACAGCATCA				
9	TTGAAGTTCGTTTCAAGACC	272	54	54	Han *et al.* (2001)
	ACGCAAAGAAAAGAAAGAAAA				
10a	ACGTAATTTTGTACTTTTTCTTCC	222	58	56.5 59	Han *et al.* (2001)
	CAATAGAAAGGAGGTGAGATTC				
10b	ATTATCCTGAGTCTTACGTC	229	54	51.5 54.5 57.5	Han *et al.* (2001)
	TAACTTAGTGTGATAATTTTGAGA				
10c	TCTTCCTCCTTCTAATCTCTCTCG	250	58	55 57 60.5	
	AAGGAACATCATGAATGTACATAGTTA				
11	CCAAAAATGTTTGAGTGAGTCT	256	60	52 55.5	Han *et al.* (2001)
	ACCATAAAACCTTTGGAAGTG				
12a	TGCATTAGGTTATTGATGATGC	299	58	54.3 56	
	TGAGAACATTGGGAGGAAGG				
12b	CTCTTGGTTGTCAGTGCTTC	261	58	54 57.5	Han *et al.* (2001)
	CAGAAAACAAACAGAGCACAT				
13	CACAGTTTATTGCATTGTTAGATTTT	385	58	57 59 61	
	CAGATGCCATGTGCTTTGAG				
14	GCTCTTCCTACTCCTTTTGG	191	58	60 61	Han *et al.* (2001)
	TTTCTGTTGCTAAGGGCATA				
15	TATGCCCTTAGCAACAGAAA	406	59	56	
	ACTTTACTGAGCGACTCTTGAA				
16	TGGATAAAGCATAATTTGTCAAGT	549	54	56.5 60	Han *et al.* (2001)
	TAGAGAAAGGTGAAAAATAAGAG				

Table 2.3: continued

Exon	Sequence (5' - 3')	Size (bp)	AT	Tm (°C)	Reference
17	GGTACGAGTGTCTGCGTATATCTG	495	58	58	
	CGAATTAAATGTAAGTTTGAAAACAA				
18	AGAAGTTGTGTACGTTCTTTTCT	367	58	54 55.5	Han *et al.* (2001)
	CTCCTTTCTACCAATAACCGC				
19a	TCATGTCACTTAGGTTATCTGG	242	54	57	Han *et al.* (2001)
	TAAAACCCACTAATACTTGAAGG				
19b	TGAGGGGAAGTGAAAGAACT	291	54	54.5 56.5 59.5	Han *et al.* (2001)
	TCCTATCCTAGTCCTGTCATGG				
20	CCACCCTGGCTGATTATCG	394	60	57 59.5 62.5	
	GCTTCTCTTACATGCCAGTTC				
21	TCAGCAAGGCCATGTTAGTA	284	58	54 58 59.5	
	CTTCCCCGCTTACTCTAATC				
22	TGCTACTCTTTAGCTTCCTAC	331	57	57 58	Han *et al.* (2001)
	CCTTAAAAGAAGACAATCAGCC				
23,1	TTTGTATCATTCATTTTGTGTGTA	282	56	56,5	Han *et al.* (2001)
	AAAAACAgCGGTTCTATGTGAAAAG				
23,2	CTTAATGTCTGTATAAGAGTCTC	268	54	53 56	Han *et al.* (2001)
	ACTTTAGATTAATAATGGTAATCTC				
24	TGACCTTTGAACTCTTTGTTTTCA	298	58	54 57.5 59.5	
	GAAAAGCTGAAAATTTAGTTGGAA				
25	CCTGTTTTATTGTGTAGATACTTCA	134	54	56	Han *et al.* (2001)
	TAAGTGGCAAGAAAATTACCT				
26	GTGTGAACAAGCCCTCCATA	374	59	55 56 58	
	GAAGATGCAAAGTAAAAAGCACT				
27a	ATGATTAGCACATTCACGGG	466	57	56.5 59	
	GCAAACTCTCCTTCTCAACC				
27b	TTTATTGTTTATCCAATTATAGACTT	296	54	54.4 55.5	
	TCCTGTTAAGTCAACTGGGAAAAAC				
28	AAA ATA AAA TTG ATT AGT GGC ATC TG	589	59	55 57.5	
	CTA GGG AGG CCA GGA TAT AG				
29	GGTTGGTTTCTGGAGCCTTT	469	58	57.5 59	
	AGCAACAAACCCCAAATCAA				
30	CAACTTCATTTGTGTTTTCTCCTAG	282	57	54 55	Han *et al.* (2001)
	CTTTGAATTCTCTTAGAATAATTGTTA				
31	TGATGTGATTTTCATTGACCA	300	58	54.5 58	
	CAGATAAATATGTGCACAAAGGAGA				
32	TGAATATACTCATCCTTTCCTGGAT	461	58	52.5 55.5 57.5	
	CATGGGACTCAAAGTTTTAGCA				
33	TCCTGCTTCTTTACAGGTTATT	348	60	56 59	Han *et al.* (2001)
	AAGTAAAATGGAGAAAGGAACTGG				
34	TTCAAAATGAAACATGGAACTTT	394	54	51.8 57.5 59.5	
	AAGTACAAAATAGCACAATAAACCAA				
35	GCATGGACTGTGTTATTGGTA	255	58	52,5	
	ATAACATTGATCATACAGTAATAGCTG				
36	ATGCATATTGTTGAAAATACAGCTA	282	59	54.5 58.5	
	TGCTTTACAACTTGAGAACCATAAA				

Table 2.3: continued

Exon	Sequence (5' - 3')	Size (bp)	AT	Tm (°C)	Reference
37	AATTCATTCCGAGATTCAGTTTAGGAG	235	58	54 56.5	
	AAGTAACATTCAACACTGATACCC				
38	AACTGCAGTGTGTTTTGAAAGAG	281	58	56 58.5 61	
	GCAACAAGAAAAGATGGAAGAG				
39	GAAAGCTACTGTGTGAACCTCATCAACC	285	62	55 57	
	GTAAGACATAAGGGCTAACTTACTTC				
40	TCAGGGAAGAAGACCTCAGGAGATGC	328	59	55,5	Han *et al.* (2001)
	TGAACTTTCTGCTCTGCCACGCAACC				
41	GTGCACATTTAACAGGTACTAT	373	62	55,5	Han *et al.* (2001)
	CTTCCTAGGCCATCTCTAGAT				
42	CTCTATTGTTTTCATCTTTCAGG	305	60	51 55 60	Han *et al.* (2001)
	CAAAAACTTTGCTACACTGACATGG				
43	TTTTCTTTTTAGTGTATTCCCATT	287	54	54.5 56	Han *et al.* (2001)
	GATTCTAAGAAATGGCTGGAA				
44	CACGTTAATTCCCTATCTTGC	268	60	58	Han *et al.* (2001)
	TGAGAAGTAGAAGACTGTATCC				
45	CATGAATAGGATACAGTCTTCTAC	269	60	56,5	Han *et al.* (2001)
	CACATTACTGGGTAAGCATTTAAC				
46	GAAATGCCCCAGAAAGTAAA	358	60	56 57	
	GTCAGTGCATTCTACAACAGC				
47	CTGTTACAATTAAAAGATACCTTGC	185	62	55,5	Han *et al.* (2001)
	TGTGTGTTCTTAAAGCAGGCATAC				
48	TTTTGGCTTCAGATGGGGATTTAC	351	58	55 57.5	Han *et al.* (2001)
	AAGGGAATTCCTAATGTTGGTGTC				
49	CTGGGAGAAACAGGCTATAC	363	58	58 59 61	Han *et al.* (2001)
	AGCAAGCTTCACACGATCT				

Marker	Location	Sequence (5' - 3')	AT	Size (bp)	Conditions	Reference
HHH202	pericentromeric	ATGAACAAGTCAAGGGACAGGCTT	63°C	A1 308	*Rsa*I, 2hx37°C	Ainsworth and Rodenhiser (1991)
	17q11.2 (D17S33)	ACTTGCCAAAGGTTACAGGGCTAC		A2 188 + 108		
D17S1824	17q11.2	ACTGAAAACTCACTCTTGTTCTGG	56°C	116-134	60W x 2h	Serra *et al.* (2001a)
		GATGTAAGTAGCATTGCCTCCC				
UT172	5' extragenic	GGTGAAAGAGCAAGACTCTGTCAC	52°C	100-120	60W x 1h30min	Shannon *et al.* (1994)
	17q12 (D17S635)	CCCCTTGATTGTAGGCACAGAAAC				
I4b	intron 4b	GCCTGGGTAACAGAGTGAGA	58°C	219	60W x 2h	Upadhyaya *et al.* (2003)
		CCGGTGCAGACTGAAAATA				
E5	exon 5	GAAGGAAGTTAGAAGTTTGTGACA	53°C	A1 220	*Rsa*I, 2hx37C	Hoffmeyer and Assum (1994)
		CAATCGTATCCTTACCAGCCAT		A2 120 + 100		
I12b	intron 12b	TTAATTGTATGCGGAGACACAC	54°C	368	60W x 4h	Unpublished
		TATTGCTGACAGAGGCAAAC				
J1J2	intron 27b	CAAGAAAAGCTAATATCGGC	54°C	404	60W x 4h	Xu *et al.* (1991)
		GGAACCTTAAGTTCACTTAG				
I27.13	intron 27b	CAGGTTTTGTGGTTTTGTTGC	67°C	377	60W x 4h	Upadhyaya *et al.* (1997)
		CACCCAAGGTCAGGAGTAGG				
EVI20	intron 27b	TAACAATTGTGGAACTGCAGCAATTATT	55°C	203	60W x 2h	Shannon *et al.* (1994)
		CCCATACCTAGTTCTTAAAGTCTG				
I38	intron 38	CAGAGCAAGACCCTGTCT	54°C	187	60W x 2h	Lazaro *et al.* (1993)
		CTCCTAACATTTATTAACCTTA				
I41	intron 41	GATTGCTGTTGTTAGGAAATAGGAC	57°C	A1 424	*Pac*I, 2hx37C	Shen and Upadhyaya (1994)
		TGCTTAACTGATCTATCAAACTCTC		A2 73 + 351		
C7CT	3'UTR	GGCTGGCACTCTGTCTCCTC	54°C	240	*Taq*I, 2hx65C	Cowley *et al.* (1998)
		CTGACTTGTTAAAGAGGAAAC		261		
EW206	3' extragenic	TGCAGTGTGGTGCATCATTCAGTG	60°C	349	*Msp*I, ON, 37°C	Rodenhiser *et al.* (1993)
	(D17S57)	GACAGGGCCAGCCATATTCCTGAT				
EW207	3' extragenic	AGGTATCAGTCAGGACCCTCTTAG	60°C	A1 587	*Hind*III, ON, RT	Rodenhiser *et al.* (1993)
	(D17S73)	CTGACACTCTGGTTTCTGTAATGTG		A2 390 + 192		
D17S802	telomeric	GCCACCTGCCCCTCAA	57°C	166-188	60W x 2h	Serra *et al.* (2001a)
	17q25.3	CTGCCAGCAGAGGCCA				

Table 2.4 : Sequences and conditions of primers used in the17q NF1 intragenic and extragenic LOH assay.

ON, overnight; *AT*, annealing temperature.

Table 2.5: Primers and condition for the microsatellite markers used in the microsatellite instability (MSI) assay, *CDKN2A* LOH assay and *TP53* LOH assay.

Marker	Location	Sequence (5' - 3')	AT	Size (bp)	Reference
BAT26	2p	TGACTACTTTTGACTTCAGCC	58°C	80-100	Papadopoulos *et al.* (1994)
(MSI)	(*MSH2*)	AACCATTCAACATTTTTAACCC			
D2S123	2p16	AAACAGGATGCCTGCCTTTA	60°C	197-227	Weissenbach *et al.* (1992)
(MSI)		GGACTTTCCACCTATGGGAC			
MYCL	1p32	TGGCGAGACTCCATCAAG	53°C	140-209	Makela *et al.* (1992)
(MSI)		CTTTTTAAGCTGCAACAATTTC			
APC	5q21/22	ACTCACTCTAGTGATAAATCG	55°C	96-122	Spirio *et al.* (1991)
(MSI)	(D5S346)	AGCAGATAAGACAGTATTACTAGTT			
Mfd15	17q11.2-q12	GGAAGAATCAAATAGACAAT	52°C	151	Weber *et al.* (1990)
(MSI)	(D17S250)	GCTGGCCATATATATATTTAAACC			
D18S58	18q22.3	GCTCCCGGCTGGTTTT	53°C	144-160	Dib *et al.* (1996)
(MSI)		GCAGGAAATCGCAGGAACTT			
p53Alu	17p13.1	GCACTTTCCTCAACTCTACA	55°C	120	Futreal *et al.* (1991)
(*TP53*)		AACAGCTCCTTTAATGGCAG			
D9S304	9p13	GTGCACCTCTACACCCAGAC	55°C	135-175	Hartmann *et al.* (2000)
(*CDKN2A*)		TGTGCCCACACACATCTATC			
D9S1748	9p21	CACCTCAGAAGTCAGTGAGT	56°C	130	Cairns *et al.* (1995)
(*CDKN2A*)		GTGCTTGAAATACACCTTTCC			
D9S942	9p21	GCAAGATTCCAAACAGTA	53°C	100	Welch *et al.* (2001)
(*CDKN2A*)		CTCATCCTGCGGAAACCATT			
D9S1751	9p21	TTGTTGATTCTGCCTTCAAAGTCTTTTAAC	54°C	150	Cairns *et al.* (1995)
(*CDKN2A*)		CGTTAAGTCCTCTATTACACAGAG			

Table 2.6: Primers and conditions for amplification of *NF1* cDNA fragments.

Primer	Sequence (5' - 3')	Size (bp)	Location	Exons	AT1	AT2
NF1 cDNA 1	aacactgggagcctgcac	425bp	77-94	1, 2, 3	58°C	55°C
	gttgcccagcaagacatttt		482-501			
NF1 cDNA 2	aagcggcctcactactattt	401bp	376-395	3, 4a, 4b	63°C	55°C
	ttgcacaatccacattgata		776-757			
NF1 cDNA 3	caggaattaactgtttgttcaga	395bp	695-717	4b, 5, 6	63°C	55°C
	ttgttttcatcaaccacgtc		1089-1070			
NF1 cDNA 4	ctgaaagcaccaaacgtaaa	412bp	981-1000	6, 7, 8, 9	63°C	55°C
	aagtgttggttgttgtgagg		1392-1373			
NF1 cDNA 5	tgattgactgccttgtttct	419bp	1338-1357	9, 10a, 10b, 10c	63°C	55°C
	cccctgttttcttggattac		1756 -1737			
NF1 cDNA 6	tctcttgtccatggtgaaac	493bp	1687- 1706	10c, 11, 12a, 12b	63°C	55°C
	ggctccaggagtacgtagtaa		2179- 2159			
NF1 cDNA 7	ggaaataccagtcaaatgtcc	428bp	2123-2143	12b, 13, 14, 15	63°C	55°C
	ttagctttgttgcttgttcc		2582- 2563			
NF1 cDNA 8	cactgaggcttgggaagata	449bp	2530-2549	15, 16	63°C	55°C
	gacccaccagatccttaaca		2978- 2959			
NF1 cDNA 9	ggctgttgtccttaatggtg	540bp	2901-2920	16, 17, 18, 19a	63°C	57°C
	ccataacccagtctgtcagg		3362-3343			
NF1 cDNA 10	gccaagagatgaaatttagg	395bp	3306-3325	19a, 19b, 20	63°C	55°C
	gtgcatgagaccactgtcta		3700-3681			
NF1 cDNA 11	ggtccttgcaatgtcaaac	477bp	3646-3664	20, 21, 22, 23.1	63°C	55°C
	aaaggatccaggagtttttg		4122-4103			
NF1 cDNA 12	agactctcttccgaggcaac	392bp	4026-4045	23.1, 23.2, 23a, 24	63°C	55°C
	ttcatacggtgagacaatgg		4417-4398			
NF1 cDNA 13	cagtaggaagtgccatgttc	402bp	4359-4378	24, 25, 26, 27a	63°C	55°C
	tatcaaaaggtcgtcttcca		4760-4741			
NF1 cDNA 14	caacagggatcataaagctg	393bp	4720-4739	27a, 27b, 28	62°C	53°C
	tttaaagcgattgctaggc		5113-5095			
NF1 cDNA 15	aaagccatattatgcaaagc	396bp	5041-5060	28, 29	63°C	57°C
	tggacagcagtagaaccaac		5436-5417			
NF1 cDNA 16	gctctcaagctagctcacaa	454bp	5375-5394	29, 30	63°C	55°C
	ctagtaactggccctcgatt		5828-5809			
NF1 cDNA 17	tctgtgtgccttaacttgtacc	428bp	5776-5797	30, 31, 32	63°C	55°C
	cactggttttgatgaaactgtc		6203-6182			
NF1 cDNA 18	aaaatatggggaagccttgg	376bp	6131-6150	32, 33	63°C	57°C
	ctctaagggagagcggacct		6506-6487			
NF1 cDNA 19	tgatgtggcagctcatcttc	365bp	6430-6449	33, 34, 35	63°C	55°C
	atgcctccatgatctccaac		6794-6775			
NF1 cDNA 20	tgacatccttggaaacagtc	416bp	6744-6763	35, 36, 37, 38	62°C	53°C
	aagtgcggtacctgctgaat		7159-7140			
NF1 cDNA 21	ctcttttgggtagctgtgg	416bp	7091-7109	38, 39, 40, 41	63°C	55°C
	cagcgacttcgaacttctt		7506- 7488			
NF1 cDNA 22	tggcctacttagcagcttta	413bp	7455-7474	41, 42, 43	63°C	55°C
	tgctactctcctcattttgg		7867- 7848			
NF1 cDNA 23	aaaaggcaagaaatggaatc	515bp	7811-7830	43, 44, 45, 46, 47	63°C	55°C
	tctggaatttgtgtttgctt		8325-8306			
NF1 cDNA 24	tttggttttaatggcttgtg	468bp	8264-8283	47, 48, 49	62°C	53°C
	aaccggatgggttcattat		8797-8779			

Gene	M/UM	Sequence (5' - 3')	Size (bp)	Conditions	Reference
RASSF1	M	F - CGAGAGCGCGTTTAGTTTCGTT	194	95°C x 15m, (95°C x 30s, 59°C x 40s, 74°C x 30s)x 35cy, 74°C x 5m	Hesson *et al.* (2004)
		R - CGATTAAACCCGTACTTCGCTAA			
	UM*	F - GGGGGTTTTGTGAGAGTGTGTTT	204	95°C x 15m, (95°C x 30s, 60°C x 40s, 74°C x 30s)x 35cy, 74°C x 5m	
		R - CCCAATTAAACCCATACTTCACTAA			
NORE1A	M*	F - CGT CGT TTG GTA CGG ATT TTA TTT TTT TCG GTT CG	204	95°C x 15m, (95°C x 30s, 62°C x 1m, 74°C x 30s)x 6cy touchdown,	Hesson *et al.* (2003)
		R - CGA CAA CTT TAA CTT CGA CGA CTT TAA CGA CTA CG		(95°C x 30s, 60°C x 1m, 74°C x 30s)x 30cy, 74°C x 5m	
	UM*	F - ATT TAT ATT TGT GTA GAT GTT GTT TGG TAT	190	as above	
		R - ACT TTA ACA ACA ACA ACT TTA ACA ACT ACA			
RB1	M	F - GGGAGTTTCGCGGACGTGAC	172	95°C x 15m, (95°C x 30s, 56°C x 1m, 74°C x 30s)x 37cy, 74°C x 5m	Gonzalez-Gomez *et al.* (2003)
		R - ACGTCGAAACACGCCCCG			
	UM	F - GGGAGTTTTGTGGATGTGAT	172	as above	
		R - ACATCAAAACACACCCCA			
CDKN2A	M	F - TTA TTA GAG GGT GGG GCG GAT CGC	151	95°C x 15m, (95°C x 30s, 62°C x 1m, 74°C x 30s)x 6cy touchdown,	Fang *et al.* (2003)
		R - GAC CCC GAA CCG CGA CCG TAA		(95°C x 30s, 61°C x 1m, 74°C x 30s)x 30cy, 74°C x 5m	
	UM*	F - TTA TTA GAG GGT GGG GTG GAT TGT	151	95°C x 15m, (95°C x 30s, 62°C x 1m, 74°C x 30s)x 6cy touchdown,	
		R - CAA CCC CAA ACC ACA ACC ATA A		(95°C x 30s, 59°C x 1m, 74°C x 30s)x 30cy, 74°C x 5m	
MLH1	M*	F - ACG TAG ACG TTT TAT TAG GGT CGC	120	95°C x 15m, (95°C x 30s, 62°C x 30s, 74°C x 30s)x 6cy touchdown,	Herman *et al.* (1998)
		R - CCT CAT CGT AAC TAC CCG CG		(95°C x 30s, 59°C x 30s, 74°C x 30s)x 34cy, 74°C x 5m	
	UM*	F - TTT TGA TGT AGA TGT TTT ATT AGG GTT GT	129	as above	
		R - ACC ACC TCA TCA TAA CTA CCC ACA			
MSH2	M*	F - TCG TGG TCG GAC GTC GTTC	132	95°C x 15m, (95°C x 30s, 60°C x 30s, 74°C x 30s)x 6cy touchdown,	Herman *et al.* (1998)
		R - CAA CGT CTC CTT CGA CTA CAC CG		(95°C x 30s, 58°C x 30s, 74°C x 30s)x 30cy, 74°C x 5m	
	UM*	F - GGT TGT TGT GGT TGG ATG TTG TTT	143	as above	
		R - CAA CTA CAA CAT CTC CTT CAA CTA CAC CA			
MGMT	M	F - CGG ATA TGT TGG GAT AGT TCGC	122	95°C x 15m, (95°C x 30s, 62°C x 40s, 74°C x 30s)x 10cy touchdown,	modified from
		R - GCA CTC TTC CGA AAA CGA AAC G		(95°C x 30s, 58°C x 40s, 74°C x 30s)x 30cy, 74°C x 5m	Esteller *et al.* (1999)
	UM*	F - GTG TTT TGG ATA TGT TGG GAT AGT TTGT	134	95°C x 15m, (95°C x 30s, 64°C x 40s, 74°C x 30s)x 10cy touchdown,	
		R - AAC TCC ACA CTC TTC CAA AAA CAA AACA		(95°C x 30s, 60°C x 50s, 74°C x 30s)x 28cy, 74°C x 5m	
RARB	M	F - TCG AGA ACG CGA GCG ATT CG	146	95°C x 15m, (95°C x 30s, 60°C x 40s, 74°C x 30s)x 10cy touchdown,	Virmani *et al.* (2000)
		R - GAC CAA TCC AAC CGA AAC GA		(95°C x 30s, 57°C x 40s, 74°C x 30s)x 30cy, 74°C x 5m	
	UM	F - TTG AGA ATG TGA GTG ATT TGA	146	as above	
		R - AAC CAA TCC AAC CAA AAC AA			

Table 2.7: Primers and condition for the methylation-specific PCR assay.
M, methylated sequence; *UM*, unmethylated sequence; *cy*, cycle.

2.3.3.2/ cDNA PCR

The PCR reaction was performed in a 20μL reaction volume as in 2.3.3.1, to which was added 1-2μL of cDNA (obtained from 2.3.4). To prevent fluctuation in heating time and block temperature, all reactions were performed on the same ABI thermocycler (Applied Biosystems, Warrington, UK).) Water was used as a negative control in each reaction.

The PCR cycling parameters, for all fragments except fragment 1 were: 94°Cx5min, (94°Cx30secs, *AT1x1min, 72°Cx1min) x 16 cycles, (94°Cx30secs, AT2x1min, 72°Cx1min) x 20 cycles, 72°Cx5min, 4°C hold

Fragment 1 cycling parameters were: 94°Cx5min, (94°Cx45secs, *AT1x45secs, 72°Cx1min) x 6 cycles, (94°Cx45secs, AT2x45secs, 72°Cx1min) x 32 cycles, 72°Cx10min, 4°C hold.

* indicates the use of a touchdown step where the annealing temperature is reduced by 0.5°C at each cycle. The annealing temperatures (AT1 & AT2), primers and fragments amplified are summarised in Table 2.6.

2.3.4/ Reverse-transcription (RT)-PCR

250ng total RNA was used in a 20μL total reaction volume. A first mix containing the RNA, random primers (0.5 g/ L), dNTPs (5mM) and purified water was heated up at 65°C for 5 min then placed on ice for 1 min. The second mix composed of 200 units Superscript II, 5x first strand buffer (250mM Tris-HCl, pH=8.3 at room temperature, 375mM KCl, 15mM $MgCl_2$), 0.1M DTT and 1μL RNAsein (ribonuclease inhibitor) was then added to the tube. The samples were left at 50°C for 1 hour, followed by a 70°C x 15 min step, on an ABI thermocycler. The concentration of the resulting cDNA was assessed using a spectrophotometer, then stored at –20°C for later use.

2.3.5/ Agarose and NuSieve agarose gel

Agarose gels were used to check PCR products. Agarose was melted in 1x TAE (40mM Tris-acetic acid [pH 8], 2Mm EDTA). Ethidium bromide (5μL) was added before the gel was poured into a horizontal gel apparatus and allowed to set. The gel was then submerged in an electrophoresis tank containing 1x TAE. A drop of loading dye (5g ficol, bromophenol blue, xylene cyanol) was added to 2μL PCR product, before being loaded on the gel. The gel was

then run at 100W for 10-20 min. The bands were visualized with UV light on a GelDoc2000 (Biorad, Hempstead, UK) fitted with a camera.

2.3.6/ PCR product purification

2.3.6.1/ *ExoI*/SAP system

Shrimp alkaline phophatase (SAP) dephosphorylates residual deoxynucleotides, preventing them from participating in the sequencing reaction, while Exonuclease I degrades unincorporated primers. A volume of 2μL "gene clean" mix (2 units *Exo*I, 0.2 units SAP) was added to 10μL PCR product and incubated at 37°C for 30 min. The enzyme reaction was then stopped by incubation at 80°C for 2 min.

2.3.6.2/ QIAquick PCR purification kit

The QIAquick kit relies on a silica-gel membrane assembly that binds DNA (100bp – 10kb) in high-salt buffer and elutes with low-salt buffer or water. It removes all primers, enzymes and impurities from the DNA. This purification kit from QIAGEN was used according to the manufacturer's instructions. Briefly, 100μL PB buffer was mixed with 20μL PCR product and applied to a QIAquick column, then centrifuged at 13000rpm for 1 min. The flow-through was discarded and 750μL of PE buffer was added to the column. This step was followed by a centrifugation at 13000rpm for 1 min, repeated once. The DNA was then eluted from the column using 50μL H_2O, left to stand for 1 min then centrifuged for 1 min at 13000rpm.

2.3.6.3/ Montage system

The Montage PCR96 Plates system was used for purification of the PCR product. 100μL purified water was added to the 22-25μL PCR product and transferred to a Montage plate by pippetting. A vacuum of 15mmHg was applied for 10 min using a Millipore vacuum pump, until wells emptied. 50μL of purified water was then added and the plate was left to stand on the bench for 10 min. The plate was then put on a Vortex Genie 2 (from Scientific Industries, New York, USA), fitted with a plate shaker, for 10 min to re-suspend the purified product. The solution was transferred to a new microwell plate and stored at –20°C for later use.

2.4 *NF1* mutation detection techniques

2.4.1/ Deletion junction-specific PCR assay

This PCR-based assay relies on the amplification of a 3.4kb chimeric fragment and uses primers specific for NF1REP-P (forward) and NF1REP-M (reverse) (Lopez-Correa *et al.*, 2001). The protocol described by Lopez-Correa and co-workers was followed and the PCR reaction executed on the ABI thermocycler (Applied Biosystems, Warrington, UK).). Briefly, a 25μL reaction was performed using the Expand Long Template PCR system with 300ng DNA, 15pmoles each primer, 0.35mM dNTPs, 10x PCR buffer 1 and 2.5U *Taq* DNA polymerase. The PCR cycling parameters were:

94°C x 3min, (94°C x 30secs, 68°C x 2.5min) x 35 cycles, 68°C x 7min, 4°C hold.

The PCR products were visualised on a 1% agarose gel. Two samples from patients with known 1.5Mb microdeletions were used as positive control in each reaction.

2.4.2/ Restriction enzyme digestion assay

For a 12.5μL PCR (as in 2.3.3.1), the reaction volume was set up as: 1U restriction enzyme, 2μL corresponding buffer and 0.2μL BSA. Reactions were left to incubate at the recommended temperature (37°C or 60°C) for 2 hours to overnight, on an ABI (Applied Biosystems, Warrington, UK) or MWG (Ebersberg, Germany) thermocycler. Incubation conditions, along with the primers used, can be found in Table 2.2.

2.4.3/ Large gel electrophoresis and silver staining

Large glass plates were thoroughly cleaned and set with fine tooth combs and spacers, before denaturing 6% polyacrylamide gels (60mL SequaGel 6, 15mL SequaGel Complete, 550μL ammonium persulfate solution) was poured and left to set for 1-2 hours. The plates were mounted on a vertical electrophoresis apparatus and TBE buffer was poured in the upper and lower tanks. The gel was preheated for 30min at 65W, while samples were prepared. The PCR products were denatured at 80C for 3.5 min with 4 μL dye solution (2mL TBE 10x, 8mL formamide, xylene blue) prior to loading on the gel. The running time of each marker is summarised in Table 2.2. The bands were finally revealed using a silver nitrate solution (350mL H_2O, 0.3 silver nitrate) and developer (700mL H_2O, 0.07g $NaBH_4$, 10g NaOH, 2250μL formaldehyde).The reaction was stopped and the bands fixed using sodium

carbonate solution (700mL H_2O, 5.24g sodium carbonate anhydrous). The gels were lifted onto Whatmann 3MM paper, covered with cling film and left to dry.

2.4.4/ DHPLC/ cDNA-DHPLC

The PCR reactions were performed in a 25μL volume and at the cycling conditions previously described (2.3.3.1 and 2.3.3.2). An additional step was required for the formation of heteroduplexes before the mutation screening was carried out. The parameters were: 8°C x 5min, *25°C x 1min. (* denotes a 0.2°C ramp).

The plate was then loaded on the machine, and the automated protocol was implemented. The melting temperatures (up to three) for each fragment are summarized in Tables 2.3 and 2.6. The principles of the technique was described in Section 1.7.4.

2.4.5/ Direct sequencing

2.4.5.1/ Big Dye reaction

The Big Dye reaction was set up in a microwell plate to a final reaction volume of 10μL: 0.75μL of Big Dye version 3.1, 1.5μL of buffer, 1.6μL primer forward or reverse and 6.15μL purified PCR product. The big dye cycling reaction was performed on an ABI (Applied Biosystems, Warrington, UK) or MWG (Ebersberg, Germany) thermocycler to these conditions: (96°C x 30secs, 50°C x 15secs, 60°C x 4min) x 30 cycles.

2.4.4.2/ Big Dye purification

The various methods are listed here in chronological order, from the earliest to the most recent.

- Isopropanol purification

The sequencing product was mixed with 80μL 75% isopropanol and left to incubate at room temperature for 15 to 30 min. The mix was then centrifuged at 4000rpm for 30 min. The supernatant was removed by gentle tapping of the plate on paper, and the pellet was re-suspended in 250μL of 70% isopropanol. A final centrifugation step at 2000rpm for 1 min was conducted with the plate upside down to finish the clean-up step. A volume of 10μL HiDi formamide was then added to the samples.

- Montage system

Big Dye purification was performed using the Montage SEQ96 plate system. 20μL of injection solution (from the Montage kit) was mixed to 10μL big dye product by pipetting and transferred to a Montage plate. A vacuum of 20Hg was applied for 3-4 min using a Millipore vacuum pump. An additional 20μL injection solution was pipetted to the plate, then a vacuum was applied for 3 min at 20mm Hg. 20μL injection solution was added to the wells and the plate was put on a shaker for 10 min. The purified product was transferred to a new microwell plate and 10μL HiDi formamide was added to each well.

2.5 Microsatellite instability and *MLH1* LOH analysis

2.5.1/ MSI

Evidence for genome-wide MSI was though using 6 polymorphic microsatellite markers. The location, type of repeat, primers and PCR conditions are summarised in Table 2.3.

2.5.2/ *MLH1* gene LOH assay

Loss of heterozygosity in *MLH1* was assessed using marker D3S1611, a CA repeat located in intron 12 of the *MLH1* gene (Annese *et al.*, 2002). The primer sequences were: forward 5'-CCC CAA GGC TGC ACT T-3'; reverse 5'- AGC TGA GAC TAC AGG CAT TTG-3' and yielded a PCR product ranging from 252-268bp. The PCR conditions were optimised to:
94°C x 5min, (94°C x 30secs, 57°C x 30secs, 72°C x 30secs) x 35 cycles, 72°C x 10min. The PCR products were visualised on a 6% polyacrylamide gel as described previously (Section 2.4.3) and required 2h30min at 60W.

2.6 Methylation analysis

2.6.1/ Bisulphite conversion

2.6.1.1/ Controls

A DNA sample from an unaffected individual was used in each reaction as a control to ensure that the primers designed to amplify bisulphate-converted DNA did not also amplify unconverted genomic DNA. The same sample was also methylated using a methylase (*Msss*I): 5 μg of sample was incubated with 10units/μL of *Msss*I, at 37°C overnight. The methylated

DNA was then subjected to bisulphite conversion (Section 2.6.1.2) and used as a control for amplification with the methylated primers (Section 2.6.2). Low-melting point agarose (LMA) alone was used as a negative control for the bisulphite conversion step.

2.6.1.2/ Bead method

Each solution was prepared on the day. The final bisulphite solution consisting of 5mL sodium bisulphite (7.2M), 1mL hydroquinone (1M), 1.5mL sodium hydroxyde (2M) and 0.45g urea (1M) was mixed gently by tube inversion and kept under light exclusion at 65°C. DNA was denatured at 95°C for a few minutes and mixed with 2% low melting point agarose (LMA) to a final concentration of 10ng/μL. A bead was formed by pippetting 80μL of the mix into pre-chilled mineral oil. The bead was placed in a new eppendorf tube and 800μL of the bisulphite solution was added, followed by an overlay of mineral oil. The tubes were incubated at 50°C for 4 hours.

2.6.1.3/ Gel purification

Gel purification of the bisulphite-converted samples was conducted after manufacturer's instruction (Qiagen Quick Gel extraction kit). Briefly, the bead was recovered and placed in a new Eppendorf tube; 3 volumes of buffer QG were added and the tube was incubated at 50°C until complete dissolution of the gel. One volume of 100% isopropanol was added to the tube before the mix was transferred to a QIAquick column and centrifugated at maximum speed for 1 min. Flow-through was discarded and the column was washed with PE buffer and two 1-min centrifugations at maximum speed. Finally, 40μL EB buffer was pipetted onto the column and left to stand for 1 min before the final 1 min centrifugation step to recover the product. Converted DNA was stored at –20°C.

2.6.2/ Methylation-specific (MS) PCR

The 20μL PCR reaction master mix was set-up as described in Section 2.3.3.1 in a microwell plate containing 2μL converted DNA (Section 2.6.1.3), unless marked with a * in Table 2.7. Primer with a * were used at ½ that normally used in the master mix (0.5pM instead of 1pM). The PCR conditions were optimised for use in the NF1 lab. All PCR reactions were subjected

to a hotstart step using HotStart *Taq* polymerase and a number of primer sets required a touch-down step for optimal results.

Primers and conditions are summarised in Table 2.7. Each PCR reaction had a positive control (*MsssI* sample) and 2 negative controls (LMA and water). To prevent fluctuations in heating time and block temperature, all reactions were performed on the same ABI thermocycler. The PCR products were visualised on 3% Nusieve gel at 80W x 30 min.

2.7 Real-time PCR analysis

Expression levels of genes also tested for hypermethylation were assessed using the real-time PCR technology. This approach relies on the fluorescent detection of SybrGreen dye-amplicon complex at a threshold value during amplification. Quantification is based on the point during cycling where the amplified PCR is first detected, rather than the product accumulated at the end of the reaction. The more a gene is expressed, the more the corresponding cDNA will be present at the beginning of the PCR, and the earlier the fluorescence will be detected. The increased fluorescence, proportional to the amplified PCR product is detected in the 7500 real-time PCR system. In this study, the 'housekeeping gene' *GAPDH* was used as an endogenous control. Sequences for the target genes' cDNA were retrieved from LocusLink and primers were designed using the Taqman probe and primer design supplied on the 7500 real-time PCR system (Table 2.8).

Gene	Sequence (5' - 3')	Location*
GAPDH	CCACCCATGGCAAATTCC	(+) 152bp
	TGGGATTTCCATTGATGACAAG	(+) 220bp
RB1	CCCCTACCTTGTCACCAATACC	(+) 2327bp
	CCAGGAATCCGTAAGGGTGAA	(+) 2402bp
MLH1	TTAATGAGCAGGGACATGAGGTT	(+) 1538bp
	TCACACAGCCCACGAAGGA	(+) 1603bp
MSH2	GCTTCGTGCGCTTCTTTCAG	(+) 53bp
	GGTCGAAAAGGCGCACTGT	(+) 115bp
CDKN2A	GCCCAACGCACCGAATAGT	(+) 111bp
	CGCTGCCCATCATCATGAC	(+) 169bp

Table 2.8: Real-time PCR primer sequences.

*Location is given relative to the ATG transcription start site on the cDNA sequence.

Reverse transcription was performed as previously described (Section 2.3.4) and the resulting cDNA concentration was assessed using a spectrophotometer. The cDNA concentration was diluted to a 50ng/μL stock solution for use in the PCR reaction. A reaction mix of 12.5μL was set up in a microwell plate and contained 6.25μL of SybrGreen, 1.25μL primers (9pM) and 0.5μL cDNA. All reactions were set up in triplicate. Results were visualised using the 7500 system software.

2.8 Schwann cell culture

2.8.1/ Dissociating cells from tumour

All media and solutions were made fresh on the day and filtered with a 0.2 M filter. The neurofibromas, received in tissue culture medium, were first separated from the surrounding skin and capsule, then washed in DMEM + 10% FCS. The tumours were then manually dissected to obtain a small piece of material from the heart of the tumour. The tumour fragment was put to incubate at 37°C and 10% CO_2 in pre-incubation medium (DMEM+10%FCS, Penstrep 1x, forskolin 2 M) for 10-14 days. The medium was changed to a fresh one every 3 days. The tissue was then transferred to a new plate with incubation medium (DMEM+10%FCS, Penstrep 1x, collagenase 160u/mL, dispase I 0.8u/mL) overnight at 37°C. On the following day, the tissue piece was completely dissolved by trituration, before being transferred to a coated plate.

2.8.2/ Schwann cell culture

2.8.2.1/ Coating a plate

Plates were first coated with poly-L-lysine (0.1mg/mL) and left for 30-60min at room temperature. The plates were then washed in PBS. The second coating was laminin (4 g/mL) at 37°C for 1 hour. The plates were then washed in PBS before receiving the tumour sample.

2.8.2.2/ Culture

After the cells being plated or freshly split, the media were changed 3 times on the first 3 days: Schwann cell medium with forskolin (DMEM+10%FCS, Penstrep 1x, IBMX 0.5mM, 10nM heregulin, 0.5 M forskolin, 2.5 g/mL insulin) on day 1, N2 medium (DMEM F12, Pentrep 1x, N2 supplement 1x) on day 2, Schwann cell medium without forskolin on day 3

(DMEM+10%FCS, Penstrep 1x, IBMX 0.5mM, 10nM heregulin, 2.5 g/mL insulin). After the first 3 days, the medium was changed every 3 days to fresh Schwann cell medium without forskolin. The essential culture mitogen heregulin is a synthetic peptide of 71 amino acids that contains the epithelial growth factor (EGF) homology domain of human heregulin β1 (common to all β-neuregulins). To split the cells, Schwann cells were detached from the support flask or well using first 1mL undiluted versene (a few seconds), followed by a PBS wash, then 2mL trypsin was added (a few minutes at 37°C). The trypsinized cells were then transferred to 10mL of DMEM+10%FCS and centrifugated for 7-10 min at 1200rpm.

2.8.3/Freezing cells dissociated from tissue or grown in culture

Cell from primary tumours were mechanically dissociated after incubation in dissociation medium, as described above. The cells and medium were then transferred to 10mL of DMEM+10%FCS and centrifugated for 7-10 min at 1200rpm. Cultured cells were first detached from the support using versene and trypsin and transferred to 10mL of DMEM+10%FCS and centrifugated for 7-10 min at 1200rpm. In both instances, the resulting pellet was re-suspended in freeze medium (DMEM+10%FCS, FCS 2x, DMSO 1x) and snap frozen on dry ice, to be later stored in liquid nitrogen.

2.8.4/ Schwann cell staining

An aliquot of cultured cells, trypsinized and pelleted, was plated on a Lab-Tek 8-wells slide and left to grow for a week, using the technique described in Section 2.8.2.2. At 70% confluence, the slide was washed in PBS and the cells were fixed for 30 min in formalin (provided by the cytogenetics department) at room temperature. The slide was washed in PBS before the cells were blocked with peroxyde (50μL/well) for 20 min in a humid chamber. At the same time, the ABC kit and solutions were prepared. The slide was then washed in PBS for 5 min, twice, and drained off. The primary antibody solution, S100, specific to the SC, was pipetted into the wells and left for 30 min in the humid chamber. At this stage, two different concentrations of antibody were used, 1/1000 and 1/5000. Concentration 1/5000 was found to yield better results. Examples of results are shown in Figure 2.1. A control was also introduced by using a solution of PBS Only. The slide was then washed in PBS for 5 min, twice, and incubated with the biotinylated secondary antibody (mouse anti-rabbit antibody) for 30 min in the humid chamber. ABC relies on a complex of avidin/biotinylated enzyme

(peroxydase in this kit), where the glycoprotein avidin binds strongly to the biotinylated antibody. The slide was washed in PBS for 5 min, twice, and incubated with the ABC solution for 30 min in the humid chamber. The slide was washed in PBS for 5 min, twice, and DAB (5mL dH_2O, 2 drops of buffer, 4 drops of DAB and 2 drops H_2O_2) was added for 10 min. The slide was washed in tap water for 5 min, twice, and counterstained in Gills for 30 secs then blued in tap water. A water-based mountant was used to fix a cover slide.

Figure 2.1: Representation of S100 immunostaining on Schwann cells.

A: S100 immunostaining of SC from neurofibroma T181.1 SC (stained brown) grew on a layer of fibroblasts (larger in shape and stained blue)

B: S100 immunostaining of SC from neurofibroma T183.1 Negative control: a solution of PBS was used in place of the S100 antibody dilution.

C: S100 immunostaining of SC from neurofibroma T183.1

CHAPTER 3: METHYLATION ANALYSIS OF CANDIDATE GENES IN NF1-RELATED TUMOURS

3.1 Introduction

In the human genome, the main epigenetic modification is the methylation of cytosine located in CpG dinucleotides. During evolution, CpG dinucleotides have been depleted to a lower frequency (four-fold less) than expected. Approximately 3-4% of cytosines are methylated in the human genome, in tissue-specific patterns (Esteller and Herman, 2002). However, there are also dense CpG dinucleotide regions, termed CpG islands (Bird, 1986). These are located in the proximal promoter regions of almost one half of all human genes, and tend to be unmethylated in normal somatic tissues, with a few exceptions such as X chromosome inactivation and imprinted genes (Baylin and Herman, 2000; Esteller and Herman, 2002).
The mechanisms underlying gene silencing by hypermethylation of promoter CpG islands is not completely understood. It has been proposed that the methylated sequence could exhibit reduced affinity for transcription factors or that steric blocking by methyl-CpG binding proteins (MDBP) could occur in a sequence-independent manner. The MDBP would not only act as a transcriptional repressor, but would also recruit protein complexes involving histone deacetylases. This model would therefore involve modifications in the dynamic structure of the chromatin, in conjunction with methylation (Jones and Laird, 1999).

In cancer, the presence of CpG islands in the promoters of tumour suppressor (TS) genes offers a possibility for gene silencing by hypermethylation (Jones and Laird, 1999). In contrast to mutational inactivation, *de-novo* methylation is thought to be a progressive event (Esteller, 2005). As reviewed in Section 1.5.2, the mechanisms involved are unclear, and could involve the "leaking" of methylation from the hypermethylated heterochromatin into the unmethylated euchromatin of the CpG islands, as well as methylation centres (Baylin, 2002; Esteller, 2005).

The past decade has seen the recognition of the importance of epigenetic modification in cancer, as attested by the growing list of TS genes silenced by hypermethylation in various tumours. Virtually all cellular pathways are targeted (Esteller, 2005). Moreover, genetics and epigenetics are thought to interact at the onset and during the evolution of cancer. Hypermethylation not only silences genes, but can also create a permissive background for

genetic damage (for example, when a mutator gene like *MLH1* is hypermethylated), and it has been proposed that epigenetics be included in the definition of Knudson's "two hit" hypothesis (Jones and Laird, 1999).

Recently, several groups have endeavoured to map hypermethylation patterns of TS genes in specific cancers. The results revealed tumour-specific gene inactivation and pathway disruption (Ueki *et al.*, 2000; Esteller *et al.*, 2001b; Morris *et al.*, 2003; Kuroki *et al.*, 2003a; Mehrotra *et al.*, 2004).

In NF1 tumours, very little is known about the process of tumour formation and progression to malignancy, after the initial inactivation of the *NF1* gene. A number of studies have implicated alterations of genes involved in the Rb and p53 pathways, and chromosomal abnormalities in NF1 MPNSTS (reviewed in Section 1.3.4). Until recently, hypermethylation studies in NF1 had been confined to the status of the *NF1* promoter, encompassed in a CpG island (Lee and Friedman, 2005). However, silencing of the *NF1* gene by hypermethylation has not so far been identified as being involved in NF1 tumorigenesis, even after quite extensive studies (Mancini *et al.,* 1999; Horan *et al.*, 2000; Harder *et al.*, 2004; Fishbein *et al.*, 2005). However, recently, one group assessed the methylation status of several TS genes in a small panel of NF1 tumours. The TS genes were found to be methylated in 4.7% to 52% of the tumours, with CpGs in the *TIMP3*, *MGMT* and *THBS1* promoters exhibiting the highest degree of hypermethylation. These results suggested that a TS gene hypermethylation pattern could also be mapped for NF1 tumours, as has been done in other cancers (Gonzalez-Gomez *et al*, 2003a). Thus, epimutation may still be important in NF1 tumorigenesis even if it is not involved in *NF1* gene inactivation itself.

The following study therefore aimed to further assess the methylation status of eight candidate genes, known to undergo hypermethylation in cancer (Table 1.1), in a large panel of NF1 tumours. The data collected here further were intended to obtain new insights into the pathways and genes involved in NF1 tumorigenesis and evolution to malignancy.

3.2 Results

3.2.1/ Methylation-specific PCR

The hypermethylation status of 8 different gene promoters was assessed in a panel of NF1 tumours composed of 12 malignant tumours (including 1 rhabdomyosarcoma –T160- and 1 medullary thyroid carcinoma or MTC -T185-), 4 plexiform neurofibromas and 11 dermal neurofibromas. Where available, the germline and somatic alterations for these tumours are summarised in Table 3.1.

Bisulphite genomic conversion was performed on each sample. This technique converts unmethylated cytosine to uracil by deamination, leaving the methylated cytosine unaffected. Low melting point agarose alone was used as negative control for each conversion reaction. Two samples repeatedly failed to convert and were removed from the study.

Methylation-specific PCR (MS-PCR) was subsequently conducted on the bisulphite-converted DNA samples. MS-PCR relies on primer sets designed to distinguish methylated from unmethylated DNA in bisulphite-converted DNA, thus taking advantage of the sequence differences resulting from bisulphite modification (Herman *et al.*, 1996). The MS-PCR primers were obtained from different publications (Table 2.7); one set of primers was modified (*MGMT* primers) to obtain larger PCR products. The fragments ranged from 120bp to 240bp, a size easily visible on a 3% NuSieve agarose gel. The primers were optimised for PCR on one specific thermocycler (ABI), which was used throughout the study to avoid temperature differences and the need for constant optimisation (Section 2.6). A positive control was obtained by bisulphite conversion of normal blood lymphocyte DNA sample artificially methylated with the enzyme *Msss*I. A non-converted normal lymphocyte DNA sample was also tested with the primers to ensure that non-converted DNA was not amplified under the conditions used. In one instance, tumour DNA from a defective bisulphite conversion did not amplify, stressing that the MS-PCR reactions only amplify converted DNA. In most cases, the MS-PCR was repeated to ensure consistency of the results. However, it became clear over the course of the study that MS-PCR is not always sensitive enough to detect hypermethylation, especially in samples with cellular heterogeneity. Thus the hypermethylation status of a sample for a particular gene was considered positive if the methylated allele amplified in at least one MS-PCR. The results from the methylation analysis for each sample are summarised in Table 3.2. Examples of MS-PCR results are illustrated in Figures 3.1, 3.2, 3.3, 3.4a, 3.5a, 3.6a, 3.7a and 3.8.

Moreover, when RNA samples were available, expression profiles for the *CDKN2A/p16*^{INK4a}, *RB1*, *MLH1* and *MSH2* genes were determined using a real-time (RT) PCR assay. The primers were designed using the "primer express" software supplied with the 7500 RT-PCR system and dissociation curves were used to assess primer specificity. Primers for *MGMT* expression analysis were designed twice but failed to yield satisfactory results and were removed from the study. Examples of the results obtained from the RT-PCR analysis can be found in Figures 3.4b, 3.5b, 3.6b and 3.7b.

3.2.2/ Methylation status of candidate genes in NF1 tumours

3.2.2.1/ The *NORE1A*, *CDKN2A* and *MSH2* genes

The *CDKN2A* locus encodes two distinct tumour suppressor (p14^{ARF} and p16^{INK4a}), and harbours two separate CpG islands so that silencing by hypermethylation of one tumour suppressor is likely to be quite independent from that of the other (Quelle *et al.*, 1995; Esteller *et al.*, 2000a). The primers used in this study were designed to assess the methylation status of the *CDKN2A/p16*^{INK4a} CpG island.

Assessment of the methylation status of the *NORE1A* (Figure 3.1), *CDKN2A/p16*^{INK4a} (Figure 3.4a) and *MSH2* (Figure 3.5a) promoters yielded consistent negative results in all tumours studied. Since the *MSss*I-methylated control amplified under the conditions used, and the results were randomly repeated, it was concluded that these genes' CpG islands did not commonly harbour hypermethylation in NF1-related tumours. *CDKN2A/p16*^{INK4a}*'s* lack of hypermethylation observed here contradicts that reported by Gonzalez-Gomez *et al.* (2003a), but is in keeping with the results of other groups (Kourea *et al.*, 1999b; Nielsen *et al.*, 1999; Berner *et al.*, 1999; Ågesen *et al.*, 2005). The observations on *NORE1A* and *MSH2* promoter methylation status in NF1-related tumours are reported here for the first time.

Additionally, expression levels of *CDKN2A/p16*^{INK4a} using real-time RT-PCR appeared dramatically reduced for two MPNSTs (T145 and T151), and to a lesser extent in T165, compared to the other tumours (Figure 3.4b). Although no hypermethylation was encountered in these tumours, *CDKN2A/p16*^{INK4a} inactivation by mutations and gross rearrangements such as deletions has been reported in NF1 and other cancers (Kourea et al., 1999b; Berner *et al.*, 1999; Nielsen *et al.*, 1999; Perry *et al.*, 2002; Perrone *et al.*, 2003; Ågesen *et al.*, 2005; Sherr, 2001; discussed in section 4.3.3.2.1) and would require further investigation. Analysis of LOH using four 9p markers revealed LOH at the *CDKN2A* locus in T145, and at an isolated

marker on 9p in T151 and T165 (section 4.2.3). *MSH2* expression appeared unchanged and relatively similar in all tumours studied (Figure 3.5b).

Table 3.1: List of tumours used in the methylation study. *NA*, not available. *MTC*, medullary thyroid carcinoma.

Patient	Blood no	Germline mutation	Tumour no	Somatic mutation/LOH	Tumour type
P23	B23	7268delCA	T23		MPNST
P130	2124	6117delG	T130		MPNST
P145	2155	5001insTG	T145	LOH	MPNST
P146	NA		T146		Meningioma
P148	2158	889-2 A>G	T148		MPNST low grade
P151	2159	6641+1 G>A	T151	LOH	MPNST
			T165a	LOH	MPNST metastase
P160	2205	288+1G>A	T160		rhabdomyosarcoma
P168	B168	G663A (T221X)	T168a	6442delA	MPNST
P169	2207	G1714A (W571X)	T169b	LOH	MPNST low grade
P184	2316		T184	4578delCTCCAGAGCAC	MPNST
P185	2318	773delA	T185	LOH	MTC
P167	2229	C3826T (R1276X)	T167		plexiform
P172	2228	5010insT	T172		plexiform
P177			T177b		plexiform
P137	2125/2142	C2446T (R816X)	T137	5894insCA	neurofibroma
P139	2128	434delTC	T139	C4637G (S1546X)	neurofibroma
P140	NA	3731delT	T140.3	LOH	neurofibroma
P141	2141	2232delA	T141.12		neurofibroma
			T141.13	5729delT	neurofibroma
P143		C6792G (T2264X)	T143.2	3124insATdelGTAG	neurofibroma
P157		7907+3A>T	T157.1a	3491delC	neurofibroma
P176	NA	large deletion	T176.1	C4812G (Y1604X)	neurofibroma
			T176.2	G5927A (W1976X)	neurofibroma
			T176.3	4119+1G>C	neurofibroma
P186	2288	large deletion	T186	2990+1G>A	neurofibroma

Gene screened	*RASSF1A*	*NORE1A*	*RARB*	*RB1*	*CDKN2A*	*MLH1*	*MSH2*	*MGMT*
Tumours								
Malignant tumours								
T23.3	U	U	U	U	U	U	U	M
T130	M	U	U	U	U	U	U	U
T145	M	U	U	U	U	U	U	M
T146	U	U	U	U	U	U	U	M
T148	U	U	U	U	U	U	U	U
T151	M	U	U	U	U	U	U	M
T160	U	U	M	U	U	U	U	U
T165	M	U	U	U	U	U	U	M
T168	U	U	U	U	U	U	U	U
T169	M	U	U	M	U	U	U	U
T184	U	U	U	U	U	U	U	M
T185	M	U	U	U	U	M	U	U
Plexiform nf								
T156	U	U	U	U	U	U	ND	U
T167	U	U	U	U	U	U	U	M
T172	U	U	U	U	U	U	U	U
T177b	U	U	U	U	U	U	U	U
T177c	U	U	U	U	U	U	U	U
neurofibromas								
T137	U	U	U	M	U	U	U	M
T139	U	U	U	U	U	U	U	M
T140.3	ND	U	U	U	U	U	U	M
T141.12	U	U	U	U	U	U	U	U
T141.13	U	U	U	U	U	U	U	U
T143.2	U	U	U	U	U	U	U	U
T157.1a	ND	U	U	U	ND	U	U	M
T176.1	U	U	U	U	U	U	U	M
T176.2	U	U	U	U	U	U	U	U
T176.3	U	U	U	U	U	U	U	M
T186	U	U	U	U	U	U	U	U

Table 3.2: Methylation status of 8 genes in a panel of 27 NF1-related tumours. *M*, hypermethylation was detected; *U*, denotes the unmethylated status; *ND*, not done.

3.2.2.2/ The *RASSF1A* gene

Hypermethylation of the *RASSF1A* promoter was observed in 6/12 malignant tumours, but not in any of the plexiform or dermal neurofibromas studied (Figure 3.2). Moreover, the positive methylation status was observed consistently in the 6 tumours, suggesting a high level of hypermethylation of the CpG Island, or a predominant number of methylated clones. The tumours were 5 MPSNTs, one of which was low grade, or MPNST metastases, and one was a medullary thyroid carcinoma (MTC). This is the first report of *RASSF1A* promoter hypermethylation being potentially involved in NF1 carcinogenesis.

3.2.2.3/ The *RB1* gene

Generally, the promoter region of the *RB1* gene was found to be unmethylated (Figure 3.6a). In two tumours, a low-grade MPNST and a dermal neurofibroma, a faint positive methylation status was observed in one of three PCRs, suggesting either a low level of hypermethylation, or a low number of methylated clones. These events cannot be distinguished without further investigation using cloning and sequencing techniques. An expression profile for the two samples could not be completed because RNA was unavailable. However, RT-PCR analysis of a small panel of samples (all negative for hypermethylation) failed to reveal major differences in expression in most tumours (Figure 3.6b), although tumour T186 demonstrated reduced *RB1* expression. The frequency of hypermethylation observed (7%, 2/27) was lower than that found by Gonzalez-Gomez *et al.* (2003a, 14%, 3/21) which is the only other study that has assessed *RB1* promoter hypermethylation in NF1 tumours.

3.2.2.4/ The *MLH1* gene

The promoter region of *MLH1* was consistently negative for hypermethylation in 26/27 of the tumours studied (Figure 3.7a), with the exception of one malignant tumour. The medullary thyroid carcinoma, T185 was found to show a positive hypermethylation status in two out of three MS-PCRs. Thus it would appear that *MLH1* inactivation by hypermethylation is not a common event in hallmark NF1 tumours (neurofibromas, MPNSTs), but may occur in rare malignancy arising in the context of NF1. Expression analysis for *MLH1* by RT-PCR yielded reduced expression levels for two malignant tumours, T151 and T185, and one dermal neurofibroma, T186 (Figure 3.7b). The *MLH1* gene can be inactivated by different mechanisms in cancer (Jones and Laird, 1999), which these results may reflect, as only T185

demonstrated *MLH1* promoter hypermethylation. Additionally, different mechanisms may generate different degrees of gene silencing, which may explain the differences in expression profiles. Nonetheless, hypermethylation in T185 may contribute to the reduced *MLH1* expression level observed. To further assess the involvement of this MMR gene in NF1 tumorigenesis, LOH was assessed for *MLH1* in 10 malignant tumours, including T185 (results presented in section 4.2.2). Evidence for LOH was found in 4 tumours that also demonstrated a degree of microsatellite instability, including T185. Thus a combination of *MLH1* LOH and promoter hypermethylation in MTC T185 may explain the reduction in expression seen by RT-PCR analysis.

3.2.2.5/ The *RARB* gene

The *RARB* gene encodes several isoforms, through alternative promoter usage and alternative splicing. The transcription of the gene yielding isoforms RARβ2 and RARβ4 is under the control of promoter P2. Loss of RARβ2, in particular, has been found in cancer, and hypermethylation of promoter P2 has been described (Virmani *et al.*, 2000; Esteller *et al.*, 2001c; Table 1.1). This is the *RARB* promoter investigated in this study. Hypermethylation of *RARB* was observed in one tumour only, T160, a rhabdomyosarcoma (Figure 3.3), out of the 27 NF1-related tumours. This would suggest that *RARB* hypermethylation is not a common event in NF1-related tumours, but may occur in rare tumours associated with the disease.

3.2.2.6/ The *MGMT* gene

The hypermethylation status of the *MGMT* promoter obtained by MS-PCR in the NF1 associated tumours presented a very heterogeneous picture (Figure 3.8). Hypermethylation was observed across the tumour panel, regardless of the tumour type. These observations were in keeping with those published by Gonzalez-Gomez and co-workers (2003a). Moreover, the hypermethylation levels also appear to be different for each tumour, offering a methylation-positive profile in 1/3 or 2/3 PCR. The number of MS-PCR performed for each tumour DNA sample was limited to three owing to severe time constraints, and the fact that such heterogeneity cannot be properly resolved by MS-PCR alone means that a more sensitive technique, such as cloning and sequencing, would be necessary. The methylation-positive malignant tumours were all MPNSTs except for one meningioma. Only one plexiform tumour exhibited hypermethylation for *MGMT*; however, the panel was small (4 tumours).

Additionally, six dermal neurofibromas were found to be hypermethylated for *MGMT*. As plexiform, not dermal, neurofibromas may give rise to MPNSTs, these results may suggest that *MGMT* inactivation may occur in NF1 tumorigenesis, rather than in the progression to malignancy. Along with the Gonzalez-Gomez study (2003a), this is the first report of *MGMT* involvement in NF1 tumours.

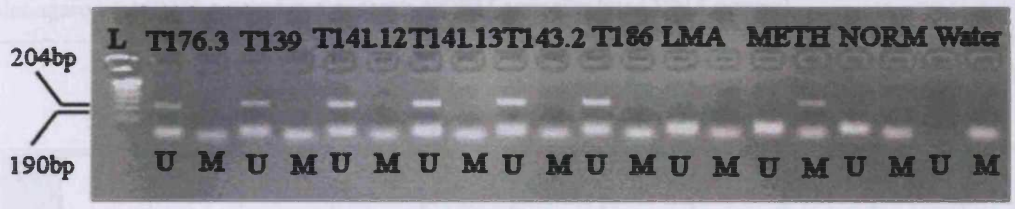

Figure 3.1: Representation of *NORE1A* methylation analysis by MS-PCR. The unmethylated fragment (U) has a size of 190bp; the methylated fragment (M) has a size of 204bp. Primer dimmers can be seen at the bottom of the gel. *L*, molecular weight marker; *LMA*, low melting point agarose; *METH*, methylated control; *NORM*, unmethylated DNA control.

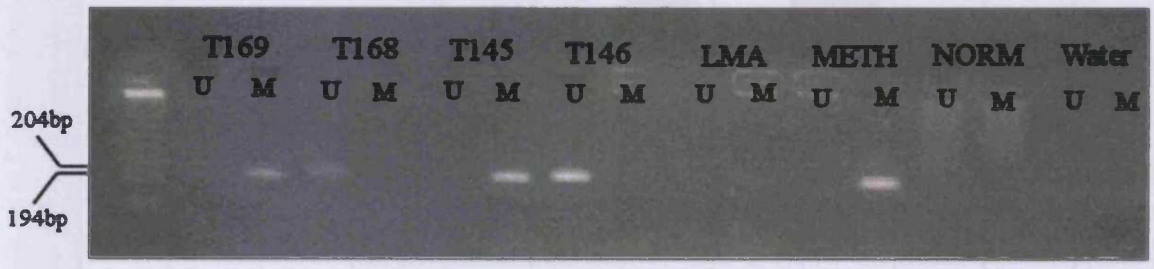

Figure 3.2: Representation of *RASSF1A* methylation analysis by MS-PCR. The unmethylated fragment (U) has a size of 204bp; the methylated fragment (M) has a size of 194bp. *L*, molecular weight marker; *LMA*, low melting point agarose; *METH*, methylated control; *NORM*, unmethylated DNA control.

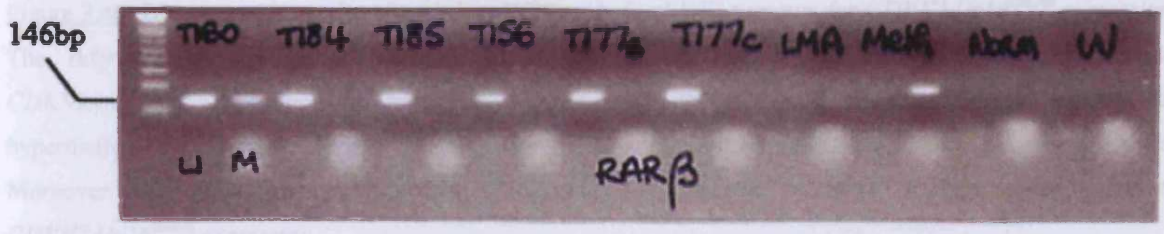

Figure 3.3: Representation of *RARB* methylation analysis by MS-PCR. Both the unmethylated fragment (U) and methylated fragment (M) have a size of 146bp. *L*, molecular weight marker; *LMA*, low melting point agarose; *METH*, methylated control; *NORM*, unmethylated DNA control.

Figure 3.4a: Representation of *CDKN2A/p16*^{INK4a} methylation analysis by MS-PCR. Both the unmethylated fragment (U) and methylated fragment (M) have a size of 151bp. *L*, molecular weight marker; *LMA*, low melting point agarose; *METH*, methylated control; *NORM*, unmethylated DNA control.

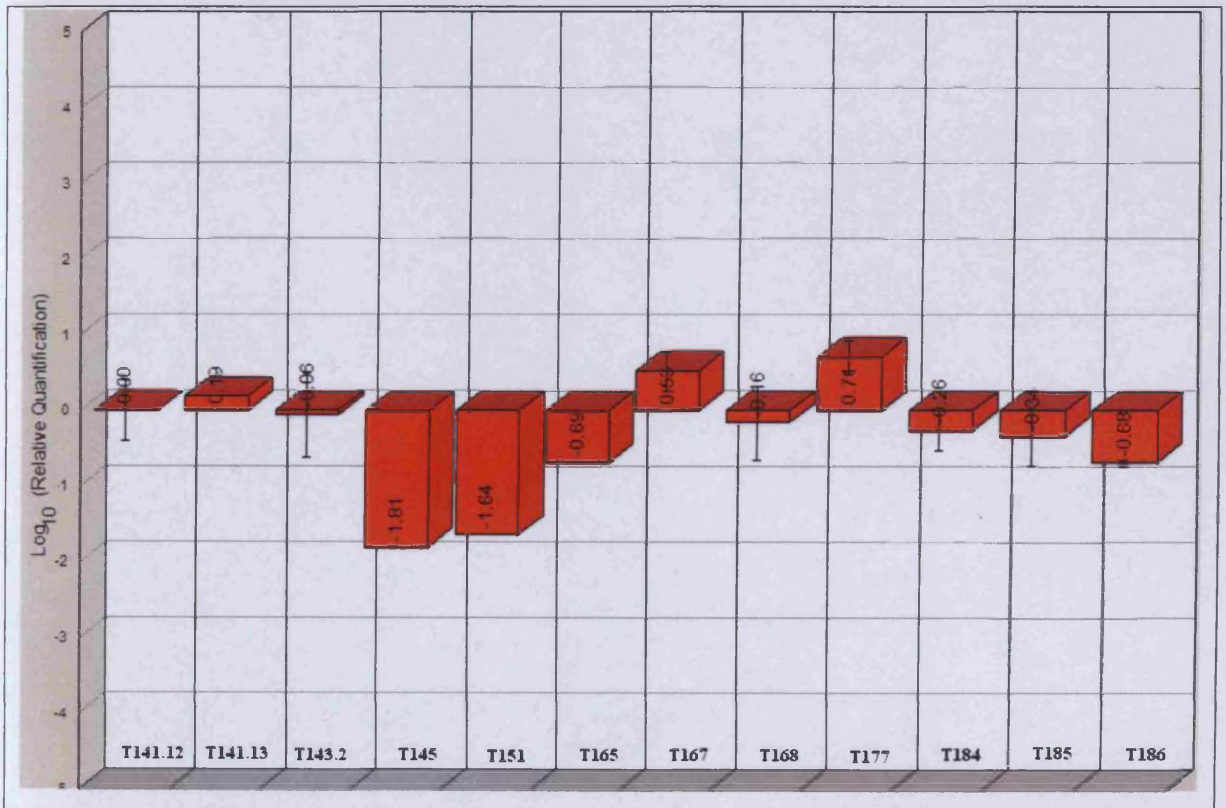

Figure 3.4b: Diagrammatic results of real-time PCR analysis of NF1 tumours for *CDKN2A/p16*^{INK4a} expression. The reference used is a dermal neurofibroma, T141.12.Three MPNSTs appear to have reduced *CDKN2A/p16*^{INK4a} expression, in particular tumours T145 and T151. The tumours were negative for hypermethylation of *CDKN2A/p16*^{INK4a}, suggesting that another inactivation mechanism may be involved. Moreover, two plexiform neurofibromas, T167 and T177, appear to show a slight upregulation of *CDKN2A/p16*^{INK4a} expression.

Figure 3.4: *CDKN2A/p16*^{INK4a} methylation and expression analysis in NF1-related tumours.

Figure 3.5a: Representation of *MSH2* methylation analysis by MS-PCR. The unmethylated fragment (U) has a size of 143bp; the methylated fragment (M) has a size of 132bp. *L*, molecular weight marker; *LMA*, low melting point agarose; *METH*, methylated control; *NORM*, unmethylated DNA control.

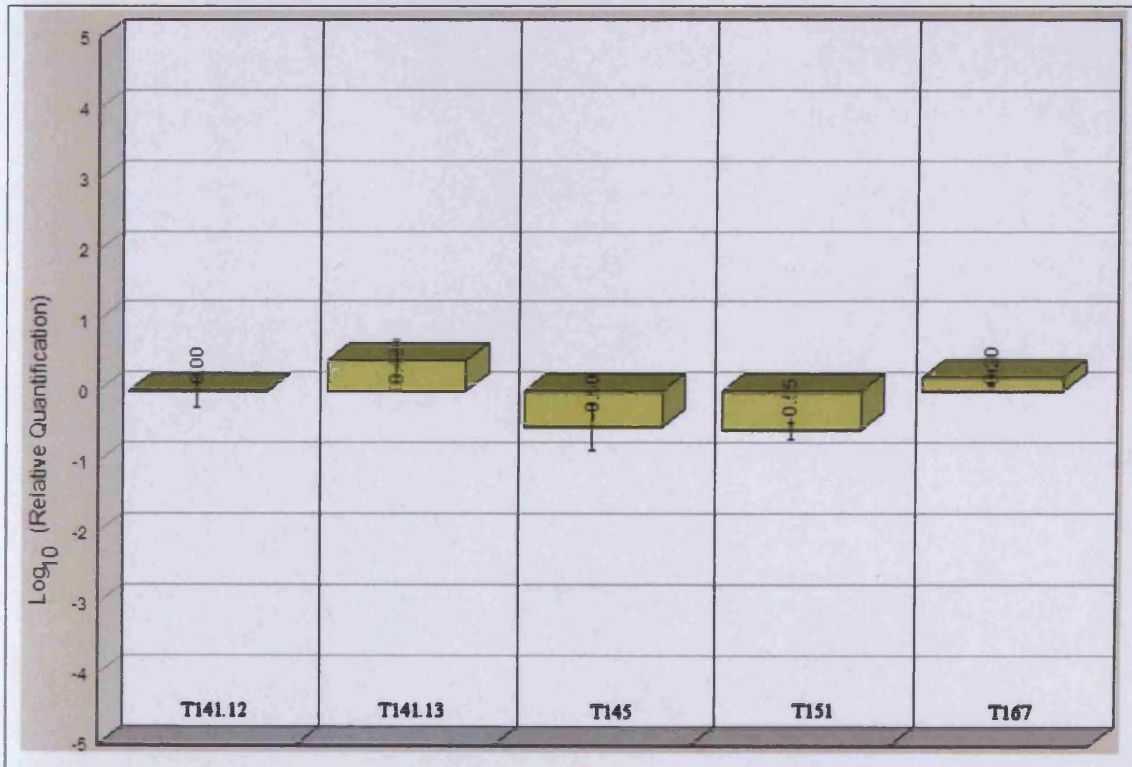

Figure 3.5b: Diagrammatic results of real-time PCR analysis of NF1 tumours for *MSH2* expression. The reference used is a dermal neurofibroma, T141.12. Expression of *MSH2* appears unchanged in NF1 tumours.

Figure 3.5: *MSH2* methylation and expression analysis in NF1-related tumours.

Figure 3.6a: Representation of *RB1* methylation analysis by MS-PCR. Both the unmethylated fragment (U) and methylated fragment (M) have a size of 172bp. *L*, molecular weight marker; *LMA*, low melting point agarose; *METH*, methylated control; *NORM*, unmethylated DNA control.

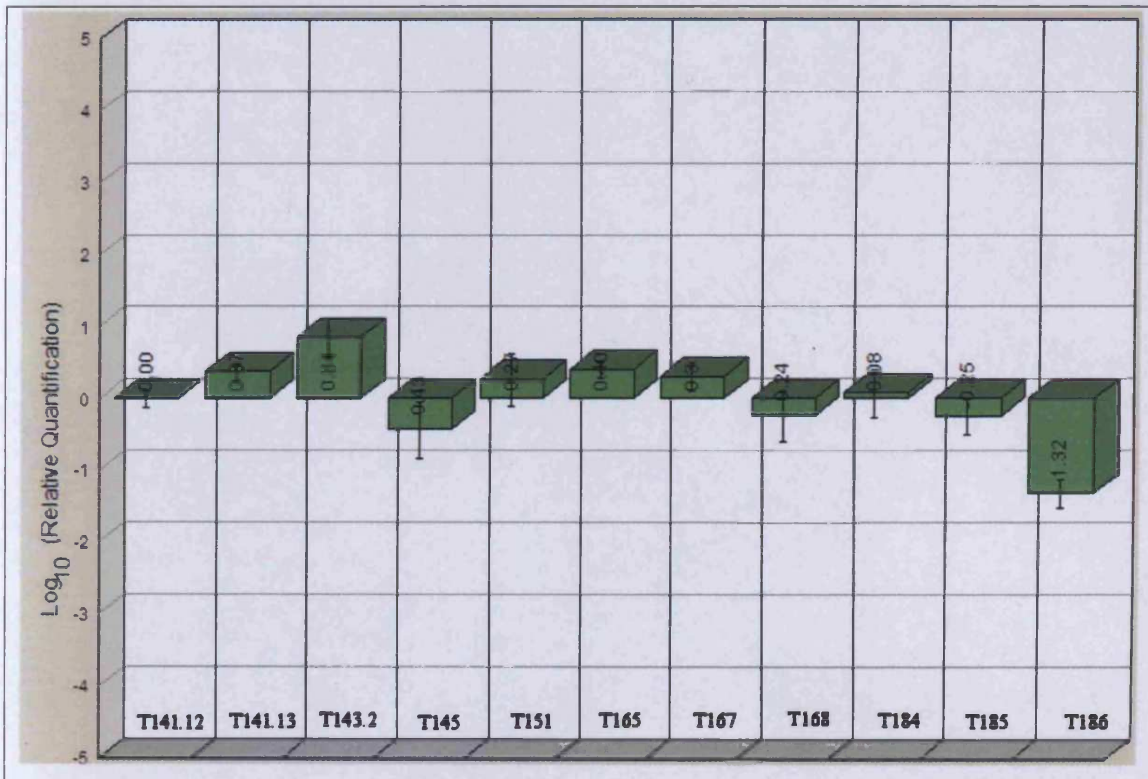

Figure 3.6b: Diagrammatic results of real-time PCR analysis of NF1 for *RB1* expression. The reference used is a dermal neurofibroma T141.12. Although the expression profile was generally the same between tumours, neurofibroma T186 exhibited a decrease in *RB1* expression.

Figure 3.6: *RB1* methylation and expression analysis in NF1-related tumours.

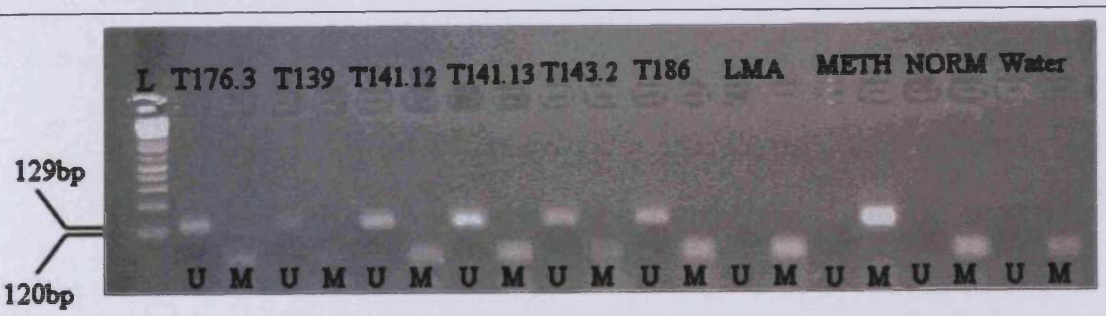

Figure 3.7a: Representation of *MLH1* methylation analysis by MS-PCR. The unmethylated fragment (U) has a size of 129bp; the methylated fragment (M) has a size of 120bp. *L*, molecular weight marker; *LMA*, low melting point agarose; *METH*, methylated control; *NORM*, unmethylated DNA control.

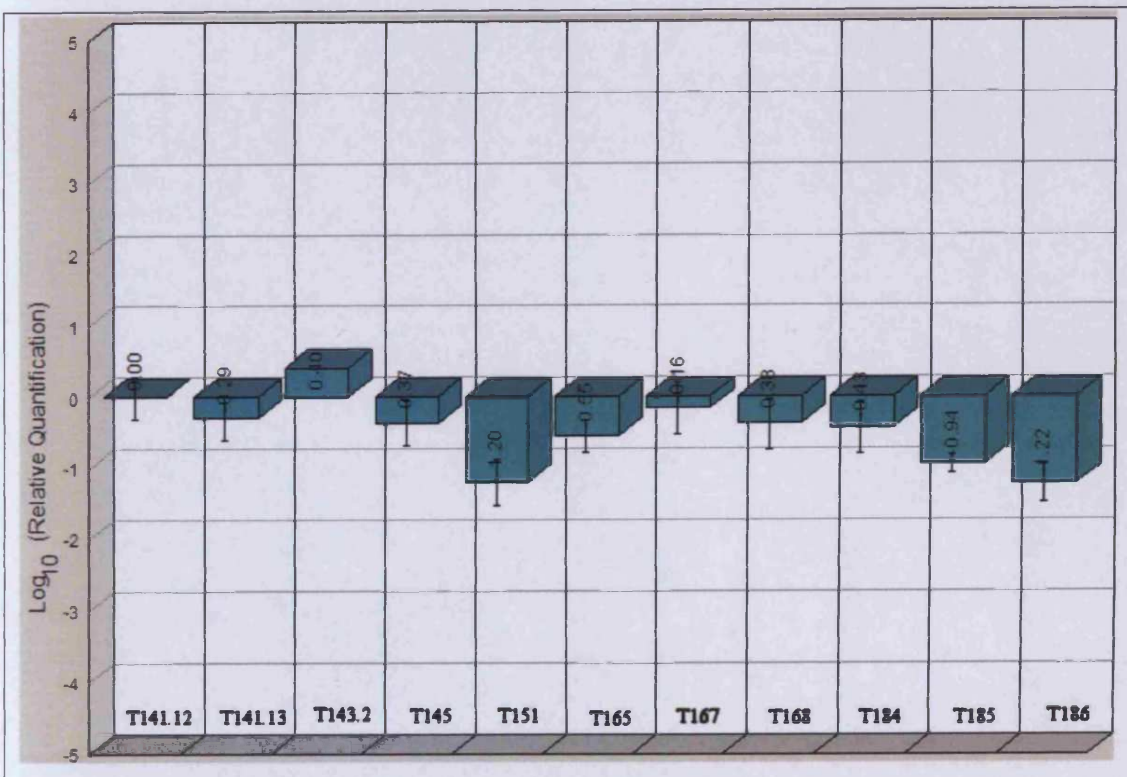

Figure 3.7b: Diagrammatic results of real-time PCR analysis of NF1 for *MLH1* expression. The reference used is a dermal neurofibroma T141.12. Expression of *MLH1* appears to be diminished in two malignant tumours, one of which, T185, exhibits hypermethylation for *MLH1*.

Figure 3.7: *MLH1* methylation and expression analysis in NF1-related tumours.

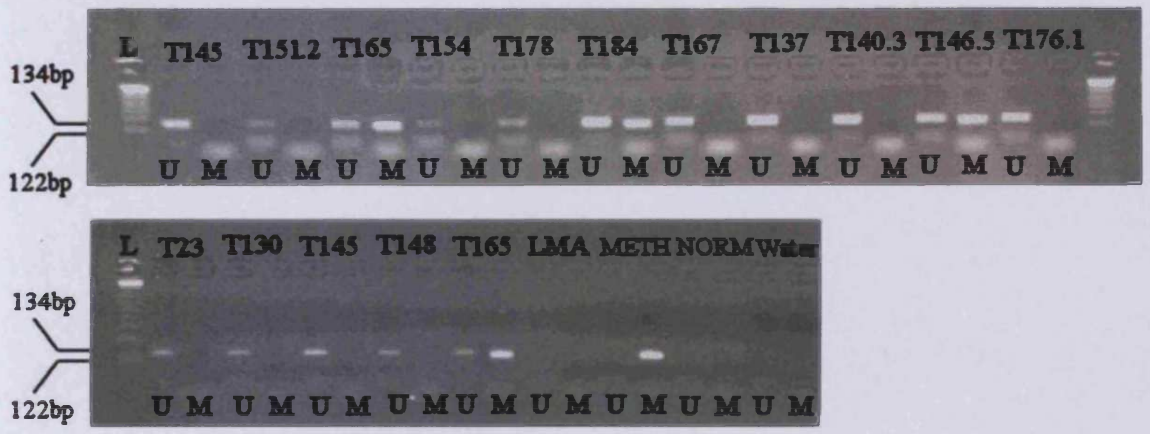

Figure 3.8: Representation of *MGMT* methylation analysis by MS-PCR. The unmethylated fragment (U) has a size of 134bp; the methylated fragment (M) has a size of 122bp. *L*, molecular weight marker; *LMA*, low melting point agarose; *METH*, methylated control; *NORM*, unmethylated DNA control.

3.2.3/ Hypermethylation profiles by NF1 tumour types

For comprehensive analysis, the results from this study were summarized by tumour types in Table 3.3.

3.2.3.1/ Malignant tumours

Hypermethylation was identified predominantly in malignant NF1 tumours, with 9/12 tumours (75%) exhibiting promoter hypermethylation for at least one gene. Moreover, 5/12 tumours were positive for hypermethylation in two gene promoters. 3/12 malignant tumours were found to be hypermethylated for both *RASSF1A* and *MGMT*: T145 is a MPNST; T151 and T165 are an MPNST and an MPNST metastasis respectively, from the same patient. 1/12 malignant tumour, T185 (MTC) showed hypermethylation for both *RASSF1A* and *MLH1*. 1/12 malignant tumour, T169, a low-grade MPNST, showed hypermethylation for both *RASSF1A* and *RB1*.

A *RASSF1A*-mediated pathway appears to be commonly disrupted by hypermethylation in malignant NF1 tumours. Tumours with hypermethylation in more than one gene, which may consequently disrupt more than one signalling pathway, may represent more evolved tumours. Such an event may also be a sign of the aggressiveness of the tumour.

Gene screened	*RASSF1A*	*NORE1A*	*RARB*	*RB1*	*CDKN2A*	*MLH1*	*MSH2*	*MGMT*	
Malignant tumours									
Rare	(+)	(-)	(+)	(-)	(-)	(+)	(-)	(+)	
MPNSTs	(+)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	CANCER
Plexiform nf	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	BENIGN
neurofibromas	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	BENIGN

Table 3.3 : Hypermethylation profile by NF1 tumour types.

The malignant tumours were divided between the rare tumours that occur in the context of NF1 (meningioma, medullary thyroid carcinoma, rhabdomyosarcoma) and are likely to be disrupted in different pathways than hallmark NF1 tumours, and MPNSTs, which arise from plexiform neurofibromas (Ruggieri and Huson, 1999). The possibility for malignant evolution is marked by the arrow. By contrast, dermal neurofibromas (referred to as neurofibromas in the table) do not undergo malignant evolution (Ruggieri and Huson, 1999). (+) indicates that a positive hypermethylation profile was seen in at least one tumour of the group. As mentioned in Section 3.2.1, a positive hypermethylation profile was considered when the methylated allele was amplified in at least one MS-PCR. (-) indicates a normal, unmethylated, profile.

This table allows to visualised genes that may be involved a) as an early event in NF1 tumorigenesis, rather than in malignant evolution (*MGMT*, *RB1*), b) in the progression of malignancy and perhaps aggressiveness (*RASSF1A*) c) in rare tumours, but not in typical NF1 tumours, suggesting the disruption of distinct cellular pathways (*RARB*, *MLH1*).

3.2.3.2/ Plexiform neurofibromas

Only 1/4 (25%) plexiform neurofibromas showed a positive hypermethylation profile in 1/3 MS-PCRs at the *MGMT* promoter, suggesting a generally low level of hypermethylation in the gene studied. One plexiform neurofibroma was assessed for methylation in two different locations (a nodular part and a matricial part), but the two samples yielded similar negative results.

Plexiform neurofibromas are thought to be the precursor tissues for MPNSTs (Riccardi, 1992; Korf et *al.*, 1997). Interestingly, no plexiform neurofibroma harboured hypermethylation at the *RASSF1A* promoter, which was found methylated in MPNSTs in this study. This could suggest that *RASSF1A* involvement in NF1 carcinogenesis occurs later in the development of the malignant tumour, rather than as an event determining the progression from the benign to malignant state. However, the number of plexiform neurofibromas studied here was small.

3.2.3.3/ Dermal neurofibromas

Only 1/11 dermal tumour, T137, showed hypermethylation for both *MGMT* and *RB1*. 7/11 dermal tumours showed some level of hypermethylation for *MGMT*, suggesting that *MGMT* may be regularly disrupted in NF1 benign tumour, or occur as an early event in tumorigenesis.

3.3 Discussion

3.3.1/ Methylation-specific PCR

MS-PCR is a reliable and fast method to obtain a global picture of gene promoter methylation in a tumour. It is also relatively inexpensive as it does not require any particular modification to the primers and amplimers can be analysed on an agarose gel. Moreover, once PCR conditions are optimised, the results are reproducible (as shown by the controls).

However, the technique carries the limitation of a PCR-based assay, in its sensitivity and by being limited to the sequence bound by each primer, which could be different from the rest of the gene promoter sequence. Thus the sequence analysed represents a small fraction of the promoter region, and a positive results by MS-PCR is dependant upon hypermethylation of that particular region, which may not reflect the status of the neighbouring sequence. Nevertheless, all but one of the primer sequences used in this study have been previously

published and their use has been shown to give an accurate depiction of the promoter hypermethylation status. Additionally, the positive methylation status observed with these primers was correlated with either the absence or down-regulation of expression, using RT-PCR or immunohistochemistry in a number of studies (Herman *et al.*, 1998; Esteller et al., 1999; Virmani *et al.*, 2000; Hesson *et al.*, 2003; Kuroki *et al.*, 2003b; Hesson *et al.*, 2004). In a study using the same primers for *MLH1* hypermethylation, it was also demonstrated that treatment by a de-methylating agent could restore expression, as well as normal activity (showing that activity was not lost due to a mutation; Herman *et al.*, 1998). Such compelling evidence for the role of epigenetic modification on the regulation of gene transcription has also been demonstrated for a number of different genes, including *RASSF1A* and *CDKN2A/p16^{INK4a}*, in which treatment with a de-methylating agent allowed re-expression after methylation (Dallol *et al.*, 2003; Fang *et al.*, 2003; Takahashi *et al.*, 2004; Chen *et al.*, 2005). While the presence of a methylated allele detected by MS-PCR in the present study may not, by itself, certify that hypermethylation and subsequent gene inactivation occur, such correlation has been demonstrated by others, thus strengthening the results of the present study..

The forward primers for both the methylated and unmethylated sequence of *MGMT* were carefully re-designed to obtain larger PCR fragments, and each moved 41bp upstream of the original fragments (Esteller *et al.*, 1999). These PCR amplimers encompassed 3 and 4 CpG dinucleotides, respectively. The *MGMT* promoter MS-PCR yielded different results on consecutive PCRs, and it cannot be ruled out that a flaw in the primers' location, combined with the limited sensitivity of MS-PCR, might account for these results. However, this was also observed when using other published primers sets to amplify whole tumour DNA, whereas the positive control DNA was constant for each PCR. While the quantity of bisulphite converted DNA template in the PCR could also be at fault, one DNA conversion usually yields enough treated DNA to assess all 8 genes, so that, should this problem occur, it would also be seen in more than one MS-PCR for a particular gene.

It should also be appreciated that NF1 tumours, in particular neurofibromas, are composed of highly heterogeneous cell populations (Peltonen *et al.*, 1988), and that degree of contamination by adjacent "normal" tissue cannot always be averted even by careful dissection. Although it is now accepted that Schwann cells (SC) are the only cells which bear the somatic mutation in the *NF1* gene, other cell types also contributes to tumour formation (Carroll and Stonecypher, 2004). It may be possible that, in neurofibromas, the hypermethylation status may differ, not only within the clones of an original tumour cell, but

also between the cellular populations. Moreover, as reviewed in Section 1.5.2, an accepted promoter hypermethylation mechanism relies on the progressive methylation of the individual CpGs in the CpG island. This model allows for clones of each cell to develop different methylation profiles over time, in clonal fashion, thereby adding to the heterogeneity of CpG methylation originally present in the cell (Baylin, 2002). Taken together, this may explain the observations of MS-PCR inconsistency, since different percentages of methylated cells may be amplified in an extremely heterogeneous cellular population. Identifying the number of hypermethylated clones or the number of methylated CpGs in the island would require a lengthy process of cloning and sequencing. For example, such an analysis was conducted on 28 *RASSF1A*-methylation-positive extrahepatic cholangiocarcinoma tumours, and a variable number of methylated CpG was observed, from 4 to 16 CpG (out of 16, Chen *et al.*, 2005). In all 28 samples, however, expression of *RASSF1A* was silenced as a result. Ideally, a similar estimation of the extent of CpG methylation and its influence on gene expression should be conducted for each gene.

As discussed in Section 3.3.2, finding suitable control samples for use in such methylation assays with NF1 tumours remains problematic. Ideally, the hypermethylation status observed in NF1 tumours should be compared to the methylation status in normal tissue, to help determine the extent of the involvement of hypermethylation in NF1 tumorigenesis. In past studies, such combined healthy tissue/malignant tissue approach has been used [non-neoplastic meningeal tissue/meningioma (Bello *et al.*, 2004), normal/cancerous thyroid tissues (Schagdarsurengin *et al.*, 2002)], and the normal tissues were often obtained following autopsy. The use of blood DNA samples as control may be suited for studies on hypermethylation in haematological cancers (Takahashi *et al.*, 2004), as similar tissues are then compared. The use of blood DNA samples as normal control to solid tumours, such as NF1 tumours, is however debatable. In the Gonzalez-Gomez study, two samples of non-neoplastic peripheral nerve sheath tissue were used as control, and no hypermethylation was identified in these samples in the 11 genes investigated, including *CDKN2A/p16*^{INK4a}, *RB1* and *MGMT* (Gonzalez-Gomez *et al.*, 2003a). As no such control was available to the present study, the hypermethylation observed was arbitrarily scored as positive when a methylated allele was observed by MS-PCR. Of note this study and the Gonzalez-Gomez study yielded similar results for *MGMT* and *RB1*.

Interestingly, and as previously reported, the unmethylated allele was almost always amplified in the MS-PCR in all tumours and genes. It has been proposed that the cellular heterogeneity, as well as cytosine hemimethylation (where only one allele is methylated) could explain the amplification of the unmethylated allele (Gonzalez-Gomez *et al.*, 2003a). By contrast, *RASSF1A*-hypermethylation-positive samples (T145, T165) rarely showed amplification of the unmethylated allele (Figure 3.2). This would suggest that *RASSF1A* hypermethylation might be bi-allelic in these tumours. As MPNSTs are almost homogeneously composed of Schwann cells, then the possibility of amplifying an unmethylated allele would be reduced to the occasional contamination by surrounding non-tumour tissue (which cannot always be avoided at dissection).

The findings of a gene hypermethylation in a cancer cell do not necessarily imply that epigenetic event was causal to the development and evolution of the tumour. As reviewed in Section 1.3.4, MPNSTs harbour a multitude of chromosomal defects and it is possible that some of these may affect genes involved in maintaining normal methylation. There is a number of examples in the literature of genetic disease that causes methylation defect: ICF syndrome (immunodeficiency and developmental defects) and mutations in the DNA methyltransferase *DNMT3B* gene, Rett syndrome (degeneration in speech and acquired motor skills) and mutations in the methyl-CpG binding protein *MECP2* gene (Robertson and Wolffe, 2000; Sections 1.5.1 and 1.5.2). In return, hypermethylation of genes responsible for genome integrity, such as *MLH1*, can result in genome-wide instability (Charames and Bapat, 2003). The interaction between genetic and epigenetic mechanisms is complex and fascinating. In the following sections, the consequences of the gene hypermethylation identified in NF1-related tumours are discussed.

In conclusion, MS-PCR is a simple technique that can easily be implemented and can give an overview of a gene's methylation status. Because of the heterogeneous nature of NF1 tumours, and the need for additional investigation of the methylation status of the genes presented here, the use of more sensitive technique could be envisaged. One such technique, recently developed, is quantitative methylation PCR (Q-MSP), which relies on the real-time PCR technology and uses fluorescent probes to detect the methylation status in bisulphite-treated DNA. The use of fluorescence increases assay specificity and sensitivity: Q-MSP can detect 1/10,000 methylated copies, whereas MS-PCR is deemed to be limited to 1/1000 (Herman *et al.*, 1996; Eads *et al.*, 2000; Jéronimo *et al.*, 2004; Cohen *et al.*, 2003). The

technology was successfully used to distinguish significant methylation levels between prostate cancers, thereby helping to stratify the tumour grade/developmental stage (Jéronimo *et al.*, 2004). The recently reported technique of quantitative multiplex-methylation-specific PCR (QM-MSP) has been devised to assess the methylation status of multiple gene promoters, with a sensitivity similar to that of Q-MSP (Fackler *et al.*, 2004).

The ability to integrate epigenetic DNA analyses into the available genomic microarray platform provides the means of rapidly obtaining quantitative and qualitative information of DNA methylation status across the entire genome. This application relating to the detection of alterations in methylation patterns on a genomic chromosomal scale has been used in cancer studies where DNA from tumour and corresponding normal tissue is compared (Pollack and Iyer, 2002; Zardo *et al.*, 2002; Tompa *et al.*, 2002).

Given the growing number of TS and regulatory genes found to be inactivated by hypermethylation, these techniques would allow the exploration of new avenues in NF1 tumour formation and progression.

3.3.2/ Real-time PCR analysis

RT-PCR is a promising alternative to cDNA microarray as a rapid and precise approach to gene expression quantitation. It requires only a small amount of total RNA, which makes it suitable for analysing small tumours and has been shown to detect even weakly expressed genes in NF1 tumours (Lévy *et al.*, 2004).

In this study, the level of expression of genes previously assayed for hypermethylation was assessed using real-time reverse transcription PCR, which allows continuous measurement of the PCR product amount using a SybrGreen fluorescent dye. The mRNA levels were normalized to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) transcript, used as a control in many RT-PCR assays (Esteller *et al.*, 2000a, 2000b; Gao *et al.*, 2004; Hesson *et al.*, 2004; Seidel *et al.*, 2005). The issue of normalization has previously been raised, in that even housekeeping genes may not always show constant mRNA levels. Although *GAPDH* remains one of the most popular control genes, it should be noted that the assay might benefit from being normalized with two or more control genes (Mocellin *et al.*, 2003).

In the present study, reference tissue was not available as there is no "normal" equivalent to these tumours, and samples had to be analysed in relation to each other. Dermal neurofibromas, which do not undergo malignant transformation, have previously been used as

"normal" controls against plexiform neurofibromas and MPNSTs (Lévy *et al.*, 2004). As this presents an obvious *caveat* for the study, the real-time RT-PCR analysis is intended as a complement to the methylation study.

Additionally, the total RNA extracted from a neurofibroma is effectively a mix of total RNAs from different cell populations. It is worth noting that a cell type may or may not express a gene, or express it to a different degree to that observed in another cell type. Thus, the use of RT-PCR on RNA extracted from neurofibroma gives an average of the expression of a particular gene, and may mask its true expression in the relevant cell type (which SC are assumed to be, given that they bear *NF1* gene inactivation). It may also be that a reduced expression of a given gene, seen in MPNSTs (homogeneously composed of SC), may be emphasized when compared with the expression in neurofibromas, because the latter are not composed exclusively of SC.

Nevertheless, as mentioned in Section 3.3.1, RT-PCR is often used to correlate epigenetic inactivation by promoter hypermethylation and reduction or loss of gene expression; the use of real-time technology allows for practicality, sensitivity and quantification of the result, compared to a gel-based detection RT-PCR (Virmani *et al.*, 2000; Dallol *et al.*, 2003; Fang *et al.*, 2003; Hesson *et al.*, 2003; Mocellin *et al.*, 2003; Takahashi *et al.*, 2004; Wong *et al.*, 2004).

In the present study, the results obtained by RT-PCR proved difficult to interpret owing to the variations (error bars) and would require additional investigation. Promoter hypermethylation appeared to correlate with a reduction of expression levels (for example, T185 with *MLH1*). With samples that show a reduced level of expression, but no promoter hypermethylation, it cannot be ruled out that another inactivation mechanism for the gene may have occurred. For example, in Section 4.2.3, LOH at the *CDKN2A* locus was described in tumours T145 and T168. A drastic reduction of *CDKN2A/p16*^{INK4a} expression was also found by RT-PCR in T145, but not T168, which may indicate that a mutation or other alteration may have occurred in the remaining wild-type allele in that particular tumour. Additionally, and as previously noted, the lack of a perfect correlation between mRNA expression and promoter hypermethylation status is not unexpected, because of the many factors involved in the control of gene expression, including chromatin conformation, availability of co-factors, and repressor and enhancer molecules (Mehrotra *et al.*, 2004).

One dermal tumour, T186, exhibited lower levels of expression in some (*RB1*, *MLH1*), but not all (*CDKN2A/p16*^{INK4a}) of the genes assessed. While this could be attributed to the quality of RNA obtained from this tumour, Almeida and co-workers have demonstrated that, even on degraded RNA (due to delays at room temperature, as would be the case with the biopsies used here), real-time RT-PCR quantification of a gene product normalized to a reference gene transcript provides a reliable estimate of RNA levels (Almeida *et al.*, 2004). Patient P186 carries a large deletion of the *NF1* gene as a germline mutation, and has very severe neurofibroma manifestations. It has been previously reported that patients with large deletions which encompass *NF1* gene and flanking 14 genes , tend to have more severe clinical features and are more prone to malignancy (Kayes *et al.*, 1994; Leppig *et al.*, 1997; Dorschner *et al.*, 2000; De Raedt *et al.*, 2003). Therefore, the low expression levels in T186 could reflect a wide range of disruptions in the tumour, possibly correlated with the loss of the *NF1* gene and potentially important surrounding sequences. This is difficult to determine because of the cellular heterogeneity found in neurofibromas.

Owing to the limited number of genes assessed, it was difficult to identify tumour-specific patterns in this study, although some malignant tumours (T151, T185) generally presented lower levels of expression, not necessarily correlated to the presence of hypermethylation. As previously mentioned, it cannot however be ruled out that such differences are in part due to the cellular composition of the tumours, and the issue would require further investigation. Moreover, a gene can be silenced by genetic and epigenetic events, and the hypermethylation study reported here cannot account for all the patterns observed.

In summary , a RT-PCR assay can be an attractive tool to assess quickly the altered expression of candidate genes in NF1 tumours, and could be combined to mutation/epigenetic alterations detection techniques to help determine the pathogenicity of these alterations. These results also emphasize the complex nature of NF1 tumorigenesis and the need for additional investigation in the genes presented here.

3.3.3/ Methylation status in NF1 tumours

3.3.3.1/ The *RASSF1A* gene

Hypermethylation of the *RASSF1A* promoter was consistently observed in 50% of malignant tumours in this study. The majority (5/6) of the tumours presenting hypermethylation for

RASSF1A are MPNSTs. This is the first report of *RASSF1A* involvement in NF1 tumorigenesis. Ras effector *RASSF1A* is a relatively new TS gene, and has been found inactivated by hypermethylation in a wide range of tumours (Table 1.1; also reviewed in Agathanggelou *et al.*, 2005). Although only recently identified, the *RASSF1A* tumour suppressor gene has rapidly gained a prominent role in human cancers and has been tied to cellular processes of cell cycle regulation, apoptosis and microtubule stability (Agathanggelou *et al.*, 2005).

RASSF1A is thought to promote apoptosis and cell-cycle arrest by inhibiting accumulation of CyclinD1 (Vos *et al.*, 2000; Ortiz-Vega *et al.*, 2002; Khokhlatchev *et al.*, 2002) and the growth inhibitory properties of *RASSF1A* are thought to be activated by the presence of oncogenic Ras (Ortiz-Vega *et al.*, 2002; Vos *et al.*, 2003). Therefore, the present results would suggest that inactivation of *RASSF1A* by hypermethylation might allow the tumorigenic cells to evade apoptosis, and continue to grow. Since positive methylation status was only observed in half the MPNSTs, such an event could occur later in the life of the tumour, and may be a marker of aggressiveness (one tumour is a MPNST metastase and one is a MTC, a rare, more aggressive tumour). Moreover, the cellular mechanisms involving *RASSF1A* are only just beginning to be unravelled and it has been suggested that *RASSF1A* may be involved in Ras-independent pathways, depending on the cell type (Kim *et al.*, 2003). Perhaps *RASSF1A* involvement in NF1 tumorigenesis is also dependant on its inactivation in one particular cell type. Schwann cells (SC) are now accepted to be the primary defective type of cells in NF1 tumours (Carroll and Stonecypher, 2004). SC growth has been linked to elevated levels of CyclinD1 (Kim *et al.*, 2000), and, in *Nf1*-deficient mice, elevated levels of Ras have been correlated with elevated CyclinD1 levels (Kim *et al.*, 1997a, b). Taken together, these observations may suggest that, in NF1 tumour SC, where loss of *NF1* function leads to unregulated Ras expression, *RASSF1A* could act as a control point to steer cell fate towards apoptosis, and inhibit cell growth by preventing accumulation of CyclinD1 (Figure 3.9). Loss of control by RASSF1A may thus occur in a subset of tumours, and present a key step to acquire the "capability to evade apoptosis", as described by Hanahan and Weinberg (2000).

Another interesting function of RASSF1A is its capacity to interact with and stabilize microtubules (Agathanggelou *et al.*, 2005). A dominant-negative RASSF1A that destabilizes microtubules and missense mutants that failed to co-localize with microtubules have been described and led to the identification of a microtubule association domain between amino acids 120 and 185 (Liu *et al.*, 2003; Dallol *et al.*, 2004; Vos *et al.*, 2004). It has been

suggested that RASSF1A's interaction with microtubules may positively affect the protein's ability to inhibit growth and induce cell cycle arrest (Dallol *et al.*, 2004; Vos *et al.*, 2004; Rong *et al.*, 2004; Agathanggelou *et al.*, 2005).

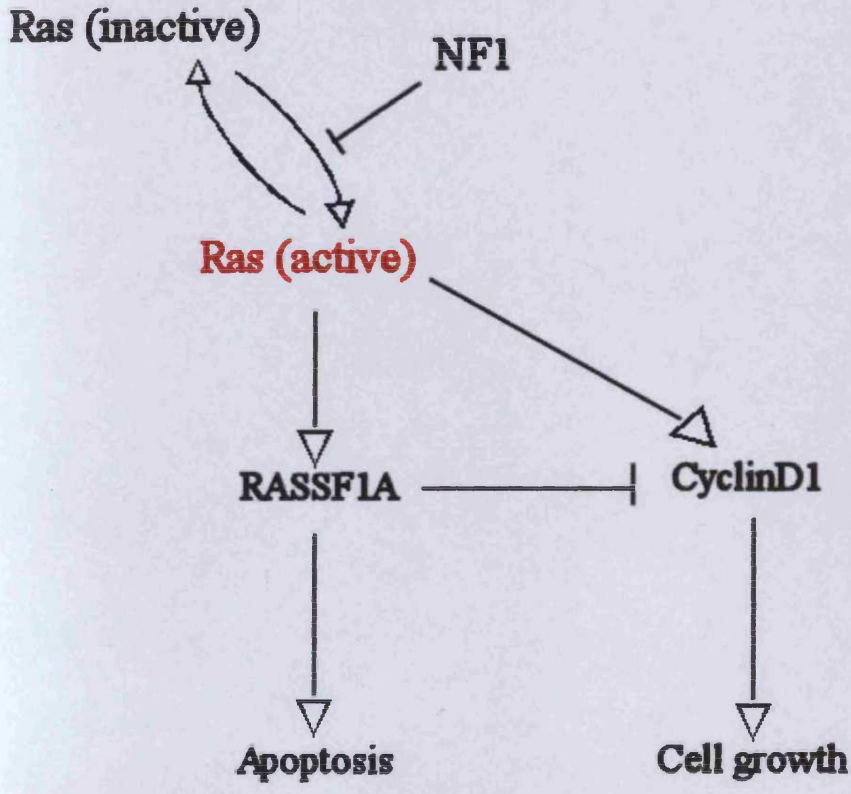

Figure 3.9: Proposed mechanism of *RASSF1A* growth suppression and interaction with *NF1*.
In NF1 tumour SC, NF1 loss would result in elevated Ras levels, and ultimately uncontrolled cell growth. In a subset of tumours, additional loss of the *RASSF1A* control would not only impair apoptosis, but also be permissible to uncontrolled cell growth.

Incidentally, neurofibromin has also been reported to interact with tubulin *in vitro* and the interaction has been shown to result in the inhibition of the *NF1*-GRD function (Bollag *et al.*, 1993). Additionally, mutations in conserved N-terminal residues were shown to cause a loss of interaction between NF1-GRD and microtubules (Xu and Gutmann, 1997). Finally, treatment of the cells with anti-mitotic agents, which cause microtubules to disassemble, was followed by the release of neurofibromin (Gregory *et al.*, 1993). Therefore, it would appear that interaction with stable microtubules (whose stability may also be positively regulated by *RASSF1A*) may have opposite effects on the two gene products; RASSF1A may require such

an interaction to inhibit cell growth and induce cell cycle arrest, whereas neurofibromin GAP activity would be inactivated by microtubule interaction.

MTC is a rare thyroid carcinoma tumour and is though to arise with increased incidence in NF1 (Lenoir, 1994). Interestingly, the only MTC in the study was found to be hypermethylated for the *RASSF1A* as well as *MLH1* promoter. In agreement with the present results, *RASSF1A* silencing by hypermethylation has been observed in 71% of thyroid carcinomas, and at a higher incidence in aggressive forms, such as MTC (80%; Schagdarsurengin *et al.*, 2002). Inactivation of *CDKN2A/p16^{INK4a}* by hypermethylation was also found in the majority of cases with *RASSF1A* silencing (Schagdarsurengin *et al.*, 2002), but not all, as would be the case for the tumour studied here. In contrast to the potential inactivation of *MLH1* in T185 (with the potential association of genomic instability at the nucleotide level), loss of RASSF1A function has been suspected to sensitise cells to activated-Ras-induced genomic instability, which involves aneuploidy, polyploidy and nuclear defects (Vos *et al.*, 2004). Haploinsufficiency of *RASSF1A*, generally in the context of inactivation of other 3p TS genes, has also been though to play a role in tumorigenesis of renal cell and head and neck squamous cells carcinomas (Morrissey *et al.*, 2001; Hogg *et al.*, 2002). Because this is the first report implicating *RASSF1A* in NF1 malignancy, it would be of particular interest to determine both the nature of the inactivating mechanisms (mutation, LOH, hypermethylation) and the extent of *RASSF1A* alteration in NF1 tumours.

Other mechanisms of inactivation for *RASSF1A* have not been investigated in the present study. Mutations in *RASSF1A* are a rare event (Dong *et al.*, 2003; Agathanggelou *et al.*, 2005 ; Chen *et al.*, 2005). However, it would be of particular interest to assess loss of heterozygosity at 3p21.3 for the tumours presenting with aberrant methylation, as inactivation of *RASSF1A* by both hypermethylation and LOH have been reported in different cancers (Agathanggelou *et al.*, 2001; Hogg *et al.*, 2002; Yu *et al.*, 2003; Chan *et al.*, 2003; Agathanggelou *et al.*, 2005; Chen *et al.*, 2005; Tischoff *et al.*, 2005).

The only rhabdomyosarcoma in this study, T160, did not exhibit hypermethylation for *RASSF1A*. Similarly, a recent study on *RASSF1A* hypermethylation in soft tissue sarcomas (STS) revealed various degrees of hypermethylation (6-50%) in the different STS types, but failed to identify any *RASSF1A* epigenetic modification in rhabdomyosarcomas (Seidel *et al.*, 2005). These results contrast, however, with an earlier study by Harada *et al.* (2002) where *RASSF1A* hypermethylation was observed in 61% of rhabdomyosarcomas.

In contrast to the results described here, a recent expression study of 489 genes by real-time RT-PCR in pooled MPNSTs (9 tumours) and plexiform neurofibromas (12 tumours) revealed no major difference in expression between benign and malignant tumours for 5/6 *RASSF* family genes (*RASSF1*, *RASSF3*, *RIN2*, *AD037* and *NORE1*) whereas *RASSF2* expression was drastically reduced in MPNSTs (Lévy *et al.*, 2004). Nevertheless, both studies would warrant a more in-depth investigation of the relation between the RASSF family of Ras effector and *NF1*.

3.3.3.2/ The *NORE1A* gene

From the *RASSF* family of Ras effectors, *NORE1A* (also known as *RASSF5*) is thought to inhibit cell growth, although the underlying mechanism is still unclear (Khokhlatchev *et al.*, 2002; Vos *et al.*, 2003; Aoyama *et al.*, 2004). *NORE1A* has also been found to heterodimerize with RASSF1A, and is thought to be involved in the Ras-mediated apoptosis pathway (Ortiz-Vega *et al.*, 2002). Hypermethylation of the *NORE1A* promoter has been described in cancer and is correlated with down-regulation of gene expression (Hesson *et al.*, 2003; Morris *et al.*, 2003).

The absence of *NORE1A* promoter hypermethylation in this panel would suggest that silencing of this gene by hypermethylation is uncommon in NF1 tumours. The hypermethylation status of *RASSF1A* and *NORE1A* gene promoters has been reported to be uncorrelated (Morris *et al*, 2003). Although many different mechanisms can account for gene inactivation, *NORE1A* mutations or LOH remain, to date, unreported but for two missense changes identified in lung tumour cell lines (Hesson *et al.*, 2003).

3.3.3.3/ The DNA repair genes

Little or no methylation was observed in the promoter of mismatch repair (MMR) genes *MLH1* and *MSH2*, suggesting that these genes are not commonly inactivated by hypermethylation in NF1 tumours. In some cancers, aberrant *MLH1* hypermethylation has been suggested as an early epigenetic event (Mukai and Sekiguchi, 2002), which does not appear to be the case in NF1 tumours, since it was not observed in any of the hallmark tumours (dermal or plexiform neurofibromas, MPNSTs). The only tumour, T185, with hypermethylation in the *MLH1* promoter is a MTC. As this tumour is a rare occurrence in NF1, it may have arisen from a different background than other NF1 tumours. The germline

mutation in this patient is a one-base deletion in exon 6, resulting in a frameshift. Therefore it may be possible that, in this patient, loss of *MLH1* function occurred as an early event and created a permissive background for the first hit in the *NF1* gene. Unfortunately, no clinical data was available for this patient. Additionally, the use of one LOH marker for *MLH1* identified LOH in T185 (Section 4.2.2), which is consistent with the theory that, in this tumour, *MLH1* has been inactivated by both hypermethylation and LOH. *MLH1* double hits in cancer have been reported as mutation/LOH, mutation/hypermethylation (Jones and Laird, 1999) and, recently, as hypermethylation/LOH (Smidgiel *et al.*, 2004, Limpaiboon *et al.*, 2005). Moreover, as discussed in Section 4.3.3.2, rare cases of homozygous mutations in *MLH1* or *MSH2* resulted in NF1-related features in the patients, suggesting some level of interaction between the *NF1* gene and the MMR genes, where the *NF1* gene could be a target for MMR defects (Ricciardone *et al.*, 1999; Raevaara *et al.*, 2004; Menko *et al.*, 2004). Finally, tumour T185 showed instability at MSI marker MYCL only (1/6 markers, Section 4.2.1). Inactivation of *MLH1* by hypermethylation has been correlated with MSI in some (Herman et *al.*, 1998; Ueki *et al.*, 2000; Esteller, 2000a), but not all cancers (Hayashi *et al.*, 2003). In the case of this MTC, a rare tumour, low MSI may be correlated with loss of *MLH1* function. No previous reports have shown *MLH1* inactivation in MTC.

Additionally, the expression profile of *MLH1* suggests that the gene may be involved in NF1 tumorigenesis, and its function may be, at least partially, lost through means other than hypermethylation. Other avenues have not been fully investigated; however LOH may be one of the inactivating mechanisms. Assessment of *MLH1* LOH using marker D3S1611 revealed an allelic loss in 4/10 malignant NF1 tumours, supporting this theory (Section 4.2.2). Tumour T151, which appears to show a decrease in *MLH1* expression, also showed LOH at the *MLH1* marker. By contrast, tumours T145 and T165, appeared to harbour LOH, but did not seem to show a marked decrease in expression. Since only one microsatellite markers was used, it cannot be ruled out that the LOH observed in these two tumours was in fact an allelic imbalance related to microsatellite instability.

Hypermethylation of the *MSH2* promoter has not been found in the present study, suggesting that such an event may be uncommon in NF1 tumours. Aberrant *MSH2* promoter methylation has rarely been reported, but has nonetheless been found in 93% of non-small cell lung cancer (Herman *et al.*, 1998; Wang *et al.*, 2003). The *MSH2* expression analysis on a panel of NF1 tumours failed to reveal any alteration in expression; however, the panel size was small. Other means of inactivation, such as *MSH2* mutations, have not been investigated in the present

study, and cannot be ruled out. Patients with *MSH2* homozygous deletion have been described to harbour NF1 features (Whiteside *et al.*, 2002; Bougeard *et al.*, 2003). It has however been suggested that loss of the MMR function, rather than any one particular gene, may affect the *NF1* gene (Whiteside *et al.*, 2002).

Another gene involved in DNA repair, *MGMT,* was found to be frequently hypermethylated in NF1 tumours, regardless of their malignancy status. These results would suggest that *MGMT* may be silenced by hypermethylation as an early event in NF1 tumorigenesis, as has been reported in other cancers (Esteller *et al.*, 2000b). The 48% hypermethylation status identified in this study is similar to the 43% identified by Gonzalez-Gomez *et al.* (2003a). The primers used in this study were modified from primers published in Esteller *et al.*, (1999), and the forward primers are approximately 40 bases upstream from those used by Gonzalez-Gomez *et al.* If the tumour type is considered, hypermethylation of the *MGMT* promoter was seen in 50% (6/12) malignant tumours (55% MPNSTs) and 47% (7/15) benign tumours. The latter is in keeping with the proportion reported by Gonzalez-Gomez *et al.* (2003a) for benign tumours (44%; 8/18). The present results show a slightly higher percentage of *MGMT* promoter hypermethylation in malignant tumours than the Gonzalez-Gomez study (33%, 1/3). However, the discrepancy could be attributed to the difference in panel size.

Additionally, *MGMT* was found hypermethylated in the only meningioma of the study. Meningiomas are non-glial tumours, also found as rare complications of NF1 (Créange *et al.*, 1999). *MGMT* hypermethylation has been observed in brain tumours, predominantly in gliomas (Esteller *et al.*, 1999; Mölleman *et al.*, 2005), but also in meningiomas (16%, Bello *et al.*, 2004).

In contrast to a report by Harada and co-workers, where hypermethylation of *MGMT* was found in 6% of rhabdomyosarcoma (1/18), the present study did not detect *MGMT* hypermethylation in the only rhabdomyosarcoma available (Harada *et al.*, 2002). A low incidence of hypermethylation in rhabdomyosarcoma (3%, 1/30) was also reported by Yeager *et al.* (2003). These results would suggest that *MGMT* is not commonly inactivated by hypermethylation in this tumour type. The lack of MGMT promoter hypermethylation has been linked to a high MGMT activity in rhabdomyosarcoma; results of significant importance in the context of therapy, as MGMT activity has been linked to rhabdomyosarcoma's poor response to chemotherapeutic agents (Yeager *et al.*, 2003).

Moreover, the presence of hypermethylation in the *MGMT* promoter, and possible inactivation, does not seem to be linked to the type of mutation in the *NF1* gene. These results suggest that *MGMT* inactivation may not have a direct influence on the mutation in the *NF1* gene. However, it may help the accumulation of mutations in other genes, as reported for the *TP53* (Esteller *et al.* 2001b; Nakamura *et al.*, 2001) or *K-Ras* genes (Esteller *et al*, 2000b). Mutations in *TP53* have been reported in NF1 malignant tumours (Nigro *et al.*, 1989; Menon *et al.*, 1990; Legius *et al.*, 1994; Birindelli *et al.*, 2001), but were not investigated in the present study.

The presence of hypermethylation in more than half the tumours studied may indicate that loss of DNA repair function is as necessary a step to the formation or evolution of NF1 tumours as for other tumours (Hanahan and Weinberg, 2000). It has been suggested that *MGMT* is the first line of defence in DNA repair, followed by the MMR function, and that loss of *MGMT* function may overload the repair system.

3.3.3.4/ The *RB1* and *CDKN2A* genes

The occurrence of *RB1* hypermethylation obtained here was somewhat lower (7.4%) than that reported by Gonzalez-Gomez and co-workers (14%; 2003a), but the discrepancies could be attributed to the sensitivity of the technique, and the heterogeneous nature of NF1 tumours. If considered by tumour type, hypermethylation of the *RB1* promoter was seen in 8% (1/12) malignant tumours (11% MPNSTs) and 7% (1/15) benign tumours. The Gonzalez-Gomez study reported a proportion of 33% (1/3) MPNSTs and 11% (2/18) benign tumours. Unfortunately, the two tumours, T137 and T169, were not available for the RT-PCR assay, which generally yielded similar levels of expression in the samples. Although first identified in retinoblastoma, *RB1* is also lost in many cancers, through epigenetic silencing, deletion or mutation (Sherr and McCormick, 2002), and the latter two mechanisms were not investigated in the present study.

Taken together, these results would suggest that silencing of *RB1* might occur occasionally in benign NF1 tumours. The loss of *RB1* in up to a third of malignant tumours could indicate a late event in the life of the tumour. Loss of *RB1* function has been associated with high-grades of malignancy (Crybs *et al.*, 1994; Nakamura *et al.*, 2001) and poor prognosis (Kishi *et al.*, 2005). However, in this study, the only MPNST (T169) that demonstrated hypermethylation was a low-grade MPNST. It may be possible that loss of *RB1* in this case may be a marker of the evolution of aggressiveness of the MPNST. Interestingly, tumour T169 was not found to

harbour LOH at *CDKN2A/p16*^{INK4a} (Section 4.2.3). It has been suggested that cells with loss of p16^{INK4a} function tend to retain wild-type Rb and *vice versa* (Sherr and Roberts, 1995). Thus the limited number of tumours with *RB1* promoter hypermethylation may represent a sub-group of tumour which harbours alteration of the Rb pathway through epigenetic inactivation of *RB1*, and not alteration of *CDKN2A/p16*^{INK4a}, often reported in NF1 malignant tumours (Section 4.3.3.2.1). In NF1 MPNSTs, deletion and loss of expression of RB1 have been reported in some studies (Berner *et al.*, 1999; Mawrin *et al.*, 2002), but not all (Kourea *et al.*, 1999a; Birindelli *et al.*, 2001; Ågesen *et al.*, 2005). This is indeed consistent with the event of mutually exclusive involvement of disruption of some genes, as a consequence of their involvement in the same pathway; examples include: *CDKN2A/p16*^{INK4a} mutation excluding alterations in *RB1* (or cyclin-D/CdK overexpression; Sherr and McCormick, 2002), and *TP53* mutations and *p14*^{ARF} mutations/deletions (Frank *et al.*, 2004).

The MTC was not found to harbour hypermethylation for the *RB1* promoter, nor was the *RB1* expression significantly decreased. In mouse models, *Rb*$^{+/-}$ mice are highly cancer prone and develop MTCs (Hu *et al.*, 1994; Williams *et al.*, 1994), and it has recently been shown that E2F3, a key downstream effector of Rb, is also involved in the metastasis of MTC from *Rb*$^{+/-}$ mice (Ziebold *et al.*, 2003). Thus it might be of significance to fully investigate the involvement of RB1 in rare complications, such as MTCs, arising in the context of NF1.

In this study, *CDKN2A/p16*^{INK4a} appeared consistently negative for hypermethylation, in contrast to the 22% hypermethylation in NF1 benign tumours identified by Gonzalez-Gomez *et al.* (2003a). The primers used in this study are identical to the set used by Gonzalez-Gomez *et al.* (2003a). In keeping with the results presented here, earlier studies also showed hypermethylation-related silencing of *CDKN2A/p16*^{INK4a} was absent in both MPNSTs and neurofibromas (Kourea *et al.*, 1999b, Nielsen *et al.*, 1999; Berner *et al.*, 1999; Ågesen *et al.*, 2005). Perrone *et al.* (2003) identified, by MS-PCR, hypermethylation of the *CDKN2A/p16*^{INK4a} promoter that was intermediary to the ones presented above: 6% hypermethylation was seen in NF1-related MPNSTs (12% including sporadic MPNSTs). However, the percentage of hypermethylation rose to 31% (NF1 and sporadic MPNSTs) when investigated using nested PCR. In other cancer studies, hypermethylation was also observed in exon 2 of *CDKN2A/p16*^{INK4a} at higher frequency than in the promoter (Konishi *et al.*, 2002), and has not been investigated in this study. Taken together, these results suggest that promoter hypermethylation is not a major mechanism for inactivation of *CDKN2A/p16*^{INK4a} in

NF1 tumours. Nevertheless, *CDKN2A/p16^{INK4a}* loss of expression is a common event in MPNSTs, and has been primarily associated with gross rearrangements of the gene and deletions (Kourea *et al.*, 1999b; Berner *et al.*, 1999; Nielsen *et al.*, 1999; Birindelli *et al.*, 2001; Perry *et al.*, 2002; Perrone *et al.*, 2003; Ågesen *et al.*, 2005; Section 4.3.3.2.1), which could explain the variations in expression observed in the real-time RT-PCR assay. Drastically reduced expression of *CDKN2A/p16^{INK4a}* was seen in MPNSTs T145 and T151. The former harboured a deletion (loss of heterozygosity, LOH) at the *CDKN2A* locus, whereas the latter appeared not to, despite showing LOH at the most centromeric marker (Section 4.2.3). These results do not however preclude the existence of other mechanisms of inactivation.

3.3.3.5/ The *RARB* gene

Only one malignant tumour, T160, harboured hypermethylation in the *RARB* promoter. Tumour T160 is a rhabdomyosarcoma, an extremely rare soft tissue sarcoma (STS) of the primitive muscle cells, and is found at increased incidence in NF1 patients (Yang *et al.*, 1995), with a prevalence of 1.4-6% in affected children (Reich *et al.*, 1999; Lampe *et al.*, 2002; Hadjustilianou *et al.*, 2002). Nuclear receptor RARβ, and isoforms RARα and RARγ, mediate the biological effects of retinoids, which includes inhibition of cell growth and induction of apoptosis in cancer cell lines (Lotan, 1995; Mangelsdorf *et al.*, 1995) through both p53-dependant (Lovat *et al.*, 1997; Toma *et al.*, 1997) and p53-independant (Dmitrovsky, 1997; Oridate *et al.*, 1997) pathways. Retinoids have been linked to inhibition of proliferation in STS cell lines (Gabbert *et al.*, 1988; Crouch and Helman, 1991) and STS cell lines resistant to the induction of apoptosis by retinoids did not express RARβ, although expression of the universal receptor RARγ was observed (Brodowicz *et al.*, 1999).

The *RARB* gene encodes several isoforms of RARβ, through use of different promoter and alternative splicing, and it is thought that the different isoforms, by allowing for a diversity in structure and pattern of expression, can control different subsets of retinoic acid-response genes, thus achieving the different effects of retinoic acids (Virmani *et al.*, 2000). Isoform RARβ2 is under the control of promoter P2, and loss of RARβ2 as well as aberrant methylation of promoter P2, has been implicated in breast cancer (Esteller *et al.*, 2001c; Yang *et al.*, 2002). Reduction in RARβ2 expression has also been correlated with hypermethylation of promoter P2 in lung (Virmani *et al.*, 2000), oesophageal (Kuroki *et al.*, 2003a), head and neck squamous cell carcinomas (Youssef *et al.*, 2004) and breast cancer metastasis (Mehrotra

et al., 2001). By contrast, *RARB* promoter hypermethylation was absent in 18 rhabdomyosarcomas and 5 rhabdomyosarcoma-derived cell lines (Harada *et al.*, 2002). The *RARB* gene is located at 3p24 and LOH at this locus has been reported in many tumours including breast, oesophageal, non-small cell lung and squamous cell carcinomas, and often correlated with reduced expression (Sekido *et al.*, 1998; Picard *et al.*, 1999; Virmani *et al.*, 2000; Zou et *al.*, 2001; Kuroki *et al.*, 2003a). Other modes of inactivation for *RARB* have not been investigated in the present study.

RARB is a relatively new TS gene, thought to be involved in the early events of tumorigenesis (Kuroki *et al.*, 2003a), and found hypermethylated in haematopoietic malignancies (Takahashi *et al.*, 2004), conditions that also occur with increased incidence in NF1 children (Shannon *et al.*, 1994; Side *et al.*, 1997) and *Nf1+/-* mice. These results could suggest that rare complications encountered in NF1, such as a rhabdomyosarcoma, may present a disruption of the same pathway, here *RARB* inactivation, as non-NF1-related tumours, but through a different mode of inactivation (here, promoter hypermethylation). T160 did not exhibit hypermethylation for any other genes in the study, nor was *RARB* hypermethylated in other tumours.

3.3.4/ Conclusion

The use of MS-PCR is a rapid and inexpensive way to obtain a global picture of separate gene methylation in a tumour. As more accurate methylation detection techniques are developed, the epigenetic map of cancer will become clearer. Additional use of expression profiling methods, such as real-time PCR, can not only complement MS-PCR results, but also provide insights into gene inactivation, and direct further study on the mechanisms involved. The results reported here add to our knowledge of NF1 tumorigenesis and progression to malignancy, mechanisms still largely unknown beyond the inactivation of the *NF1* gene. It is also the first report of *RASSF1A* involvement in NF1 malignancy. Additionally, the results outlined a tumour-specific gene inactivation, in multiple tumours arising in the context of a single condition. The data gathered here supports the idea that NF1 tumorigenesis is a complex multistep process, and emphasises the need for further investigation into candidate genes. Finally, the discovery of genes inactivated by hypermethylation in NF1 tumours could provide a unique opportunity to identify targets for therapy, as, unlike micro-lesions, aberrant methylation is a reversible process.

CHAPTER 4: MICROSATELLITE INSTABILITY AND CANDIDATE GENES FOR INVOLVEMENT IN MALIGNANCY IN NF1 TUMOURS

4.1 Introduction

Microsatellites are short (1-6bp), polymorphic sequences tandemly repeated in the human genome. Owing to the repetitive nature of microsatellites, errors in DNA replication can occur; in normal cells, the integrity of the genome is monitored by different mechanisms, and such nucleotide incorporation errors are repaired by the DNA mismatch repair (MMR) system. In cells where the MMR system is deficient, the expansion/deletion is not repaired, and, defects in microsatellite sequences can accumulate. Microsatellite instability (MSI) is defined as "a change of any length due to either insertion or deletion of repeating units, in a microsatellite within a tumour compared to normal tissue" (Boland *et al.*, 1998), and describes the presence of allelic shifts in different loci.

Microsatellite instability was first described in hereditary non-polyposis colorectal cancer (HNPCC), a familial condition that features defects in the MMR genes in up to 90% of the patients, and in sporadic colorectal cancer (Aaltonen *et al.*, 1993, Ionov *et al.*, 1993; Thibodeau *et al.*, 1993; Bishop and Hall, 1994; Hoang *et al.*, 1997). The two major MMR genes involved in HNPCC are *MLH1* and *MSH2*, found mutated in the majority of patients (Liu *et al.*, 1996; Arzimanoglou *et al.*, 1998). Since then, MSI has been observed in many cancers including lung (Shridhar *et al.*, 1994; Peltomäki *et al.*, 1993), bladder (Gonzalez-Zulueta *et al.*, 1993), cervical (Ou *et al.*, 1999), ovarian (Sood *et al.*, 1996; Cai *et al.*, 2004), pancreatic (Han *et al.*, 1993), gastric (Han *et al.*, 1993; Peltomäki *et al.*, 1993) and breast cancer (Siah *et al.*, 2000; Jönsson *et al.*, 1995) and acute myeloid leukaemia (Faulkner *et al.*, 2004).

Defects in the MMR pathway allow the accumulation of mutations in both non-coding (microsatellite) and coding regions, as the cell acquires a "mutator phenotype" (Loeb, 1991; Thibodeau *et al.*, 1996; Halling *et al.*, 1999). Thus accumulation of errors in key genes may result in the activation of oncogenes or inactivation of tumour suppressor genes and drive tumour progression (Loeb, 1998), by not only allowing the cell to advance through the multistep process of tumorigenesis but also promoting cellular proliferation at every stage (Kinzler and Vogelstein, 1996). Repeat sequence alterations in coding gene regions are likely

to result in frameshifts that truncate proteins. Markowitz *et al.* (1995) first reported inactivating mutations of the TGF growth factor beta receptor type II (*TGFB2R*) gene (TGF RII) in colorectal tumour with MSI; the mutation occurred within a poly $(A)_{10}$ repeat track (Wang *et al.*, 1995).

In NF1, MSI was first investigated by Ottini *et al.* (1995), who uncovered microsatellite alterations in 50% of neurofibromas as compared to normal tissue, suggesting a role for MSI in NF1 tumorigenesis. However, later reports failed to identify MSI in benign NF1 tumours (Serra *et al.*, 1997; Luijten *et al.*, 2000). A relationship between the *NF1* gene and the MMR system was also suggested by recent reports of individual harbouring a homozygous inactivation of an MMR gene (*MLH1*, *MSH2*, *MSH6* or *PMS2*). Interestingly, these patients manifested clinical signs of NF1, such as CALS, axillary freckling and neurofibromas, as well as haematopoietic cancers (Wang *et al.*, 1999; Ricciardone *et al.*, 1999; Graham *et al.*, 2001; Trimbath *et al.*, 2001; Vilkki *et al.*, 2001; Whiteside *et al.*, 2002; Bougeard *et al.*, 2003; Hedge *et al.*, 2003; De Vos *et al.*, 2004; Gallinger *et al.*, 2004; Menko *et al.*, 2004; Raevaara *et al.*, 2004).

Whilst *NF1* gene inactivation is required for neurofibroma formation (Serra *et al.*, 2001a), loss of neurofibromin alone cannot account for the tumours' transformation to malignancy. Alteration at *TP53* (Nigro *et al.*, 1989; Menon *et al.*, 1990; Glover *et al.*, 1991; Legius *et al.*, 1994; Jhanwar *et al.*, 1994; Luria *et al.*, 1997; Rasmussen *et al.*, 2000; Birindelli *et al.*, 2001; Leroy *et al.*, 2001; Koga *et al.*, 2001, section 4.3.3.2.2), *CDKN2A* (Berner *et al.*, 1999; Kourea *et al.*, 1999; Nielsen *et al.*, 1999; Birindelli *et al.*, 2001; section 4.3.3.2.1) and other genes (Section 1.3.4) have been reported in MPNSTs but not neurofibromas, suggesting that these genes are involved in the malignant progression of NF1 tumours.

The present study aimed to assess the extent of MSI in a large panel of NF1-related tumours, and help define the involvement of the MMR pathway. In particular, the *MLH1* gene was assessed for loss of heterozygosity in a selected tumour panel. Finally, the *TP53* and *CDKN2A* genes were assessed for LOH in NF1-related malignant tumours.

4.2 Results

4.2.1/ Microsatellite instability in NF1-related tumours

A total of 151 tumours were screened for MSI; the tumour types were represented thus: 16 malignant tumours (including 1 rhabdomyosarcoma and 1 medullary thyroid carcinoma, MTC), 5 plexiform neurofibromas and 130 dermal neurofibromas. The study was conducted on paired blood lymphocyte/tumour DNAs and/or multiple tumour DNAs.

Microsatellite instability was sought in NF1-related tumours by using 6 polymorphic microsatellite markers as previously described (Upadhyaya *et al.*, 2004), using large 6% polyacrylamide gels and silver staining. The markers used were located on chromosomes 1p (*MYCL*, a tetra-pentanucleotide repeat), 2p (BAT26, a mononucleotide repeat; D2S123, a dinucleotide repeat), 5q (*APC*, a dinucleotide repeat), 17q (D17S250, a dinucleotide repeat) and 18q (D18S58, a dinucleotide repeat). Earlier DNA samples, from B23 to T128.30 (Table 4.1) were originally screened for Mfd15 and D2S123 by Dr Song Han.

Additionally, in the 16 malignant tumours, markers D9S1751, D9S942, D9S1748 and D9S304 were used to determine LOH at the *CDKN2A* locus and in the 9p region in general, whereas LOH at *TP53* was assessed using marker p53alu. These markers also bear importance in the MSI study, as they were found to be unstable in some tumours.

Instances of MSI were repeated to ensure accuracy in their determination. Results for the analysis on every patient for the 6 MSI markers is summarised in Table 4.1. Additional MSI in the *CDKN2A* and *TP53* markers is listed in Table 4.2. Representative examples of the MSI observed are collected in Figures 4.1, 4.2 and 4.3. Part of this MSI study has been published (Upadhyaya *et al*, 2004).

Out of 135 benign tumours, only 3 dermal neurofibromas exhibited an allelic shift in 1/6 marker (one in D18S58, one in APC and one in Mfd15). None of these tumours demonstrated LOH in the *NF1* gene, and the associated somatic mutation is not known. Additionally, one dermal neurofibroma, T142.2, exhibited instability in 3/6 markers (D18S58, D2S123 and MYCL). However, this tumour seems to represent an isolated case of high genomic instability, and the loss of heterozygosity (LOH) study for the *NF1* gene also showed instability in two *NF1* intragenic markers (J1J2, EVI20), as reported in Section 6.2.4.1.

By contrast, 7/14 MPNSTs and 1/1 MTC displayed different degrees of instability. One MPNST, T116, harboured the highest MSI, with instability in 4/6 markers (66%). Moreover, the same tumour also manifested instability at additional markers D9S304, D9S1751 and p53alu. Tumour T116 harboured a somatic deletion in exon 10c (1541delAG) predicted to result in a truncated transcript but the germline mutation was not identified (mutation analysis conducted by Dr Song Han; Upadhyaya *et al.*, 2004).

MPNST T87 displayed instability in 3/6 markers (50%), and also in additional markers D9S304, D9S1751 and p53alu. No mutations in the *NF1* gene have been identified in this patient.

One malignant tumour, T145 exhibited instability in 2/6 markers (33%), in markers D18S58 and *APC*. Tumour T145 also demonstrated instability in marker p53alu. The germline mutation in this patient was a 2bp deletion in exon 28 (Section 6.2.4.1.3) and the tumour harboured a LOH encompassing the *NF1* gene (Section 6.2.4.1).

Finally, four MPNSTs (T151, T165, T169 and T178) and the MTC (T185) showed instability in 1/6 marker (16%), either in D18S58 or *MYCL*. Of the four MPNSTs, only T151 also demonstrated additional instability in marker p53alu. All five tumours but T178 (no identified mutation) harboured some degree of LOH in the *NF1* gene (section 6.2.4.1). Tumours T151 and T165 belonged to patient P151, who presented with a splice site-related germline mutation in intron 35 (Section 6.2.4.1.1). Tumours T169 and T178 were donated by patients P169 and P178, respectively; these two patients are brothers and presented the same germline nonsense mutation in exon 11 (Section 6.2.4.1.2). Patient P185 harboured a 1bp germline deletion in exon 6 (Section 6.2.4.1.4).

Microsatellite instability was generally seen as either compressions (shortening of repeats, Figures 4.1 and 4.3), or expansions (lengthening of repeats, Figures 4.1, 4.2 and 4.3), both strong indicators of MSI. Occasional additional alleles and allelic imbalances (Figures 4.1 and 4.2) were also observed and can be considered as indirect indicators of MSI (Dams *et al.*, 1995).

These results would suggest that MSI is more common in malignant NF1 tumours than in benign NF1 tumours. As only half of the MPNSTs harboured some degree of instability, MSI may occur at a later stage in the development of the tumour and reflect tumour evolution or aggressiveness.

Table 4.1: Results from the MSI study on 151 NF1-related tumours using 6 microsatellite markers. Samples that were successfully analysed are indicated with a *. *I* on a red background denotes instability. *ND*: repeated PCR was unsuccessful.

		Markers						
Blood/Tumours		D18S58	Mfd15	D2S123	MYCL	BAT 26	APC	
Blood	B23	*	*	*	*	*	*	Neurofibromas
Tumours	T23.3	*	*	*	*	*	*	
	T23.4	*	*	*	*	*	*	
	T23.6	*	*	*	*	*	*	
	T23.7	*	*	*	*	*	*	
	T23.8	*	*	*	*	*	*	
	T23.9	*	*	*	*	*	*	
	T23.10	*	*	*	*	*	*	
Blood	B30	*	*	*	*	*	*	
Tumour	T30	*	I	*	*	*	*	
Blood	B34	*	*	*	*	*	*	
Tumour	T34.2	*	*	*	*	*	*	
Blood	B37	*	*	*	*	*	*	
Tumours	T37.1	*	*	*	*	*	*	
	T37.2	*	*	*	*	*	*	
Blood	B38	*	*	*	*	*	*	
Tumour	T38	*	*	*	*	*	*	
Blood	B56	*	*	*	*	*	*	
Tumour	T56	*	*	*	*	*	*	
Blood	B60	*	*	*	*	*	*	
Tumour	T60	*	*	*	*	*	*	
Blood	B63	*	*	*	*	*	*	
Tumours	T63.2	*	*	*	*	*	*	
	T63.8	*	*	*	*	*	*	
Blood	B65	*	*	*	*	*	*	
Tumour	T65	*	*	*	*	*	*	
Blood	B68	*	*	*	*	*	*	
Tumours	T68.1	*	*	*	*	*	*	
	T68.2	*	*	*	*	*	*	
	T68.3	*	*	*	*	*	*	
Blood	B74	*	*	*	*	*	*	
Tumours	T74.1	*	*	*	*	*	*	
	T74.3	*	*	*	*	*	*	
Blood	B81	*	*	*	*	*	*	
Tumour	T81.1	*	*	*	*	*	*	
Blood	B88	*	*	*	*	*	*	
Tumours	T88.1	*	*	*	*	*	*	
	T88.2	*	*	*	*	*	*	
Blood	B89	*	*	*	*	*	*	
Tumours	T89.1	*	*	*	*	*	*	
	T89.2	*	*	*	*	*	*	
Blood	B90	*	*	*	*	*	*	
Tumour	T90	*	*	*	*	*	I	
Blood	B98	*	*	*	*	*	*	
Tumours	T98.3	*	*	*	*	*	*	
	T98.5	*	*	*	*	*	*	
	T98.6	*	*	*	*	*	*	
Blood	B99	*	*	*	*	*	*	
Tumour	T99	*	*	*	*	*	*	

Table 4.1: continued

Blood/Tumours		Markers						
		D18S58	Mfd15	D2S123	MYCL	BAT 26	APC	
Blood	B100	*	*	*	*	*	*	Neurofibromas
Tumour	T100	*	*	*	*	*	*	
Blood	B101	*	*	*	*	*	*	
Tumours	T101.2	*	*	*	*	*	*	
Blood	B106	*	*	*	*	*	*	
Tumours	T106.1	*	*	*	*	*	*	
	T106.2	*	*	*	*	*	*	
	T106.5	*	*	*	*	*	*	
Blood	B109	*	*	*	*	*	*	
Tumours	T109.1	*	*	*	*	*	*	
	T109.4	*	*	*	*	*	*	
Blood	B111	*	*	*	*	*	*	
Tumours	T111.1	*	*	*	*	*	*	
	T111.2	*	*	*	*	*	*	
Blood	B113	*	*	*	*	*	*	
Tumour	T113	*	*	*	*	*	*	
Multiple tumours	T128.1	*	*	*	*	*	*	
	T128.2	*	*	*	*	*	*	
	T128.3	*	*	*	*	*	*	
	T128.4	*	*	*	*	*	*	
	T128.5	*	*	*	*	*	*	
	T128.6	*	*	*	*	*	*	
	T128.7	*	*	*	*	*	*	
	T128.8	*	*	*	*	*	*	
	T128.9	*	*	*	*	*	*	
	T128.10	*	*	*	*	*	*	
	T128.11	*	*	*	*	*	*	
	T128.12	*	*	*	*	*	*	
	T128.13	*	*	*	*	*	*	
	T128.14	*	*	*	*	*	*	
	T128.15	*	*	*	*	*	*	
	T128.16		*	*	*	*	*	
	T128.17	*	*	*	*	*	*	
	T128.18	*	*	*	*	*	*	
	T128.19	*	*	*	*	*	*	
	T128.20	*	*	*	*	*	*	
	T128.21	*	*	*	*	*	*	
	T128.22	*	*	*	*	*	*	
	T128.23	*	*	*	*	*	*	
	T128.24	*	*	*	*	*	*	
	T128.25	*	*	*	*	*	*	
	T128.26	*	*	*	*	*	*	
	T128.27	*	*	*	*	*	*	
	T128.28	*	*	*	*	*	*	
	T128.30	*	*	*	*	*	*	
Blood	2125/2142	*	*	*	*	*	*	
Tumours	T133.1	*	*	*	*	*	*	
	T133.2	*	*	*	*	*	*	
	T133.3	*	*	*	*	*	*	
	T133.4	*	*	*	*	*	*	
	T137	*	*	*	*	*	*	
	T142.1	*	*	*	*	*	*	

able 4.1: continued

Blood/Tumours		Markers						
		D18S58	Mfd15	D2S123	MYCL	BAT 26	APC	
	T142.2	I	*	I	I	*	*	Neurofibromas
	T142.3	*	*	*	*	*	*	
Blood	2126	*	*	*	*	*	*	
Tumours	T135.1	*	*	*	*	*	*	
	T135.2	*	*	*	*	*	*	
Blood	2127	*	*	*	*	ND	*	
Tumours	T136.1	*	*	*	*	*	*	
	T136.2	*	*	*	*	*	*	
Blood	2128	ND	*	*	*	*	*	
Tumour	T139	ND	*	*	ND	*	*	
Multiple tumours	T140.1	*	*	*	*	*	*	
	T140.2	*	*	*	*	*	*	
	T140.3	*	*	*	*	*	*	
	T140.4	*	*	*	*	*	*	
	T140.5	*	*	*	*	ND	*	
Blood	2141	*	*	*	*	*	*	
Tumours	T141.1	*	*	*	*	*	*	
	T141.2	*	*	*	*	*	*	
	T141.3	*	*	*	*	*	*	
	T141.4	*	*	*	*	*	*	
	T141.5	*	*	*	*	*	*	
	T141.6	*	*	*	*	ND	*	
	T141.7	ND	*	*	*	ND	*	
	T141.8	*	*	*	*	*	*	
	T141.9	*	*	*	*	*	*	
	T141.10	*	*	*	*	ND	*	
	T141.11	*	*	*	*	*	*	
	T141.12	*	*	*	*	*	*	
	T141.13	*	*	*	*	*	*	
	T141.14	*	*	*	*	*	*	
	T141.15	ND	*	*	*	*	*	
	T141.16	*	*	*	*	*	*	
Multiple tumours	T143.1	*	*	*	*	*	*	
	T143.2	*	*	*	*	*	*	
	T143.3	*	*	*	*	*	*	
	T143.4	*	*	*	*	*	*	
	T143.5	*	*	*	*	*	*	
	T143.6	*	*	*	*	*	*	
	T143.7	*	*	*	*	*	*	
Multiple tumours	T144.2	*	*	*	*	*	*	
	T144.3	*	*	*	*	*	*	
Blood	2156	*	*	*	*	*	*	
Tumour	T147	*	*	*	*	*	*	
Blood	2157	*	*	*	*	*	*	
Tumour	T149	*	*	*	*	*	*	

Table 4.1: continued

Blood/Tumours		Markers						
		D18S58	Mfd15	D2S123	MYCL	BAT 26	APC	
Multiple tumours	T150.1	*	*	*	*	ND	*	Neurofibromas
	T150.2	*	*	*	*	ND	*	
	T150.4	*	*	*	*	ND	*	
Blood	2174	*	*	*	*	*	*	
Tumours	T152.1	*	*	*	*	*	*	
	T152.2	*	*	*	*	*	*	
Blood	2175	*	*	*	*	*	*	
Tumour	T153	*	*	*	*	*	*	
Multiple tumours	T157.1A	*	*	*	*	*	*	
	T157.2A	*	*	*	*	*	*	
Blood	2227	*	*	*	*	*	*	
Tumour	T159	*	*	*	*	*	*	
Blood	2288	*	*	*	*	*	*	
Tumour	T186	*	*	*	*	*	*	
Multiple tumours	T176.1	*	*	*	*	*	*	
	T176.2	*	*	*	*	*	*	
	T176.3	*	*	*	*	*	*	
Blood	B108	*	*	*	*	*	*	Plexiform neurofibromas
Tumour	T108.12	*	*	*	*	*	*	
Blood	B110	*	*	*	*	*	*	
Tumours	T110.1	*	*	*	*	*	*	
	T110.2	*	*	*	*	*	*	
Blood	2170	ND	*	*	ND	*	ND	
Tumours	T156.1B	*	*	*	*	*	*	
	T156.1D	*	*	*	*	*	*	
Blood	2229	*	*	*	*	*	*	
Tumour	T167	*	*	*	*	*	*	
Blood	B87	*	*	*	*	*	*	Malignant NF1 tumours
Tumour	T87		*	*		*		
Blood	B115	*	*	*	*	*	*	
Tumour	T115	*	*	*	*	*	*	
Blood	B116	*	*	*	*	*	*	
Tumour	T116		*			*		
Blood	B118	*	*	*	*	*	*	
Tumour	T118	*	*	*	*	*	*	
Blood	2124	*	*	*	*	*	*	
Tumour	T130.2a	*	*	*	*	*	*	
Blood	2155	*	*	*	*	*	*	
Tumour	T145.2d		*	*	*	*		
Blood	2158	*	*	*	*	*	*	
Tumour	T148.3b	*	*	*	*	*	*	
Blood	2159	*	*	*	*	*	*	
Tumours	T151.3	*	*	*	I	*	*	
	T165a	*	*	*	I	*	*	
Blood	2176	*	*	*	*	*	*	
Tumour	T154.1b	*	*	*	*	*	*	
Blood	2207	*	*	*	*	*	*	
Tumour	T169	*	*	*	I	*	*	
Blood	2230	*	*	*	*	*	*	
Tumour	T178	I	*	*	*	*	*	

Table 4.1: continued

Blood/Tumours		Markers D18S58	Mfd15	D2S123	MYCL	BAT 26	APC	Malignant NF1 tumours
Blood	B168	*	*	*	*	*	*	
Tumour	T168	*	*	*	*	*	*	
Blood	2205	*	*	*	*	*	*	
Tumour	T160	*	*	*	*	*	*	
Blood	2316	*	*	*	*	*	*	
Tumour	T184	*	*	*	*	*	*	
Blood	2318	ND	*	*	*	*	*	
Tumour	T185	*	*	*	I	*	*	

Table 4.2: Summary of LOH/MSI at 9p and TP53 markers in NF1 malignant tumours.

UI, uninformative for LOH; *LOH*, loss of heterozygosity for the marker.

Blood/Tumours		Markers D9S304	D9S1748	D9S942	D9S1751	p53Alu
Blood	B23	*	*	*	*	*
Tumours	T23.11	*	*	*	*	LOH
	T23.12	*	*	*	*	*
Blood	B87	*	*	*	*	*
Tumour	T87	I	*	*	I	I
Blood	B115	*	*	*	*	*
Tumour	T115	*	*	*	*	*
Blood	B116	*	*	*	*	*
Tumour	T116	I	*	*	I	I
Blood	B118	*	*	*	*	UI
Tumour	T118	*	*	*	*	UI
Blood	2124	UI	*	*	*	*
Tumour	T130.2a	UI	*	*	*	*
Blood	2155	*	*	*	*	*
Tumour	T145.2d	*	LOH	LOH	LOH	I
Blood	2158	UI	*	*	*	UI
Tumour	T148.3b	UI	*	*	*	UI
Blood	2159	*	*	*	*	*
Tumours	T151.3	LOH	*	*	*	I
	T165a	LOH	*	*	*	*
Blood	2176	*	*	*	*	*
Tumour	T154.1b	*	*	*	*	*
Blood	2207	*	*	*	*	*
Tumour	T169	*	*	*	*	*
Blood	2230	*	UI	*	*	*
Tumour	T178	*	UI	*	LOH	*
Blood	B168	*	*	*	*	*
Tumour	T168	LOH	LOH	LOH	LOH	LOH
Blood	2205	*	*	*	*	UI
Tumour	T160	*	*	*	*	UI
Blood	2316	*	*	*	*	UI
Tumour	T184	*	*	*	*	UI
Blood	2318	*	*	*	*	*
Tumour	T185	*	*	*	*	*

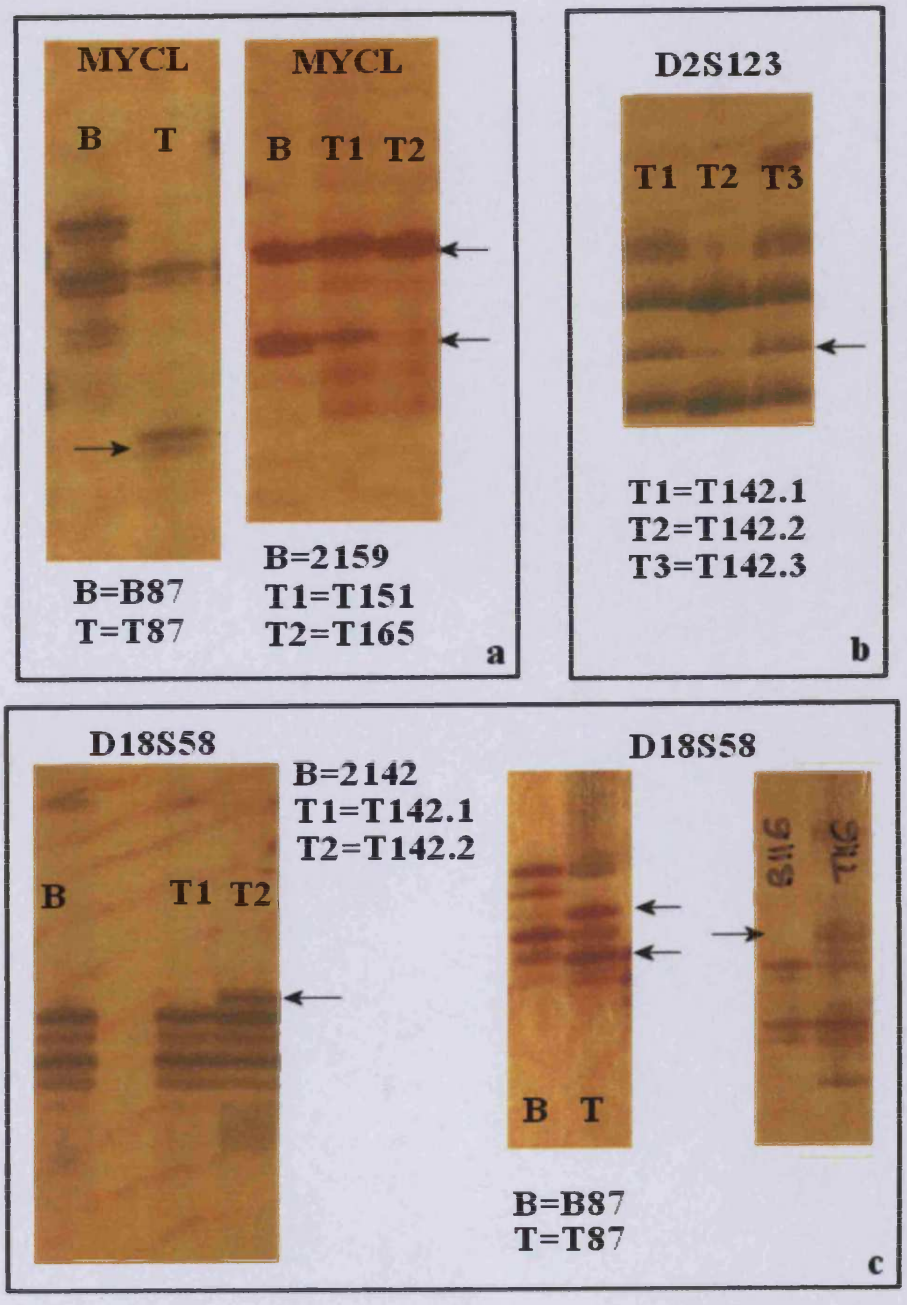

Figure 4.1: Representation of MSI in microsatellite markers in NF1 tumours

a. marker MYCL (1p): arrow points to a compression in tumour T187, to an allelic imbalance in tumour T151 and to an expansion in tumour T165, as compared to the corresponding blood sample.

b. marker D2S123 (2p): arrow points to an allelic imbalance in neurofibroma T142.2 as compared to other tumours from the same patient.

c. marker D18S58 (18q): tumour T142.2 showed an additional band (although the presence of 3 bands may also be due to the cellular heterogeneity of the tumour). In T87, the two alleles have shifted, and it cannot be determined whether these are due to compressions or expansions. Arrow points to a compression in tumour T116.

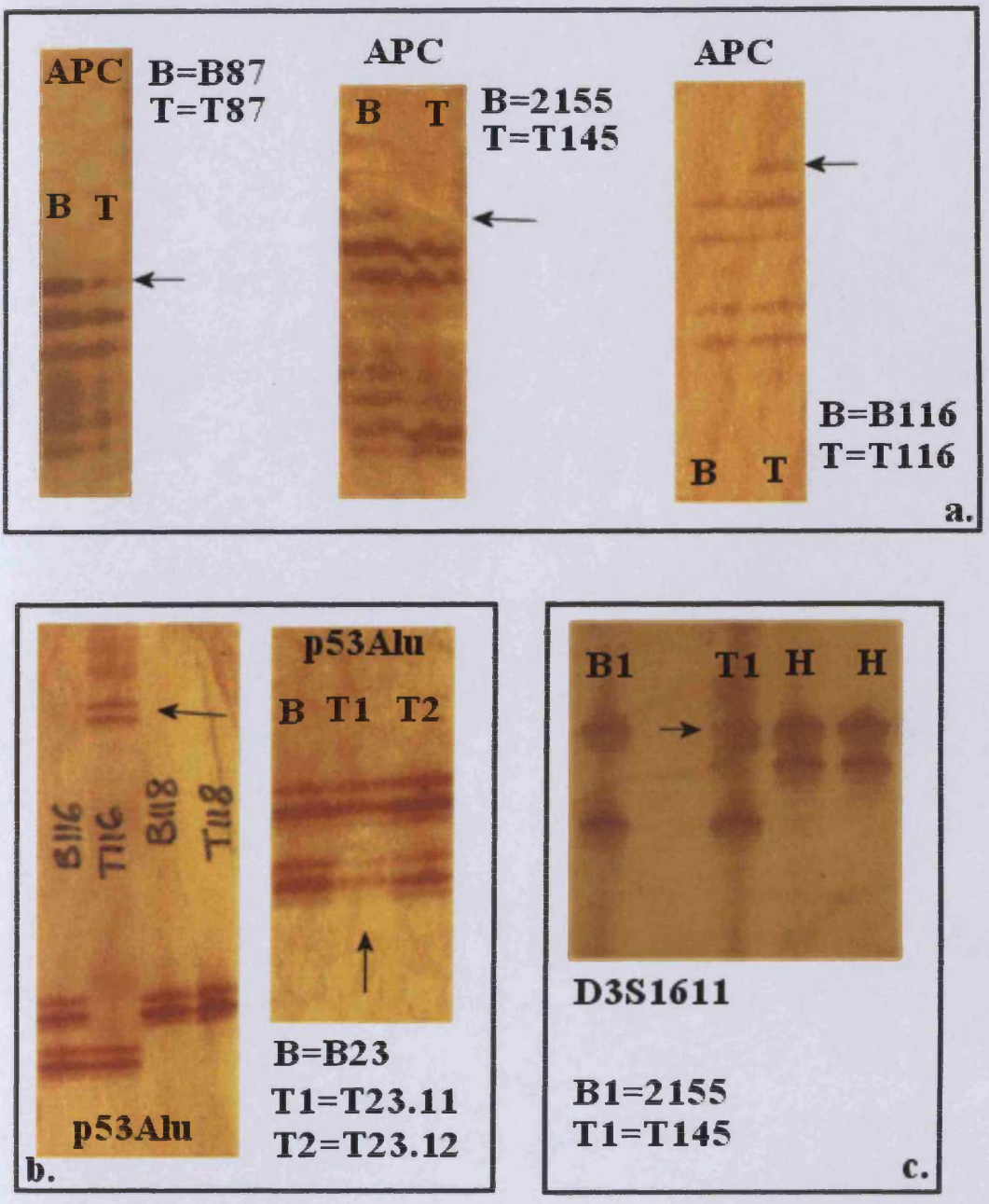

Figure 4.2: Representation of LOH and MSI in NF1 tumours.

a. marker APC (5q): tumour T87 shows an allelic loss in the marker. Arrow points to an allelic imbalance in tumour T145; there is also an additional band on the bottom of the gel. Tumour T116 shows an additional band.

b. marker p53Alu: arrow points to a compression in tumour T116, whereas LOH can be seen in tumour T23.11

c. marker D3S1611: arrow points to a LOH in the top band of tumour T145, compared to the corresponding blood. *H*, heterozygous samples.

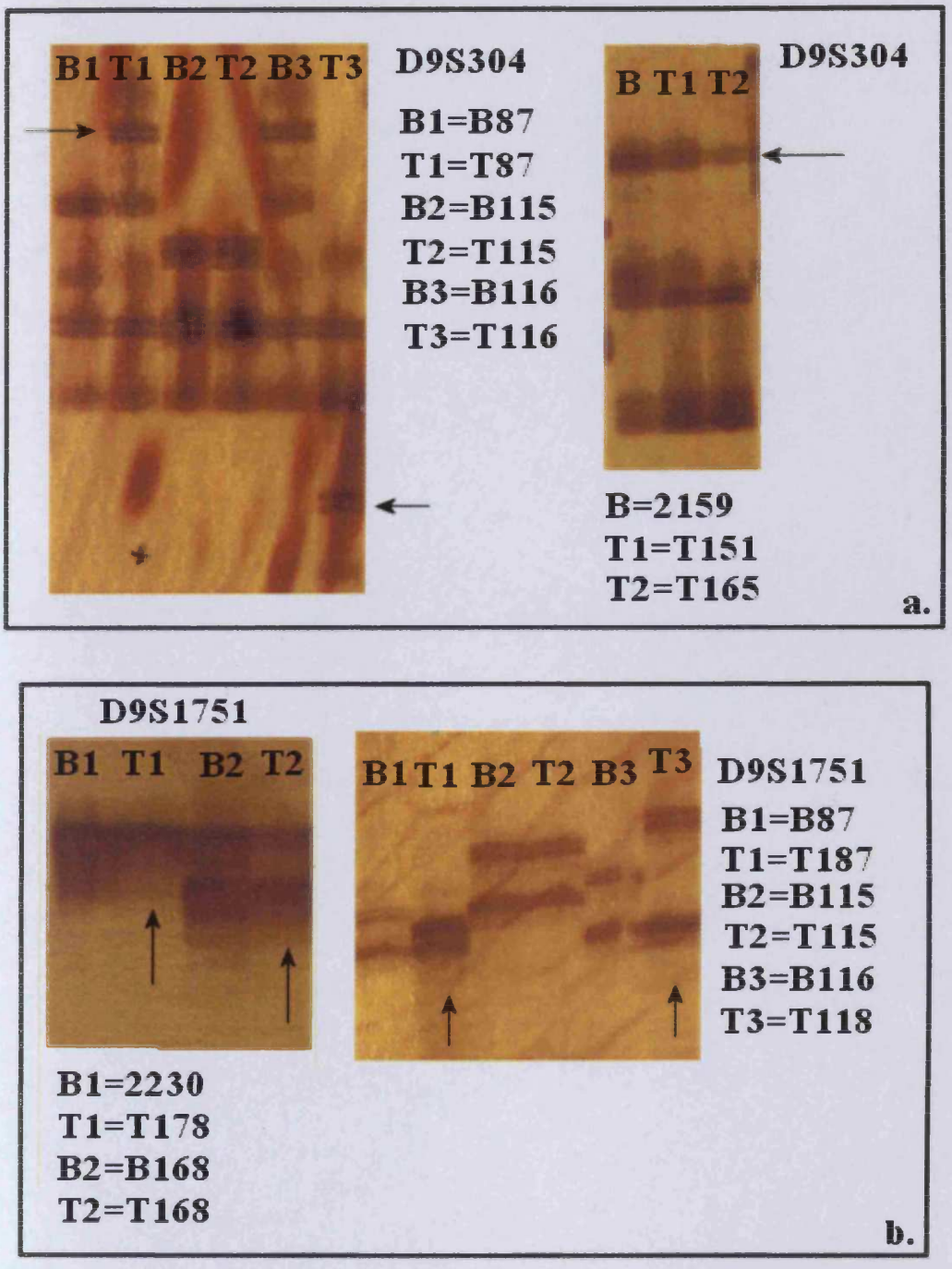

Figure 4.3: Representation of LOH and MSI at 9p markers in NF1 tumours

a. marker D9S304: arrow points to an expansion in tumour T87, and to a compression in tumour T116. By contrast, tumours T151 and T165 show a dosage difference indicative of LOH.

b. marker D9S1751: arrow points to LOH in both tumours T178 and T168. In T187, compression can be seen, whereas tumour T118 exhibited an expansion at the same marker.

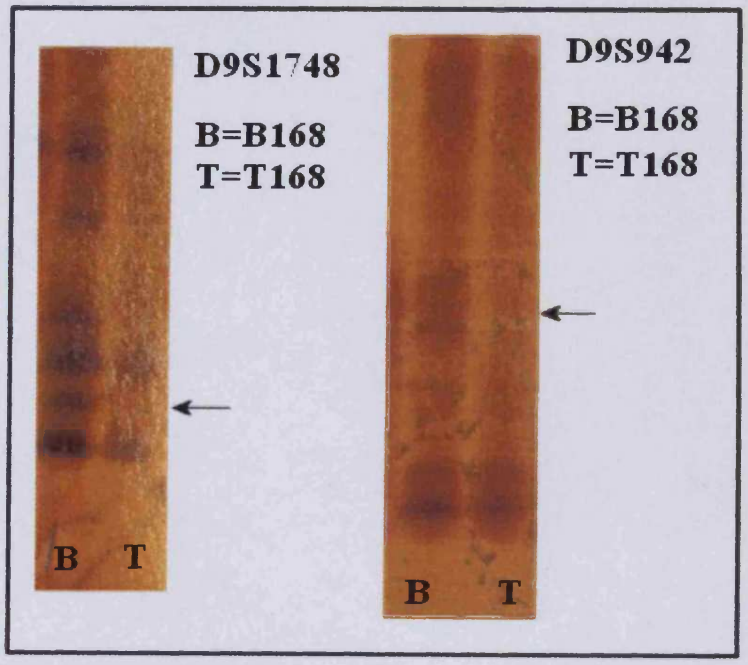

Figure 4.4: Representation of LOH at two near-*CDKN2A/p16*^{INK4a} markers in an NF1 MPNST, as indicated by the arrow.

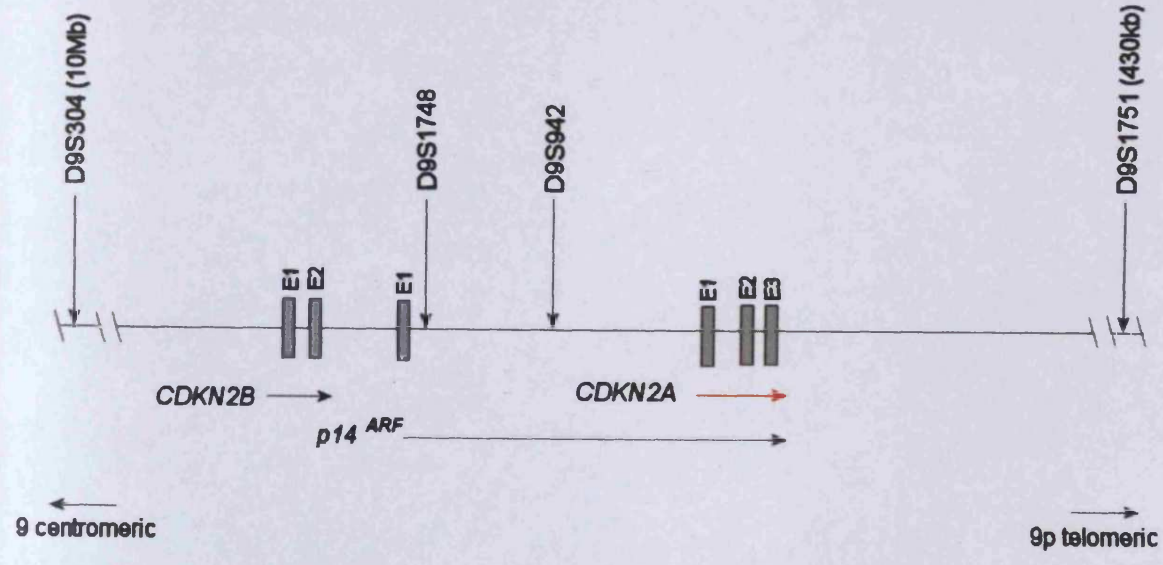

Figure 4.5: Representation of 9p21, the *CDKN2A* and *CDKN2B* loci, and relative marker distribution.

4.2.2/ LOH at the *MLH1* locus

Loss of heterozygosity at *MLH1* was sought in 10 malignant tumours from the original panel (Section 4.2.1; Table 4.3) using marker D3S1611 (Limpaiboon *et al.*, 2002; Smidgiel *et al.*, 2004). Older samples were no longer available, and could not be tested. Marker D3S1611 is located in intron 12 of *MLH1* (Annese *et al.*, 2002) and has previously been used as the sole marker to determine LOH in *MLH1* (Limpaiboon *et al.*, 2002; Limpaiboon *et al.*, 2005). Results are summarized in the following table (Table 4.3) and an example of LOH at D3S1611 can be found in Figure 4.2.

Table 4.3: Results from LOH study at MLH1 in 10 NF1 malignant tumours.
METH, positive methylation status at the *MLH1* promoter (Section 3.2.2.4); *NI*, marker was not informative.

Tumour	MSI	LOH	METH
T145	**2/6**	**YES**	NO
T151	**1/6**	**YES**	NO
T165	**1/6**	**YES**	NO
T185	**1/6**	**YES**	**YES**
T178	1/6	NI	NO
T169	1/6	NI	NO
T130	NO	NO	NO
T160	NO	NI	NO
T168	NO	NO	NO
T184	NO	NO	NO

Of 11 tumours, four (T145, T151, T165 and T185) exhibited LOH for *MLH1*. Interestingly, these four tumours had also displayed some degree of instability in the MSI analysis. Additionally, MPNST T151 appeared to show a reduction of expression for *MLH1* in the real-time RT-PCR assay, and MTC T185 displayed both reduced expression and methylation of the *MLH1* promoter (Section 3.2.2.4). The *MLH1* gene can be inactivated by different mechanisms in cancer (Jones and Laird, 1999), and the LOH results observed in tumours T151 and T185 are consistent with the reduction in expression observed in Section 3.2.2.4. By contrast, tumours T145 and T165 did not appear to show a drastically reduced expression, which may indicate that the allelic imbalance observed is in fact related to MSI. Alternatively,

the marker is located in intron 12, and the LOH observed may not affect the gene transcript. The results presented here are based on one informative marker alone, and it therefore cannot be ruled out that the apparent LOH observed in the samples may in fact be associated with instability, especially since these tumours displayed MSI at other markers. However, marker D3S1611 has been used in previous studies to determine both LOH and instability (Limpaiboon *et al.*, 2002). Although the issue of *MLH1* inactivation would require further investigation, the apparent correlation between *MLH1* LOH and microsatellite instability in the NF1 tumours is intriguing.

Two MPNSTs (T169 and T178), which displayed instability in one marker, could not be assessed for LOH in *MLH1*, as D3S1611 was not informative for these tumours. The remaining malignant tumours (T130, T160, T168 and T184), which had been found to be negative for MSI, did not harbour LOH for *MLH1* at the marker locus used. Finally, *MLH1* LOH was investigated in 13 NF1 benign tumours (taken from the mutation detection panel in Section 6.2.4). The marker was uninformative in one tumour, and showed a normal profile in the remaining samples.

4.2.3/ LOH at the *CDKN2A* and *TP53* loci

Allelic loss on 9p, and at the *CDKN2A* locus in particular, was assessed using markers D9S1751, D9S942, D9S1748 and D9S304. Markers D9S1751 and D9S304 are ~430 kb distal and ~10 Mb distal to *CDKN2A*, respectively, and markers D9S942 and D9S1748 are both on 9p21, closely linked to the *CDKN2A* locus (Cairns *et al.*, 1995; Hartmann *et al.*, 2000; Welch *et al.*, 2001; Figure 4.5). D9S942 is a highly polymorphic marker located within 20kb of the *CDKN2A/p16^{INK4a}* gene (Ohta *et al.*, 1996) and D9S1748 is located adjacent to exon 1 (of p14^{ARF}) on the locus (Mao *et al.*, 1995). In previous studies, D9S942 and D9S1748 have successfully been used to detect *CDKN2A* locus LOH in urinary bladder cancer (Berggren *et al.*, 2003), melanoma (Cachia *et al.*, 2000) and renal carcinoma (Schraml *et al.*, 2001).

The analysis was conducted on the 16 malignant tumours from Section 4.2.1 and results are summarized in Table 4.2.

Tumour T168 showed allelic loss at all four markers, suggesting that deletion of the majority, or entirety of the chromosome 9 short arm occurred in this tumour.

MPNST T145 harboured LOH at markers D9S1751, D9S942 and D9S1748. Thus the tumour may have lost the two genes at the *CDKN2A* locus and $p15^{INK4B}$ (*CDKN2B*), to which D9S1748 is also close. The deletion may span most of the short arm of chromosome 9p, but not all of it, as D9S304, closest to the centromere (9p13), is heterozygous.

Two MPNSTs from the same patient, T151 and T165, both harboured LOH at marker D9S304, but not at any of the three other markers. This would suggest that the *CDKN2A* locus is not altered in these tumours. The apparent allelic loss at marker D9S304 may in fact be a sign of MSI. Distinguishing between LOH and allelic loss associated with instability can be difficult when using isolated markers. Loss of heterozygosity (LOH) would usually span a larger region of the chromosome, whereas MSI-associated loss would be restricted to the marker used. Thus the use of additional markers closely flanking the marker showing allelic loss would allow one to distinguish between the two events (Dams *et al.*, 1995).
Interestingly, three malignant tumours (T145, T151 and T165) displayed reduced levels of expression for $CDKN2A/p16^{INK4a}$, to different degrees, in the real-time RT-PCR assay, and there was no evidence of hypermethylation at the $CDKN2A/p16^{INK4a}$ promoter (Section 3.2.2.1). These results were discussed in Section 3.3.3.4 and may indicate other means of gene inactivation in these three tumours, such as a mutation, or hypermethylation in other parts of $CDKN2A/p16^{INK4a}$ (exon 2, for example; Konishi *et al.*, 2002), or as seen previously in NF1 tumours, homozygous deletion (HD) of exon 2 only (Perrone *et al.*, 2003), all of which would not have been detected in the two studies (Section and 3.2.2.1).

Tumour T178 harboured LOH at marker D9S1751 only, and this cannot be ruled out as instability. Although marker D9S1748 was not informative for this patient, the heterozygosity of marker D9S942 would suggest that the *CDKN2A* locus is not altered by deletion in this tumour.

The p53Alu marker is located in the first intron of the *TP53* gene (Futreal *et al.*, 1991). Results from this study are summarized in Table 4.2 and an example of LOH at p53Alu can be found in Figure 4.2. Of the 16 malignant tumours, two, T23.11 and T168, displayed LOH at the *TP53* locus using the intragenic p53Alu marker. Tumour T23.11 is a MPNST that exhibited LOH at the *NF1* gene from intron 41 to the 3' flanking region (LOH study conducted by Dr Song Han; Upadhyaya *et al.*, 2004).

4.3 Discussion

4.3.1/ Detection of microsatellite instability

4.3.1.1/ Acrylamide gel/ silver staining technique

As commented upon in Section 6.3.1.2, regarding the LOH assay, the use of large vertical gels and silver staining can be labour intensive and time-consuming. However, as a PCR-based technique, it does not require any particular modification of the primers and is also relatively inexpensive.

With regard to the MSI study, a number of *caveats* inherent to the use of this technique have been documented (Oda *et al.*, 2002) and include migration and *Taq* DNA polymerase errors. The latter has a tendency for causing slippage within repetitive sequences, and also possesses a terminal deoxynucleotidyl transferase (TDR) activity, which can add one base-pair to PCR products. Thus, the use of *Taq* DNA polymerase may bias the results, particularly in the case of mononucleotide repeats. The instability reported here was repeatedly seen in at least two independent runs, and was therefore included in the results.

While the technique used here was probably the most efficient way to determine MSI at the time of this study, the inherent limitations in sensitivity of the method must be taken into account. Furthermore, in the case of dermal neurofibromas, the PCR amplifies DNA from a heterogeneous cell population and, although the tumour tissues were carefully dissected, some level of contamination by "normal" surrounding tissue cannot be excluded. As a result, MSI-associated allele loss, for example, may be less evident and hence the level of MSI in these tumours may be underestimated.

The current improved technique relies on the analysis of fluorescent PCR products and subsequent automated analysis on a genetic analyser equipped with software for fragment analysis (Dietmaier *et al.*, 1999; Maehara *et al.*, 2001; Faulkner *et al.*, 2004; Cai *et al.*, 2004). The simplicity of the PCR-based technique, coupled with the sensitivity of fluorescence labelled products, allows the ready detection of novel alleles present in as few as 5% of cells (Olipitz *et al.*, 2001). The technique can also be performed as a multiplex fluorescence-based PCR assay, to improve on screening time (Broaddus *et al.*, 2004). Such an approach could be considered for markers that are less easy to read on a gel, such as mononucleotide repeats. This type of technology would also help to circumvent the "human error" associated with reading a gel and allow quantification of the result, which may prove particularly useful in the

case of NF1 tumours. It would certainly represent an attractive alternative to large gels and silver staining.

4.3.1.2/ Markers relevance

Since MSI is a hallmark of HNPCC, it stands to reason that the markers used for scoring MSI, and the cut-off points to determine MSI, reflect the studies performed on this disease. Owing to the differences in experimental approaches, a consensus protocol was drafted in 1997 at the National Cancer Institute (NCI) workshop. The guideline recommended the use of 5 markers for detection of MSI in HNPCC (Boland *et al.*, 1998). However, MSI has also been observed in other tumour types, and the utility/relevance of the HNPCC guidelines in the context of other cancers has yet to be determined. It has been suggested that the five marker panel may not detect MSI in tumours that fall outside the usual tumour spectrum of HNPCC (Broaddus *et al.*, 2004). It is possible that markers informative for one tumour type may be less informative in another. For example, screening with BAT26 has been proposed as a rapid method to determine MSI and MMR defects in colorectal cancer, as it is highly informative in these tumours (Hoang *et al.*, 1997; Hatch *et al.*, 2005). The same marker, however, did not prove as informative for MSI in cervical cancer (Ou *et al.*, 1999), breast cancer (Siah *et al.*, 2000) or acute myeloid leukaemia (Faulkner *et al.*, 2004). Since this marker is located in the *MSH2* gene, it has been proposed that instability of BAT26 may be implicated in tumours with accumulation of mutations directly related to MMR genes and therefore this marker may not be suitable for other cancers with microsatellite instability (Ou *et al.*, 1999).

In the present study, BAT26 did not detect MSI in NF1 tumours. This echoes the results obtained by Luijten et *al.* (2000b) on a small panel of NF1-related tumours with the same marker. Their results were also contrasted against an MSI-positive duodenal carcinoma, for which BAT26 showed instability.

Another example is D2S123: reported unstable in a majority of HNPCC cases (Aaltonen *et al.*, 1993), the marker was deemed less informative in other cancers (Arzimanoglou *et al.*, 1998). Markers Mfd15 and D2S123, both (CA) dinucleotide repeats, were also rarely informative for MSI in the present study, each showing instability in one tumour. By contrast, two other (CA) dinucleotides, D18S58 and *APC*, were instable in 6 and 4 tumours respectively. Marker *MYCL*, a tetra-pentanucleotide repeat, was the most informative for MSI in this panel. It has been proposed that tri- and tetra-nucleotide repeats may be more effective in detecting MSI in some cancers (Jonsson *et al.*, 1995). On the other hand, this type of

marker has also been reported to have a spontaneous mutation rate up to 50 fold greater than that of dinucleotide repeats (Arzimanoglou *et al.*, 1998).

Clearly, the choice of markers used may impact on the MSI detection rate and the more microsatellite markers that are used, the more often MSI is likely to be found. The markers used in this study had proved most informative in other cancers and provided a starting point to approach MSI in NF1 tumours, out of the hundreds of microsatellite markers available. They also represented different types of repeat sequences, which may help us to gain a better understanding of which microsatellite markers could be used in the specific context of NF1 tumours.

4.3.2/ Microsatellite instability in NF1 tumours

In this study, different degrees of MSI were observed in half of NF1-related malignant tumours (8/16, 50%). By contrast, a large panel of benign NF1 tumours showed rare events of instability (4/135, 3%), usually in one microsatellite marker only.

In many cancers, the definition of MSI has yet to be standardized and depends on the number of markers used and on the interpretation of the instability observed. In HNPCC, the recent classification drafted by the NCI workshop recommends the use of five "core" markers and allows for additional markers, by defining MSI thus: MSI-High (MSI in $\geq$2 of 5, or > 30% markers), MSI-Low (MSI at 1of 5, or <10% markers), or MSI stable (no MSI) (Boland *et al.*, 1998). The same criteria have been applied to MSI analysis in ovarian carcinoma (Cai *et al.*, 2004). Some studies consider that MSI at one (or more) loci to be of significance, while others rely on a percentage of markers tested (Ou *et al.*, 1999; Siah *et al.*, 2000; Faulkner *et al.*, 2004). Notably, Dietmaier *et al.* (1997) proposed that MSI must be observed in at least 20% of a selected panel of markers, for a tumour to be considered as exhibiting MSI.

If applied to this study, and considering only the six markers used to test all tumours, MSI was observed in 1/135 benign tumours (0.8%) and in 3/16 malignant tumours (19%) [or 3/14 MPNSTs (21%)]. Additionally, 3/135 benign tumours (2%) and 5/16 malignant tumours (31%) [or 4/14 MPNST (29%)] would be classified as showing uncertain MSI (<20% of markers).

It has also been suggested that microsatellite alterations in as few as one marker may be sufficient to identify a tumour as having MSI (Arzimanoglou *et al.*, 1998). The reasoning behind this suggestion is that once instability occurs, it can happen randomly across the

genome, and that a marker might not detect MSI at one locus, whereas the adjacent locus may be unstable (Arzimanoglou *et al.*, 1998). Admittedly, however, given a sufficient number of markers, instability can even be found in normal samples (Parsons *et al.*, 1995).
In the present study, whether the cut-off point of 20% of markers, or the simple acceptance that instability in one marker heralds MSI are considered, the fact that MSI was seen in a large proportion of MPNSTs is statistically significant (Chi-square test: χ^2=43.26, p≤0.001, 1 degree of freedom).

There have been few MSI studies conducted in NF1 tumours, and none examined as large a number of tumours as reported here. Ottini *et al.* (1995) reported 50% of MSI in NF1 neurofibromas, which contrast with the apparently lower incidence reported here. However, most of the reported instability cases just reach the 20% threshold for definite microsatellite instability (Dietmaier *et al.,* 1997). The number and type of markers used also differ; the Ottini study relied on dinucleotide repeats only whereas this report utilized different types of microsatellite repeats. Of the four (CA) repeats used here, all recommended in HNPCC guidelines, two proved extremely informative, while the other two were found unstable in only one tumour each. Therefore the different markers used may significantly account for the discrepancies between the two studies.
By contrast, Serra *et al.* (1997) failed to identify any MSI in a panel of 60 neurofibromas. A panel of 9 NF1-related MPNSTs was assessed for MSI by Berner *et al.* (1999), using 16 dinucleotide repeats markers (8 of which were on chromosome 9). Only one tumour was found to be unstable for one maker. A more recent investigation, on a small panel of neurofibromas, employing four of the six markers used here (D2S123, BAT26, *APC* and Mfd15) did not detect any instability in the benign tumours (Luijten *et al.*, 2000), in keeping with the observations from the present study.
Although there appears to be discrepancies between the few MSI studies in NF1, this is likely due to the use of different markers, as is seen for MSI reports in other cancers (Arzimanoglou *et al.*, 1998).

Of the two rare tumours, only the MTC showed instability for one marker. Microsatellite instability reports in thyroid carcinomas have been conflicting (Vermiglio *et al.*, 1995; Soares et *al.*, 1997; Gimm, 2001; Stoler *et al.*, 2002; Vaish *et al.*, 2003), and little is known regarding MTC in particular.

No MSI has been found in rhabdomyosarcomas, a result consistent with the report that MSI is rare in soft tissue sarcomas (Suwa *et al.*, 1999; den Bakker *et al.*, 2003). Instability has however been found in paediatric rhabdomyosarcoma using a set of 57 markers, and affecting a limited number of loci; marker D2S123 was found to harbour LOH in 2/14 and MSI in 3/32 samples (Visser *et al.*, 1996).

Until now, the lack of standardized guidelines, small case series and variation in microsatellite panel selection have impaired our assessment of MSI in NF1. This study offers an assessment of six markers, previously recommended in colorectal cancer, for their use in NF1-related tumours. Also reported here is the significant difference between malignant and benign NF1 tumours in harbouring microsatellite instability.

Finally, it should be mentioned that another type of instability can be found in tumour cells: chromosomal instability (CIN). Whereas MSI-positive tumours have a normal karyotype, CIN tumours are grossly aneuploid and can be characterised by higher rates of chromosomal gain/loss, which can be identified by FISH, or of LOH. CIN has been found in many cancers, including colorectal (non-MSI tumours), breast, lung, prostate and pancreatic tumours, and it has been suggested to be a more general form of instability than MSI (Texeira da Costa and Lengauer, 2002). Both types of instability (CIN and MSI) tend to be mutually exclusive. In NF1 tumours, there have been a number of cytogenetic analyses assessing the full karyotype, and, strikingly, such chromosomal aberrations have been found in plexiform neurofibromas and MPNSTs, but not (or rarely) dermal neurofibromas.

In a majority of MPNSTs, karyotypic and FISH analysis revealed numerical (haplotriploidy, tetrasomy, pentasomy...) and structural aberration (gain/loss) involving a combination of at least seven chromosomes. Aberrations have been reported on virtually every chromosome (Jhanwar *et al.*, 1994; Mertens *et al.*, 1995; Mertens *et al.*, 2000; Perry *et al.*, 2002; Frank *et al.*, 2003; Bridge *et al.*, 2004; Frahm *et al.*, 2004a). Plexiform neurofibromas harboured alterations at chromosomes 7 and 17 in one study (FISH on de-paraffined section, Perry *et al.*, 2002) and Schwann cells derived from 4 out of 6 plexiform neurofibromas, presented complex abnormalities at various chromosomes (Wallace *et al.*, 2000). By contrast, no karyotypic aberrations were found in Schwann cells derived from 7 dermal neurofibromas (Wallace *et al.*, 2000). Complex karyotypes in dermal neurofibromas have been rarely reported (Riccardi and Elder, 1986; Glover *et al.*, 1991; Mertens *et al.*, 2000), and it is

difficult to determine whether the cell type karyotyped was indeed Schwann cells, considering that Schwann cells have only recently been accepted as the progenitor cell type, and that an appropriate culture technique was only devised a few years ago (Carroll and Stonecypher, 2004; Rosenbaum *et al.*, 2000; Serra *et al.*, 2000).

Other studies have used comparative genomic hybridisation (CGH), a new technology that relies on the differences in fluorescent signals between a sample DNA and a control DNA, simultaneously hybridised to a normal chromosome spread or array. Thus, it allows genome-wide detection of chromosomal gain and losses (Kallioniemy *et al.*, 1992; Pinkel and Albertson, 2005). Analysis revealed similar results of multiple aberrations in MPNSTs but not –or rarely and to a lesser extent - in neurofibromas (Lothe *et al.*, 1996; Mechtersheimer *et al.*, 1999; Schmidt *et al.*, 2000). By contrast, a recent CGH study revealed gain/losses of chromosomes 4, 5, 13, 17, 19 and 22 in more than half of plexiform and dermal neurofibromas (Koga *et al.*, 2002). It should however be noted that in the Koga study, the DNA was extracted from whole neurofibroma tumours (thus from a mix of cell types) and not from Schwann cells.

Nevertheless, the implications of these results for NF1 malignancy are two fold. Firstly, the large number of MPNSTs – and to a lesser extent, plexiform neurofibromas – harbour chromosomal abnormalities, which may indicate that CIN drives alteration of key genes and pathways to tumour progression more frequently than MSI does. Secondly, the variability of chromosomes affected in malignant tumours would suggest that there is no one "set" of gene alterations (bar a few recurrent ones, such as the *CDKN2A* locus), but that the acquisition of key cellular functions (for example, unlimited replicative potential) may be achieved through the disruption of different genes in different tumours, with similar end results, as described by Hanahan and Weinberg (2000).

4.3.3/ Evolution of malignancy and modifier genes

4.3.3.1/ Microsatellite instability and the *MLH1* locus in NF1 tumours

The increased microsatellite instability in malignant NF1-related tumours brings to light several points. Firstly, instability was found in up to one half of the malignant tumours, but rarely in neurofibromas; this would suggest that genomic instability (heralded by MSI) seems to occur at a later stage in the progression of malignancy, and not as either a necessary or sufficient step in tumour formation. An accumulation of mutations is usually seen in tumours

during the evolution of malignancy (Kinzler and Volgelstein, 1996). The acquired characteristic of genome instability is a means to achieve these additional mutations, in order for the tumour cell to gain hallmark capabilities of cancer, as described by Hanahan and Weinberg (2000): evasion of apoptosis, limitless replicative potential, self-sufficiency in growth signals, insensitivity to growth signals, sustained angiogenesis and tissue invasion and metastasis.

Secondly, the presence of MSI in a tumour may indicate the involvement of the mismatch repair (MMR) pathway in that particular cancer. There are six human MMR genes (*MLH1*, *MSH2*, *MSH3*, *MSH6*, *PMS1* and *PMS2*) involved in maintaining genome stability (Charames and Bapat, 2003). In HNPCC, the MMR defects can in majority be attributed to heterozygous germline mutations in the *MLH1* and *MSH2* genes, followed by a second somatic lesion in the affected tissue, and up to 92% of the tumours harbour MSI (Liu *et al.*, 1996; Arzimanoglou *et al.*, 1998). By contrast, approximately 20% of sporadic colon cancers show MSI, attributable in majority to the somatic epigenetic inactivation of *MLH1* (Herman *et al.*, 1998; Arzimanoglou *et al.*, 1998; Kuismanen *et al.*, 2000). Furthermore, a large number of non-colon cancers have been found to exhibit variable degrees of instability, and mutation in *MLH1* and *MSH2* are rarely reported in these tumour types (Arzimanoglou *et al.*, 1998). Non-colonic, non-HNPCC tumours have previously been divided in two groups: tumours with instability at di and mono-nucleotides, as found in gastric and endometrial neoplasm, and tumours with tri and tetra-nucleotides instability, as found in lung, bladder, head and neck cancer (Boland *et al.*, 1998).

The type of repeats has also been linked to the mechanism underlying MSI. For example, breast tumours harbour low levels of MSI, mainly in tetra-penta-nucleotide repeats, compared to the multiple di-nucleotide, multiple loci instability in colorectal cancer (Jönsson *et al.*, 1995). It has been proposed that instability in tetra-penta nucleotides may be repaired by genes distinct from the two MMR genes in colon cancer (CC), or could involve distinct mechanisms, such as mutations affecting the exonuclease activity of DNA polymerase (Da Costa *et al.*, 1995; Glebov *et al.*, 1994). Other genes involved in genome maintenance and/or replication, or genes that regulate MMR protein function and expression, may also underlie MSI in non-CC tumours (Andrew and Peters, 2001).

These observations are specific to each tumour tissue and dependent upon the loci analysed. It is difficult to classify NF1 tumours according to these guidelines. If the latter are considered,

in NF1 tumours, the type of repeats found unstable may argue against the involvement of MMR genes. In the present study, the highest degrees of instability were observed in one tetra-penta repeat and two dinucleotide repeats, whereas BAT26, which can identify tumours with defective repairs in CC (Hoang *et al.*, 1997; Hatch *et al.*, 2005), did not show instability in NF1 tumours. Marker D2S123, which is linked to the *MSH2* gene (Aaltonen *et al.*, 1993; Peltomaki *et al.*, 1993), harboured instability in only one malignant tumour. By contrast, most malignant tumours harbouring some degree of MSI also showed LOH at the *MLH1* loci, suggesting the involvement of at least one MMR gene in NF1 tumours. Evidently, it cannot be ruled out that these observations of LOH were in fact MSI at the marker locus used, and additional flanking markers should be evaluated for LOH. However, an analysis of the *MLH1* gene expression by RT-PCR (Section 3.2.2.4) seemed to indicate a reduction in expression in some of the tumours also harbouring LOH of the gene.

These results would suggest that the *MLH1* gene might be, at least in part, involved in the process of microsatellite instability in NF1 tumours. As no LOH for *MLH1* is observed in benign tumours and in malignant tumours not displaying instability, it seems possible that the loss of *MLH1* occurred later in the progression of malignancy, and, by allowing replication errors to go unrepaired, aided the accumulation of errors in the cells. Thus, the different degrees of instability observed in the malignant tumours may represent different stages of the tumour life, where accumulation of instability occurs over time.

Additionally, marker D2S123, linked to *MSH2*, showed allelic imbalance in benign tumour T142.2. Without the use of additional markers, it cannot be ruled out that this marker profile may be indicative of a larger deletion in 2p. It may therefore be possible that the unusually high instability seen in this tumour, at both *NF1*-intragenic and extragenic markers, is due to an alteration in the MMR gene, *MSH2*.

Thirdly, a relationship between the *NF1* gene and the MMR genes, in particular *MLH1*, has been evidenced by several observations. A number of reports have linked constitutive MMR gene deficiency and the *NF1* gene. As reviewed in Section 1.6.3, patients with homozygous mutations in *MLH1*, *MSH2*, *MSH6* or *PMS2*, and thus deficient in MMR activity, were found to exhibit NF1 features and haematological malignancies and other cancers (Wang *et al.*, 1999; Ricciardone *et al.*, 1999; Graham *et al.*, 2001; Trimbath *et al.*, 2001; Vilkki *et al.*, 2001; Whiteside *et al.*, 2002; Bougeard *et al.*, 2003; Hedge *et al.*, 2003; De Vos *et al.*, 2004; Gallinger *et al.*, 2004; Menko *et al.*, 2004; Raevaara *et al.*, 2004). In *MLH1* homozygous patients, the NF1 features included CALS, axillary freckling, Lisch nodules, gliomas and

neurofibromas (Wang *et al.*, 1999; Ricciardone *et al.*, 1999; Vilkki *et al.*, 2001; Gallinger *et al.*, 2004; Raevaara *et al.*, 2004). Although the patients may present NF1 features, it has been remarked that they do no strictly satisfy the NIH criteria for NF1 (Gallinger *et al.*, 2004); however, the diversity of NF1 hallmark features, even in related individuals, is striking. For example, in three children from the same family, a boy had only several CALS whereas his first sister showed minimal axillary freckling, 3 CALS and a Lisch nodule, and his second sister had a plexiform neurofibroma, 8 CALS and Lisch nodule (Gallinger *et al.*, 2004). These results suggest that the *NF1* gene may represent a preferential mutational target in MMR deficiency. Because inactivation of the *NF1* gene is required for neurofibroma formation (Serra *et al.*, 2001), it would mean that MMR-deficiency allowed for two somatic hits in the *NF1* gene. Admittedly, the high level of instability in the cells may also have disrupted other genes in the *NF1* gene pathway. The patients were not screened for *NF1* mutation in these studies (Wang *et al.*, 1999; Ricciardone *et al.*, 1999; Vilkki *et al.*, 2001; Gallinger *et al.*, 2004; Raevaara *et al.*, 2004). Nevertheless, the first somatic hit must have occurred very early on in development, as would be suggested by the presence of different disease features in a single patient – CALS, neurofibroma and Lisch nodules – all from neural crest origin (Huson, 1998). This is also interesting in light of the high mutation rate exhibited by the *NF1* gene (Ruggieri and Huson, 1999).

Furthermore, the *NF1* gene has also been found altered in up to 50% of MMR-deficient cell lines, HNPCC tumours exhibiting a MSI phenotype, and $Mlh1^{-/-}$ mouse embryonic fibroblasts in a study by Wang *et al.* (2003). Interestingly, the type of *NF1* mutations (nucleotide substitutions, deletions in mononucleotide repeats) and position in the gene indicated the lack of a "hotspot" for MMR deficiency in the *NF1* gene.

In mouse model, $Nf1^{+/-}$ mice are prone to the development of myeloid leukaemia and other tumours (Jacks *et al.*, 1994). Interestingly, $Nf1^{+/-}/Mlh1^{-/-}$ mice develop myeloid leukaemia (and not lymphomas as observed in $Mlh1^{-/-}$ mice) and die earlier than either $Nf1^{+/-}$ or $Mlh1^{-/-}$ mice, suggesting that *Mlh1* loss accelerates *Nf1* inactivation and tumorigenesis (Gutmann *et al.*, 2003). Immunohistochemistry of the tumours revealed the lack of *Nf1* expression and high MSI was also observed in the tumours.

Of the recent study on MMR genes in NF1 patients, no mutation in *MLH1* or *MSH2* (by heteroduplex analysis) or evidence of MSI were found in the lymphocyte DNA of 20 NF1 patients with a *de novo NF1* mutation (Wang *et al.*, 2003), suggesting that constitutional MMR deficiency is not frequently involved in *de novo* NF1 cases.

It would be tempting, however, to speculate that the loss of *MLH1* and MMR function in general, or at the very least haploinsufficiency, would accelerate the appearance of the somatic hit on the remaining wild-type allele of the *NF1* gene in a subset of NF1 tumours. If this were the case, *MLH1* alterations should also be seen in some neurofibromas. Haploinsufficiency of genes involved in the maintenance of genomic stability may not initiate tumour formation, but render the cell genetically unstable, allowing for the appearance of additional mutations (Santarosa and Ashworth, 2004). Examples of haploinsufficiency for these genes are rare and most come from mouse models. Mutant *Msh2*$^{-/-}$ mice have been found to be more prone to tumours, and cells derived from *Msh2*$^{-/-}$ mice have shown a decrease in sister-chromatid exchange induced by methyl-nitrosourea, compared to wild-type mice (Bouffler *et al.*, 2000). Another group reported that while *Msh2*$^{-/-}$ mice showed an overall mutation frequency similar to that of wild-type mice, differences were observed in mutational spectrum (Zhang *et al.*, 2002). Mutant *Mgmt*$^{-/-}$; *Mlh1*$^{+/-}$ mice have also been shown to present an altered response carcinogen, but retain a normal level of spontaneous mutations, and the haploinsufficient effect was thought to be related to the levels of Mlh1 protein available in the cell and capacity to form heterodimers (Takagi *et al.*, 2003). The examples of haploinsufficiency remain few, and would require further investigation.

Finally, an interesting observation on *Mlh1* tissue-specific function was made from the mouse model. In different cell types with *Mlh1* deficiency, the type and rate of accumulation of somatic mutations was addressed. Notably, the results suggested that T cells relied on MMR genes for point mutation repair, whereas fibroblasts dependent on the MMR system for both repair of mispairing and suppression of mitotic recombination (MR; Shao *et al.*, 2004). MR is thought to be one of the primary mechanisms for LOH *in vivo* (Gupta *et al.*, 1997) and has been shown to be the mechanism underlying LOH in some NF1 tumours (Serra *et al.*, 2001b.). Three of the MPNSTs in this panel, T145, T151 and T165 were found to have potentially large deletion of the *NF1* gene and flanking regions (Section 6.2.4.2.1). Tumour T145, in particular, may harbour LOH resulting from MR. Thus, it is interesting that these three tumours also manifest LOH at the *MLH1* marker. The hypothesis of factors influencing the somatic mutation in the *NF1* gene has been put forward to explain a "trend" of similar somatic alterations in tumours from the same patient (Serra *et al.*, 2001b; discussed in Section 6.3.3).

In the present case, haploinsufficiency of *MLH1* could be one of such modifying factors to the appearance of the somatic mutation in the *NF1* gene (LOH through MR), whereas complete loss of *MLH1* could accelerate malignancy, by permitting mutations of genes in key

pathways. Similar effects of haploinsufficiency vs. complete loss have been reported with other genes in tumorigenesis (Santarosa and Ashworth, 2004). *MLH1* LOH was not found in a control panel of 13 benign tumours, including two plexiform neurofibromas (T156, assessed both in a matricial and in a nodular section of the tumour and T167), which are thought to be the precursors to MPNSTs (Ruggieri and Huson, 1999). However, other inactivating mechanisms may be involved. Investigation of *MLH1* hypermethylation was conducted in NF1 tumours, but only revealed hypermethylation in the MTC (Section 3.2.2.4). Additionally, it had been suggested that the MMR complex, rather than one particular gene, seems to interfere with the *NF1* gene (Whiteside *et al.*, 2002), and several MMR may be disrupted in NF1 tumours. As the MMR genes act by forming heterodimers (Charames and Bapat, 2003), haploinsufficiency of one or more genes may be sufficient to affect genome integrity and malignant evolution in NF1 tumours. In any case, the relationship between the *NF1* gene and the MMR genes is intriguing and deserves to be more thoroughly investigated.

Of the two rare tumours, and as mentioned above, only the MTC showed both LOH and methylation for *MLH1*. The implications of these results were discussed in Section 3.3.3.3.
The rhabdomyosarcoma did not exhibit instability and was uninformative for the marker at *MLH1*. It would be of interest to extend the study using additional *MLH1* markers, because alterations of the gene have been reported in this tumour type. Of note, a rhabdomyosarcoma from an HNPCC patient harboured a germline mutation and LOH in *MLH1*, and a somatic alteration (a 4bp deletion in poly T tract) in the *NF1* gene (Wang *et al.*, 2003). Alternatively, while MSI is rare in rhabdomyosarcoma, and none was found in the present sample, occurrence of instability in paediatric rhabdomyosarcoma has been linked to MMR system defects (Visser *et al.*, 1996).

4.3.3.2/ Other candidate genes in NF1 malignancies: the *CDKN2A* and *TP53* genes

4.3.3.2.1/ The *CDKN2A* locus

The *CDKN2A* locus encodes two distinct tumour suppressors, $p16^{INK4a}$ and $p14^{ARF}$, involved in the Rb and p53 pathways, respectively (Figure 4.5). The $p16^{INK4a}$ protein inhibits cyclin D-dependant kinases CDK4 and CDK6, which can inactivate the Rb protein by phosphorylation. Thus $p16^{INK4a}$ has a key role in maintaining Rb in its active state, and subsequent control of progression to the S phase and proliferation. The $p19^{ARF}$ ($p14^{ARF}$ in human) inhibits Mdm2

(MDM2 or HDM2 in human) by directly associating with this protein, thereby preventing it from binding to, and degrading p53, a key protein in DNA damage-mediated cell cycle arrest and apoptosis (Quelle *et al.*, 1995; Sherr, 2001; Sherr and Cormick, 2002).

The use of four microsatellite markers on the short arm of chromosome nine (9p) revealed both LOH and instability. Additionally, in three cases (T151, T165 and T178), because apparent LOH was found in only in one marker, away from the *CDKN2A* locus, microsatellite instability cannot be ruled out.

An interesting LOH profile was seen in two MPNSTs, T145 and T168, which sustained LOH for 3/4 and 4/4 markers, respectively. The results suggest the occurrence of deletions spanning more than the *CDKN2A* locus, and possibly encompassing the entire short arm (9p) in the case of T168.

Deletion of the entire 9p has commonly been found in cancer and in NF1 MPNSTs. In cancer, LOH and homozygous deletion (HD) are the primary mean of inactivation of *CDKN2A/p16^{INK4}*, followed by promoter hypermethylation, whereas intragenic mutations are rare (Kamb *et al.*, 1994; Cairn *et al.*, 1995; Williamson *et al.*, 1995; Foulkes *et al.*, 1997; Chin *et al.*, 1998; Cachia *et al.*, 2000; Esteller *et al.*, 2000a; Sherr, 2001; Esteller *et al.*, 2002; Tokugawa *et al.*, 2002; Berggren *et al.*, 2003). In NF1, previous studies have reported alterations at 9p21, 9p deletions and loss of gene expression in up to 87% of MPNSTs, but not neurofibromas (Lothe *et al.*, 1996; Berner *et al.*, 1999; Kourea *et al.*, 1999b; Nielsen *et al.*, 1999; Mertens *et al.*, 2000; Schmidt *et al.*, 2000; Birindelli *et al.*, 2001; Perry *et al.*, 2002; Perrone *et al.*, 2003; Zhou *et al.*, 2003; Bridge *et al.*, 2004; Frahm *et al.*, 2004; Ågesen *et al.*, 2005).

Deletion and HD of *CDKN2A* have been investigated using FISH (Perry *et al.*, 2002; Perrone *et al.*, 2003), Southern blot and comparative multiplex PCR (Kourea *et al.*, 1999b; Nielsen *et al.*, 1999; Perrone *et al.*, 2003; Ågesen *et al.*, 2005), but rarely with microsatellite markers. In two such analyses, LOH was identified in 5/8 MPNSTs (62.5%) with 3 markers (at 9p21, 9p22 and 9p23; Frahm *et al.*, 2004), and 4/11 MPNSTs (36%) with 8 markers on 9p (Berner *et al.*, 1999). In the present study, 11% of malignant tumours (12.5% MPNSTs) appear to show a deletion at 9p21.

Compared to an intragenic mutation, LOH might well be expected to confer a greater selective advantage to the tumour because two tumour suppressor genes involved in two distinct key cellular pathways would be inactivated. Evidently, loss of p16^{INK4a} and p14^{ARF} individually would lead to tumorigenicity. However, in a mouse model null for

CDKN2A/p16^{INK4a} and haploinsufficient for p19^{ARF}, an increase in tumorigenicity was observed, stressing the cooperative and overlapping functions of these cell cycle regulators (Sharpless *et al.*, 2001). Additionally, *p19*^{ARF}*-/-/Nf1*$^{+/-}$ mouse models develop multiple tumours and metastases, a phenotype different from that seen with *p19*^{ARF} null mice, suggesting that the two tumour suppressors cooperate in tumorigenesis (King *et al.*, 2002). The question of which tumour suppressor, *CDKN2A/p16*^{INK4a} or *p14*^{ARF}, is the most important in tumour evolution, is likely to be specific to a particular cancer, and the means of inactivation (targeted promoter methylation vs. large homozygous deletion, for example) may give clues as to the answer (Sharpless *et al.*, 2001). Methylation of the *CDKN2A/p16*^{INK4a} promoter was not seen in this study (Section 6.2.2.1), in accordance with previous reports (Kourea *et al.*, 1999b, Nielsen *et al.*, 1999; Berner *et al.*, 1999; Ågesen *et al.*, 2005), but reduced expression was however seen in a few tumours (section 6.2.2.1). Methylation of the *p14*^{ARF} promoter has not been investigated in this study, and rarely found in NF1 tumours (Perrone *et al.*, 2003).

In the present study, it may mean that haploinsufficiency or loss of one tumour suppressor (*CDKN2A/p16*^{INK4a} or *p14*^{ARF}, or combination thereof) could cooperate with the inactivation of the *NF1* gene in progression to malignancy, in at least two tumours.

In MPNST, T145 and T168, the extensive LOH also implies that loci other than the *CDKN2A* locus and its two tumour suppressors may also be altered by the deletion. The *CDKN2A* locus is adjacent to the *CDKN2B* locus (Figure 4.5), which encodes another cyclin-dependant kinase inhibitor involved in the Rb pathway, p15^{INK4b}. Similar to *CDKN2A/p16*^{INK4a} in sequence and function, p15^{INK4b} is affected in the majority (but not all) of *CDKN2A* deletions (Kamb, 1995). In NF1 malignant tumours, homozygous deletion (HD) of *CDKN2B* has been found in conjunction with HD of *CDKN2A*, and occasionally alone, and lack of (or low) expression of p15^{INK4b} was also found (Berner *et al.*, 1999; Perrone *et al.*, 2003).

A "double-target" hypothesis has been formulated where the simultaneous silencing of two or more genes can be explained by homozygous deletion (HD) in cancer cells (Jen *et al.*, 1994). In MPNSTs, it has also been suggested that there is not a preferential target for deletion in the three (p15^{INK4b}, p14^{ARF}, p16^{INK4a}) tumours suppressors (Perrone *et al.*, 2004).

While the *CDKN2A* locus, and perhaps the adjacent *CDKN2B*, could be heterozygously deleted in tumours T145 and T168, whether or not they are homozygously inactivated remains to be determined. In the present study, six of the MPNSTs (T23.11, T23.12, T87, T115, T166 and T118) have also been investigated for mutations in all three exons, by

DHPLC (work by Dr Song Han, published in Upadhyaya *et al.*, 2004). A common polymorphism was observed in one tumour and in the corresponding blood lymphocyte DNA, but no other mutations were identified. Mutation detection was not undertaken in the most recently acquired tumours, and although mutations in *CDKN2A/p16^{INK4a}* are rare, the LOH status of the two MPNSTs, T145 and T168, would warrant further investigation regarding other means for inactivation of the two tumour suppressors, aside from promoter hypermethylation.

Finally, the lack of LOH at the *CDKN2A* locus in the other tumours does not preclude the possibility of other deletions, as HD has been found to target exclusively exon 2, in some NF1 tumours (Perrone *et al.*, 2003). It would be interesting to assess the status of *RB1* and its expression in these tumours, since cells with loss of p16^{INK4a} function tend to retain wild-type Rb and *vice versa* (Sherr and Roberts, 1995).

Both rare tumours, the rhabdomyosarcoma (T160) and the MTC did not show LOH at the 9p markers. Loss or reduction of *CDKN2A/p16^{INK4a}* and *p14^{ARF}* expression has been reported in rhabdomyosarcomas and derived cell lines (Iolascon *et al.*, 1996; Obana *et al.*, 2003; Sharp *et al.*, 2002), and is thought both to facilitate growth and inhibit myogenic differentiation (Urashima *et al.*, 1999; Sharp *et al.*, 2002). By contrast, LOH of *CDKN2A/p16^{INK4a}* has been estimated to be rare in thyroid carcinomas, and was not found in a panel of 18 MTC (Schulte *et al.*, 1998). Moreover, no mutations in *CDKN2A/p16^{INK4a}* have been reported in a panel consisting of one MTC and other thyroid carcinomas (Yane *et al.*, 1996), suggesting that *CDKN2A/p16^{INK4a}* may only be rarely involved in MTCs.

4.3.3.2.2/ The *TP53* gene

In normal cells, tumour suppressor p53 is a short-lived protein, largely due to the negative feedback-loop control with mdm2 (MDM2), which binds to p53, inhibiting its transcriptional activity and mediating its degradation through ubiquitination. The p53 protein is activated and stabilized in response to a number of stress signals, plays a key role in both apoptosis and DNA-damaged induced cell-cycle arrest, and also contributes to the DNA repair processes. Tumour suppressor *TP53* is found to be altered in more than half of human cancers and, in the majority of remaining cases, it has been compromised through indirect mechanisms, such as MDM2 overexpression or *p14^{ARF}* silencing (Levine, 1997; Oren, 1999; Haupt *et al.*, 2003).

In the present study, microsatellite marker p53Alu identified both LOH and instability. In the two rare tumours (MTC and rhabdomyosarcoma), the *TP53* marker was only informative for the MTC, and no LOH was seen. This is in keeping with previous results, where no LOH was found in a panel of 29 MTC, including 9 sporadic (Herfarth *et al.*, 1997). Although *TP53* alterations appear to be involved in other thyroid carcinomas, there is little information on its involvement in MTCs and *TP53* mutations are also rare in these tumours (Yana *et al.*, 1992; Herfarth *et al.*, 1997; Velasco *et al.*, 1997; Gimm, 2001). By contrast, either aberrant or absent p53 has been reported in a quarter of rhabdomyosarcomas (Felix *et al.*, 1992).

In NF1, mutations and deletions at the *TP53* gene locus, as well as an accumulation of the p53 protein have been reported in a up to 100% of MPNSTs but not in neurofibromas (Nigro *et al.*, 1989; Menon *et al.*, 1990; Glover *et al.*, 1991; Xu *et al.*, 1992; Jhanwar *et al.*, 1994; Legius *et al.*, 1994; Kindblom *et al.*, 1995; Lothe *et al.*, 1995; Halling *et al.*, 1996; Luria *et al.*, 1997; Kluwe *et al.*, 1999; Kourea *et al.* 1999a; Mertens *et al.*, 2000; Rasmussen *et al.*, 2000; Schmidt *et al.*, 2000; Birindelli *et al.*, 2001; Leroy *et al.*, 2001; Koga *et al.*, 2002; Mawrin *et al.*, 2002; Zhou *et al.*, 2003; Frahm *et al.*, 2004a).
In studies specifically assessing LOH with various microsatellite markers on 17p, *TP53* LOH was found in 33-100% of MPNSTs (Nigro *et al.*, 1989; Menon *et al.*, 1990; Glover *et al.*, 1991; Xu *et al.*, 1992; Legius *et al.*, 1994; Lothe *et al.*, 1995; Rasmussen *et al.*, 2000; Frahm et al., 2004a). In the present study, LOH at *TP53* was seen in 12.5% of MPNSTs, thus in an apparently lower proportion than previously reported. However, LOH was assessed here with only one marker (in intron 1), and other deletions in the gene cannot be ruled out. Additionally, 6 tumours (T23.11, T23.12, T87, T115, T116 and T118) were screened for mutations in "hotspot" exons 4-6 of *TP53*, but no mutation was found (work conducted by Dr Song Han; Upadhyaya *et al.*, 2004). Mutations occurring in the remaining wild-type allele of *TP53* (with LOH on the other allele) have been reported in some cases (Nigro *et al.*, 1989; Menon *et al.*, 1990; Legius *et al.*, 1994).

These results suggested that *TP53* may be involved in malignant progression in NF1 and additional evidence comes from mouse models. Transgenic mice carrying null mutations of the *Nf1* and *p53* gene in *cis* were generated (Cichowski *et al.*, 1999; Vogel *et al.*, 1999). These mice, essentially haploinsufficient for both genes, developed tumours characteristic of knockout mice for each gene, and also developed soft tissue sarcomas. Not only were the latter tumours highly similar to their human counterpart (MPNST), but they also demonstrated

LOH of both *Nf1* and *p53* remaining alleles, indicating the cooperation of both tumour suppressors in malignant progression. It has been suggested that p53 may play a role in cell proliferation in tumour progression, as it has been found altered in high-grade MPNSTs (Zhou *et al.*, 2003)

Considering the previous reports on NF1 MPNSTs, and the fact that *TP53* and *CDKN2A/p16*^{INK4a} are the two most disrupted tumour suppressor genes in human cancers, it is perhaps not surprising that 3/16 (19%) of NF1 MPNSTs in this panel harbour alterations at one or two of these genes. Interestingly, tumour T168 harboured LOH at both 9p and the *TP53* locus. It has been suggested that deletion may occur randomly and reflect chromosomal instability, perhaps resulting from the inactivation of caretaker genes responsible for chromosomal stability (Tokugawa *et al.*, 2002). By contrast, no MSI was found in this tumour, which may suggest that chromosomal instability drove the deregulation of key processes in this tumour. *NF1* gene inactivation in this tumour however results from both germline and somatic microlesions. Additionally, because LOH in this tumour was identified on the majority or whole of 9p, it would suggest that the p53 pathway could be disrupted at two points: at p14^{ARF}, which function is to inhibit MDM2 that in turn inhibits p53, and at *TP53* expression. In this case, if *TP53* were at all expressed, the protein would be subjected to excessive inhibition. Alternatively, p14^{ARF} could have an additional role, independent of p53, in tumorigenesis, as is suspected in bladder cancer, where loss of p53 and p14^{ARF} is also seen (Berggren *et al.*, 2003).

It is not known whether both genes are homozygously inactivated in this tumour, but haploinsufficiency of *p53* has been shown to play a role in cancer and cooperative haploinsufficiency of different genes in one pathway has also been described (Santarosa and Ashworth, 2004). Ohmiya and co-workers proposed that the deregulation of major cell processes, such as cell growth and survival, in MSI cells could be achieved by multiple mono-allelic mutations accumulating in different genes involved in the same pathway (Ohmiya *et al.*, 2001). Therefore MPNST T168 may have acquired the key functions of cellular proliferation (through disruption of *CDKN2A/p16*^{INK4a} and *CDKN2B*), and evasion of apoptosis (through disruption of *p14*^{ARF} and *TP53*).

Finally, and as reviewed in Section 1.3.4, additional proteins have been found altered and/or exhibit aberrant expression in NF1 MPNSTs. These include Rb, p27 (a cyclin-dependent

kinase inhibitor), CDK4 (a cyclin-dependant kinase) and Cyclin D1 from the Rb pathway, p21 and MDM2 (HDM2) from the p53 pathway, EGFR, CD44, BLPB and PTEN (Kourea *et al.*, 1999; Birindelli *et al.*, 2001; Mawrin *et al.*, 2002; Perry *et al.*, 2002; Miller *et al.*, 2003; Su *et al.*, 2003; Zhou *et al.*, 2003; Bridge *et al.*, 2004; Ågesen *et al.*, 2005).

4.3.4/ Conclusion

The following conclusions can be drawn from the results reported here:

(i) Microsatellite instability occurs at higher incidence in NF1 malignant tumours,

(ii) NF1 tumours cannot be classified by the instability of a particular type of repeat, until a large panel of both benign and malignant tumours have been assessed using markers informative for this particular tumour tissue,

(iii) Further investigation ought to be conducted on the *MLH1* gene (and other MMR genes) for LOH and other inactivating mechanism to determine the extent of its involvement in NF1-related malignant tumour formation and MSI.

(iv) Additional markers located in candidate genes can be used to unravel parts of the multistep process of NF1 tumorigenesis and should be combined with other methods to assess the extent of the inactivation of these genes.

MPNSTs are amongst the most aggressive neoplasms, and no effective therapy is currently available (Ferner and Gutmann, 2002). Understanding the mechanisms underlying malignant progression is the first step towards the development of an effective treatment.

CHAPTER 5: IDENTIFICATION OF SOMATIC MUTATIONS DERIVED FROM SCHWANN CELL CULTURE

5.1 Introduction

The hallmark tumour of NF1, neurofibroma, is composed of multiple cell types. Schwann cells (SC), normally found in close proximity to the axons in the peripheral nervous system, are involved in the formation of neurofibromas and may constitute as much as 80% of the benign tumour (Peltonen *et al.*, 1988). It is now accepted that SC are also the primary neoplastic cells in NF1 tumours, and several observations have been made that support this theory. Firstly neurofibroma-derived SC demonstrated altered levels of Ras (Sherman *et al.*, 2000) and lack of neurofibromin expression (Rutkowski *et al.*, 2000). Secondly, neurofibroma-derived SC have demonstrated invasive and infiltrative growth, as well as angiogenic properties compared to normal SC, and were shown to have chemotactic properties (Sheela *et al.*, 1990; Muir *et al.*, 2001). Thirdly, in the mouse model, ablation of *Nf1* function in SC alone was sufficient to induce tumour formation, with the cooperation of a heterozygous microenvironment (Zhu *et al.*, 2002). Finally and perhaps most importantly, neurofibroma SC have been shown to sustain the somatic hit in the *NF1* gene, as LOH or as discrete gene lesions (Kluwe *et al.*, 1999; Serra *et al.*, 2000; Upadhyaya *et al.*, 2004).

Recently, an improved culture technique for neurofibroma-derived SC has been developed, and this has permitted the isolation of *NF1* heterozygous (*NF1*+/-) and homozygous (*NF1*-/-) SC from a benign tumour (Rosenbaum *et al.*, 2000; Serra *et al.*, 2000).

Identifying the somatic mutation in the *NF1* gene has been hampered by the same obstacles encountered in screening for a germline mutation: the large size of the gene, the lack of mutational hotspots and the presence of highly homologous pseudogenes. In somatic mutation screening, however, an added difficulty presents itself in the form of marked cellular heterogeneity with the DNA carrying a somatic mutation being greatly diluted by the presence of wild type DNA, in the heterozygous background. Isolation or enrichment of the *NF1*-/- SC population through culture could therefore provide a DNA sample with minimal interference from the *NF1* heterozygous cell population for somatic screening purposes. The aim of this chapter has been to assess the advantages of SC culture in the quest for somatic mutations of the *NF1* gene.

5.2 Results

5.2.1/ Schwann cell culture

5.2.1.1/ Obstacles encountered in cell culture and optimisation

The SC culture technique used in this study is that developed by Eric Legius' group in Belgium, where the protocol have been successfully used for several years. The method is basically that published by Serra *et al.* (2000). The methodology was brought back and adapted to the cell culture facilities available in the NF1 lab. Tumours were received regularly from other hospitals and as a result of patient donation. A number of SC cultures have been carried out over two years, and have resulted in the 5 cell lines used in this study. Following are the observations and optimized conditions resulting from the study.

SC are surface adherent cells, and an important step in their culture is to use a plate coated with laminin. This glycoprotein has been reported to be essential for SC survival, even under serum-free medium conditions, provided the cells are at high density (Cheng *et al.*, 1998). Moreover, laminin is an important factor in the enrichment of SC in the culture, due to the preferential attachment of SC to laminin (Muir *et al.*, 2001). After dissociation of the cells from the tumour, the SC obtained must be in sufficient number to be in contact with each other for the cells to grow normally. Sparsely growing cells have been observed to exhibit a more elongated form.

SC can be overgrown by fibroblasts, a much faster growing type of cells. To minimise introduction of fibroblasts into the culture, the first step is to carefully choose the type of tumours used. Larger neurofibromas allow for a tissue sample to be taken from the centre of the tumour, away from the skin layer from which most fibroblasts originate. Tumours with a well-defined capsule also allow better dissection of the tumour. However, neurofibromas are heterogeneous tumours composed of many different cell types involved in peripheral nerve, including a proportion of fibroblasts. In the culture medium, IBMX is used to inhibit the growth of sparse fibroblasts, but will have little effect once fibroblasts have overgrown the culture. SC can also grow preferentially on a layer of fibroblasts, as previously reported (Muir *et al.*, 2001). The use of laminin should however circumvent SC growth on fibroblasts, so such occurrence could be an indicator of a defective laminin coating.

Some cells still respond mildly to culture, with slow growth. The repeated use of the 3-medium phase (Section 2.8.2.2) once a week (Hilde Brems, personal communication) has yielded some success in accelerating the growth. Little is known about the mechanisms involved in SC growth and it could be argued that the use of the 3-medium phase, and therefore repeated use of forskolin, could influence the *NF1+/-* SC population, as well as the *NF1-/-* SC population.

SC are very sensitive to the variations in the medium and in the environment. It was observed that SC directly frozen after dissociation generally did not survive being revived. A change in the medium component can impair growth, and cells should be monitored closely after the use of a new medium or solution. Small aliquots of each solution in the culture should be used as to minimise the number of freezings / thawings.

5.2.1.2/ Schwann cell staining

As SC precursors, derived from the neural crest multipotent cells, differentiate to become immature SC, the S100 protein is expressed. This protein is specific to the SC in the peripheral nerve (Mirsky and Jessen, 1996).

The first cells stained (T175.2) in this study were aliquoted from a pellet of cultured cells. A drop was dried on a slide and fixed with formalin, and then the staining method described in (Section 2.8.4) was used. Although the presence of SC was identified by the difference in staining colour, the shape of the cells was not retained for observation.

As 8-wells slides became available, an aliquot of cultured SC was left to grow on the slide prior to staining. This method allowed retention of the shape of the SC as well as having colour staining (T181.1, T181.2, T181.3, and T183.1; Figure 2.1).

The S100 antibody optimal concentration was determined by staining with two different concentrations, 1/1000 and 1/5000, the latter dilution allowing better visualization of the SC. Detection of primary antibody was then achieved using the appropriate ABC kit, which utilises a peroxidase.

5.2.1.3/ Tumours and SC in this study

All five SC lines used stained positive for S100. Neurofibroma T175.2 was a small but well-defined tumour encased in a capsule. The sample cultured was taken from the centre of the

tumour. Cells T175.2 were first frozen immediately after their initial dissociation, in order for them to be revived at a later date. Once plated, the cells demonstrated a typical SC bipolar shape and fast adherence to the laminin-coated plate. The SC showed rapid growth and could be split every 10 days. The SC used in this study were from passage P2.

The three cell lines T181.1, T181.2 and T181.3 were isolated from 3 small dermal neurofibromas derived from the same patient. All three tumours had a very well-defined capsule. These cells were plated immediately after dissociation, and each demonstrated rapid growth, similar to T175.2. SC from passage P2 were used for RNA extraction.

Neurofibroma T183.1 was a larger, firm (typical of neurofibromas) tumour encased in a well-defined capsule. A piece from the centre of the tumour was used for dissociation, and cells were cultured immediately. The cell demonstrated steady growth that was first impaired by a defective laminin coating. The SC were re-plated for optimal growth. The SC used for RNA extraction were passage P1, and P2 cells were grown on a labtek slide for staining.

5.2.1.4/ RNA extraction

In all 5 cell lines, RNA extraction was conducted immediately after trypsinization of the cells. Pelleted cells were immediately incubated in trizol and kept on ice, thereby reducing the time-frame for RNA degradation. One advantage of extracting RNA from live cells was the ability to obtain a high yield of RNA.

5.2.1.5/ *In silico* analysis

The mutations identified here were subjected to the same *in silico* analysis as described in section 6.2.2. Briefly, nucleotide and amino acid conservation between human, rat, *Fugu* and *Drosophila* was assessed and alterations were checked for equivalents in *NF1* pseudogene sequences. Analysis based on the sequence alteration caused by the mutation were also conducted: disruption/creations of exonic splicing enhancers (ESEs) and cryptic splice sites were sought using ESEfinder (www.exon.cshl.org/ESE; Cartegni *et al.*, 2003), RESCUE-ESE (www.genes.mit.edu/burgelab/rescue-ese) and a splice-site prediction program (http://www.fruitfly.org/seq_tools/splice.html); Complexity of the sequence surrounding the mutation was also assessed (http://wwwmgs.bionet.nsc.ru/mgs/programs/lzcomposer; Chuzhanova et *al.*, 2002).

SC/Fragment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
T175.2	*	*	S(G)	*	*	*	S(G)	*	*	*	*	M(G)	*	*	*	*	M(S)	*	*	*	*	*	*	*
T181.1	*	*	*	*	*	*	*	*	*	*	*	*	*	M(S)	*	*	*	*	*	*	*	*	*	*
T181.2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
T181.3	*	*	*	*	*	*	*	*	*	*	*	*	*	Mi(G)	*	*	*	*	M(S)	*	*	*	*	*
T183.1	*	M(G)	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	M(S)	*	*

Table 5.1 : Summary of Schwann cells (SC) cDNA screening by direct sequencing.
G or M in brackets denotes a germline or somatic alteration. *S*, silent alteration; *Mi*, missence alteration; *M*, disease-causing mutation. * denotes a successful sequencing.

Patient	Blood	Tumour SC	Exon	Genomic mutation	Aminoacid mutation	Type/effect	References
P175	B175		**23.2**	**C4084T**	**R1362X**	Nonsense / truncation	Upadhyaya *et al.*, 1997 Messiaen *et al.*, 2000 Eisenbarth *et al.*, 2000 Han *et al.*, 2001 Kluwe *et al.*, 2003 De Luca *et al.*, 2004
			13	A2034G	P678P	polymorphism	Fahsold *et al.*, 2000
			5	A702G	L234L	polymorphism	Mattocks *et al.*, 2004
		T175.2	**31**	**C5817A**	**C1939X**	Nonsense / truncation?	This report
P150	NA			90kb 5' end deletion		large deletion	Upadhyaya *et al.*, 1990
		T181.1	**27b**	**T4706G**	**L1569X**	Nonsense / truncation	Upadhyaya *et al.*, 1997
		T181.2					
		T181.3	**34**	**6409delT**	**L2178X**	Deletion / truncation?	This report
			27b	A4711C	I1571L	Missense / ?	This report
P183	B183		**4a**	**373 ins ATGTGT del G**	**V166X (x4b)**	Indel / truncation?	This report
		T183.1	**42**	**7449 delTGCAGCCACC**	**R2498X**	Deletion / truncation?	This report

Table 5.2: Alteration identified in Schwann cells' cDNA by direct sequencing.
NA: not available. Disease-causing mutations are in bold.

5.2.2/ Mutation analysis

5.2.2.1/ Schwann cell cDNA screening

The 12kb of *NF1* cDNA was divided in 24 fragments (Table 2.6) corresponding to sizes compatible with both DHPLC and direct sequencing screening methods. Each fragment spans on average about 3 exons and ranges from 365bp to 540bp. The use of 6 touchdown PCR cycles was found to allow better amplification and the annealing temperature was slightly modified for a number of fragments to optimise the PCR reaction. PCRs were conducted on the same ABI thermocycler to reduce temperature differences. Direct sequencing of the fragments was preferred over DHPLC in this panel, due to the small series number, and the sensitivity and rapidity of the technique. Of note, no sequencing artefacts were observed in this panel, whereas such splicing aberrations were identified in cDNA from whole tumours (section 6.2.1.5). Thus, it would appear that the use of RNA from "fresh" cells could circumvent the "aged blood" effect (Wimmer *et al.*, 2000; Ars *et al.*, 2003) encountered when handling RNA from tumours that were not snap-frozen immediately after excision. However, RNA from the whole tumour samples corresponding to the cultured SC were not used in the present study, thus it was not possible to perform a direct comparison between fresh and frozen samples, and to pin down the "aged blood" RNA effect.

The screening records and sequence changes identified in all 5 SC cDNA from the 3 patients are summarised in tables 5.1 and 5.2.

5.2.2.2/ Germline alterations

The germline mutation of patient P175 was identified in T175.2 SC cDNA in fragment 12 and was confirmed by direct sequencing of blood (B175) lymphocyte DNA (Figure 5.1). The C>T transition at nucleotide 4075 resulted in the appearance of a stop codon in exon 23.2. This mutation, C4084T (R1362X), has previously been reported in 7 unrelated NF1 patients as a germline mutation and also as a somatic mutation in a dermal neurofibroma; it is expected to result in a truncated protein (Upadhyaya *et al.*, 1997; Messiaen *et al.*, 2000; Eisenbarth *et al.*, 2000; Han *et al.*, 2001; Kluwe *et al.*, 2003; De Luca *et al.*, 2004). The recurrence of the mutation is likely to be due to its location in a hypermutable CpG dinucleotide (Cooper and Youssoufian, 1988).

Moreover, two silent mutations were identified in fragments 3 and 7 and confirmed in blood DNA, in exons 5 and 13 respectively. The G702A (L234L) change is a reported

polymorphism for exon 5, with an heterozygosity of approximately 53% (Mattocks *et al.*, 2004; discussed in section 6.2.2.6.1). The silent mutation in exon 13, A2034G (P678P) has previously been reported with an allelic frequency of over 30% for each allele (Fahsold *et al.*, 2000). An indel has been reported at the same nucleotide (2034delAinsCG; Origone *et al.*, 2002), which could suggest that the multiple CGA repeats in the surrounding (39bp) region have acted as a template for these alterations.

Interestingly a parallel investigation using DHPLC to analyse T175.2 whole tumour DNA and B175 lymphocyte DNA failed to yield a result (Dr Gill Spurlock, personal communication).

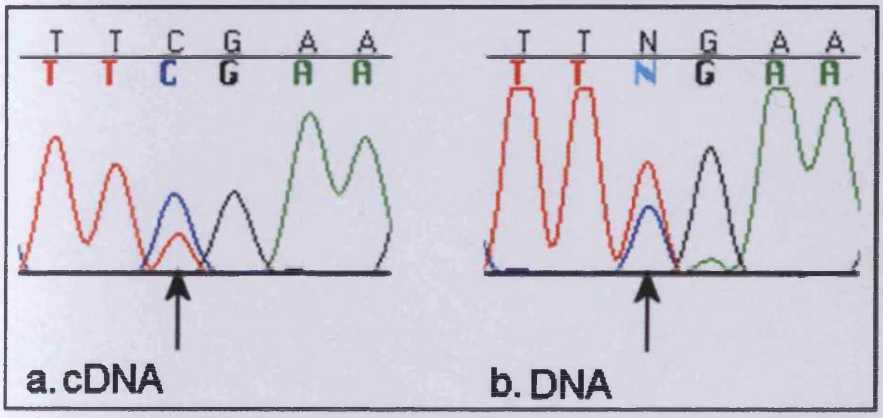

Figure 5.1: Chromatogram of germline mutation C4084T (R1362X) from patient P175.

a. Forward sequence of fragment 12 in T175.2 SC cDNA.

b. Forward sequence of exon 23.2 in B175 lymphocyte DNA.

Patient P181 has donated many tumours over the years to the NF1 Research Lab. Tumours from the same patients have therefore previously been screened: results of the mutation screen on tumours T150.1, T150.2 and T150.4 can be found in Section 6.2.4.2. The germline mutation in patient P181 is a deletion of 90kb in the 5' region of the *NF1* gene, previously reported by Upadhyaya and co-workers (Upadhyaya *et al.*, 1990).

In patient P183, a complex indel alteration was identified in fragment 2 and confirmed in blood (B183) lymphocyte DNA as the germline mutation in exon 4a (Figure 5.2). This mutation, 373insATGTGTdelG, is predicted to result in the formation of a stop codon at codon 166 (V166X) in exon 4b (assuming splicing is not altered by the frameshift) and has

not been previously reported. The mechanism underlying the mutation is unclear, and it could not be determined whether the deletion or the insertion is likely to have occurred first.

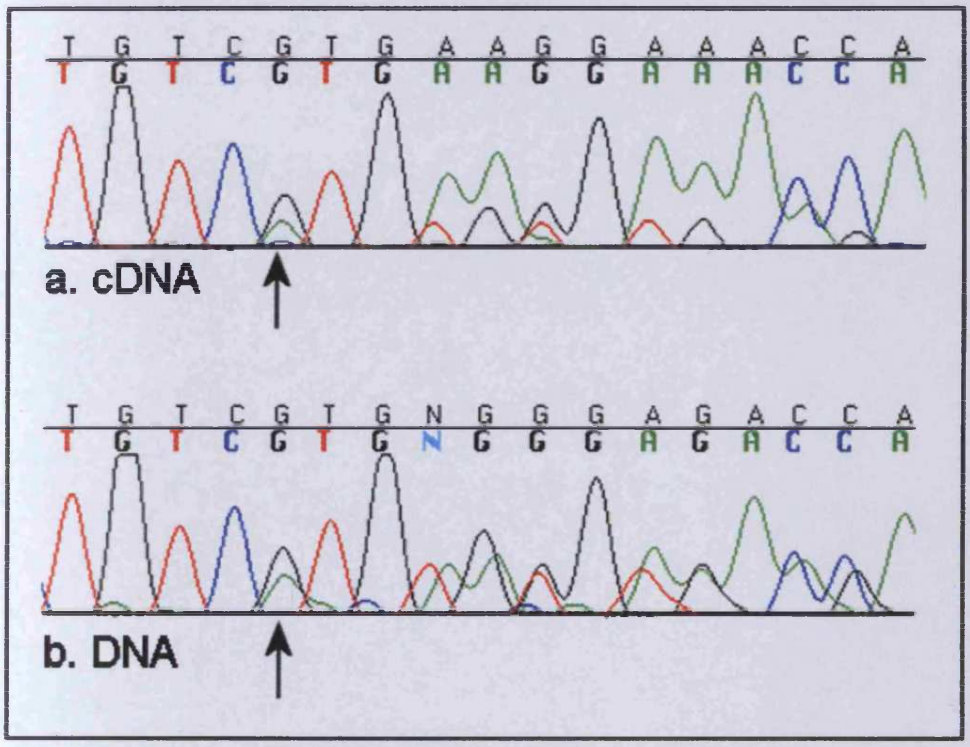

Figure 5.2: Chromatogram of germline mutation 373insATGTGTdelG from patient P183.

a. Forward sequence of fragment 2 in T183 SC cDNA.

b. Forward sequence of exon 4a in B183 blood lymphocyte DNA.

5.2.2.3/ Somatic alterations

In patient P175, a second nonsense mutation, C5817A (C1939X) was identified in fragment 17 in T175.2 SC cDNA (Figure 5.3). The C>A transversion in exon 31 was also confirmed in T175.2 whole tumour DNA. It was observed that the sequencing peak was weaker in the whole DNA as compared to SC cDNA sequencing. This could be explained by cellular heterogeneity in the whole neurofibroma (Peltonen *et al.*, 1988). C5817A was not found by direct sequencing in B175, confirming its somatic nature, and has not previously been reported.

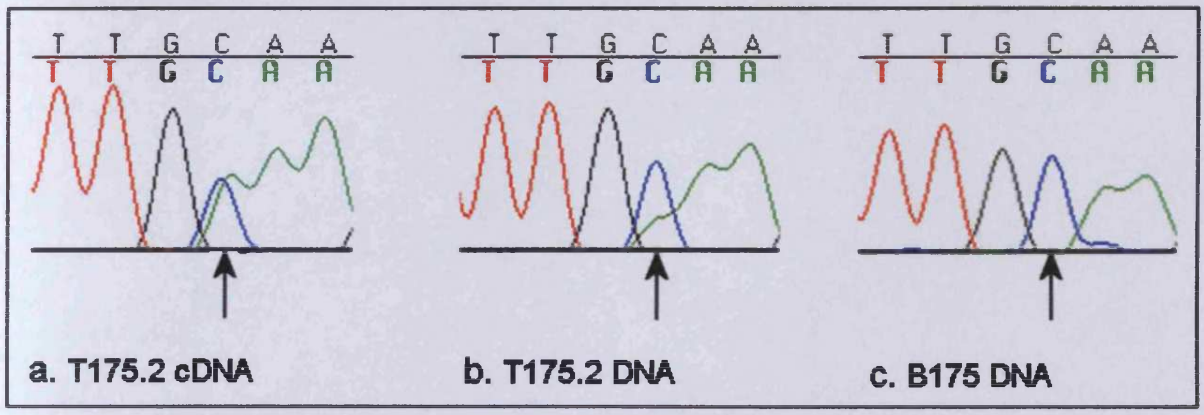

Figure 5.3: Chromatogram of somatic mutation C5817A (C1939X) from tumour T175.2. The absence of the substitution in blood DNA confirms the somatic nature of the alteration.

a. Forward sequence of fragment 17 in T175.2 SC cDNA.

b. Forward sequence of exon 31 in T175.2 whole tumour DNA.

c. Forward sequence of exon 31 in B175 blood lymphocyte DNA.

In T181.1 SC cDNA, a T>G transversion was identified in fragment 14, but was not found in T181.2 or T181.3 SC cDNA (Figure 5.4), suggesting that it is the somatic mutation in this tumour. T4706G (L1569X) in corresponding exon 23.2 has previously been reported as a germline mutation in a patient with a gastrointestinal neurofibroma, and was also found in other affected family members presenting typical NF1 features (Upadhyaya *et al.*, 1997). The mutation is predicted to truncate the neurofibromin to a size of 1568 amino acids. The substitution also creates a perfect symmetric repeat in the surrounding sequence (TtA/AGT > TgA/AGT; Cooper and Krawczak, 1993).

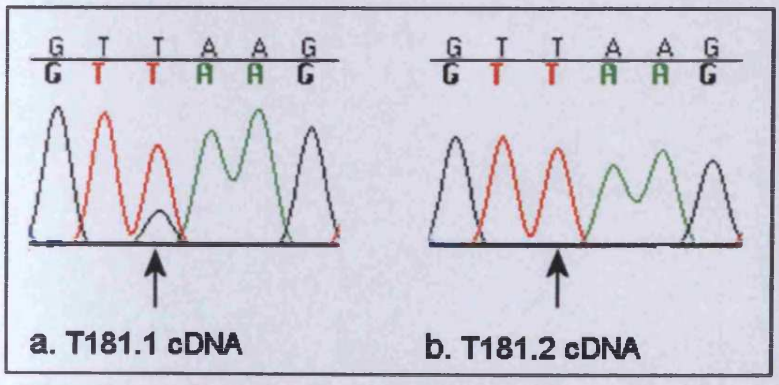

Figure 5.4: Chromatogram of somatic mutation T4706G (L1569X) from tumour T181.1. The absence of the substitution in T181.2 confirms the somatic nature of the alteration.

a. Forward sequence of fragment 14 in T181.1 SC cDNA.

b. Forward sequence of fragment 14 in T181.2 SC cDNA

A small one base deletion, 6408delT, was identified in fragment 19 of T181.3 SC cDNA but not in T181.2 or T181.3 SC cDNA from the same patient (Figure 5.5). This mutation in exon 34 is expected to result in the appearance of a stop codon at residue 2178 (L2178X) in the same exon, and is a novel mutation.

Additionally, a somatic missense mutation was also identified in exon 27b; this previously unreported alteration A4711C (I1571L) is predicted to result in a conservative substitution of nonpolar amino acids of closely related side chains and was thus less likely to modify the protein function. The 4711A nucleotide is conserved in rat and *Drosophila*, but not *Fugu*, whereas the isoleucine is conserved in all three organisms.

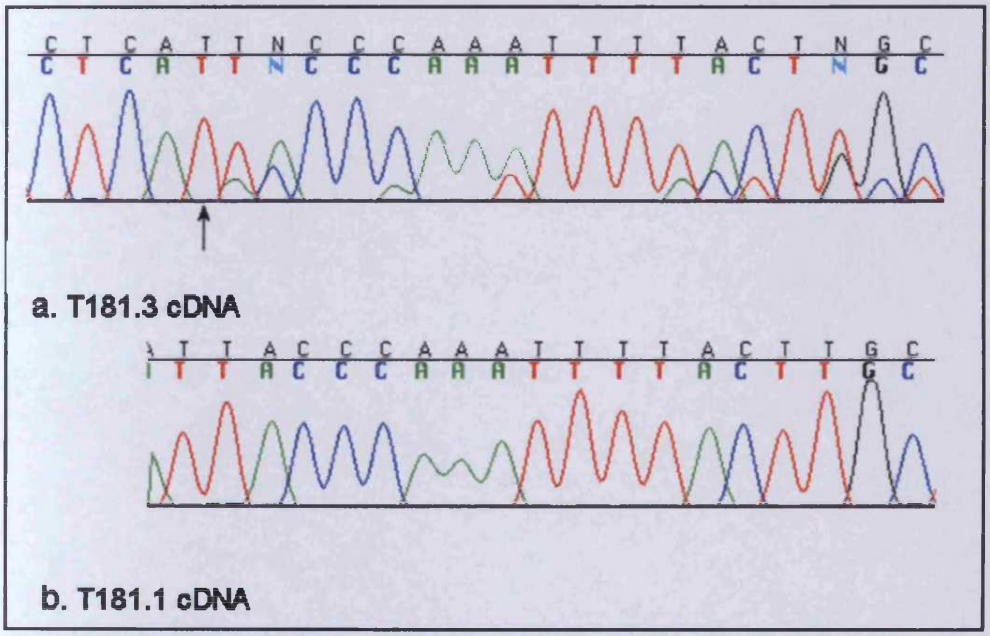

Figure 5.5: Chromatograms of somatic mutation 6408delT from tumour T181.3. The absence of the substitution in T181.1 confirms the somatic nature of the alteration.

a. Forward sequence of fragment 19 in T181.3 SC cDNA.

b. Forward sequence of fragment 19 in T181.1 SC cDNA

No mutation was identified in T181.2 SC cDNA. It is possible that the somatic mutation in this tumour is located in an intron in or close to a donor or acceptor splice site, or involves a deletion (including LOH) or gross rearrangement that could not be identified by the technique used in this study.

In patient B183, a 10bp deletion, 7448delTGCAGCCACC, was found in fragment 22 of T183.1 SC cDNA. This mutation in exon 42 was not found in blood lymphocyte DNA (B183), but confirmed in T183.1 whole tumour DNA (Figure 5.6) and is expected to result in the appearance of a stop codon in the same exon (R2498X). This alteration has not previously been reported. The region is flanked by inverted repeats (GGATA...13bp...TATCC), which may have mediated the deletion (Cooper and Krawczak, 1993).

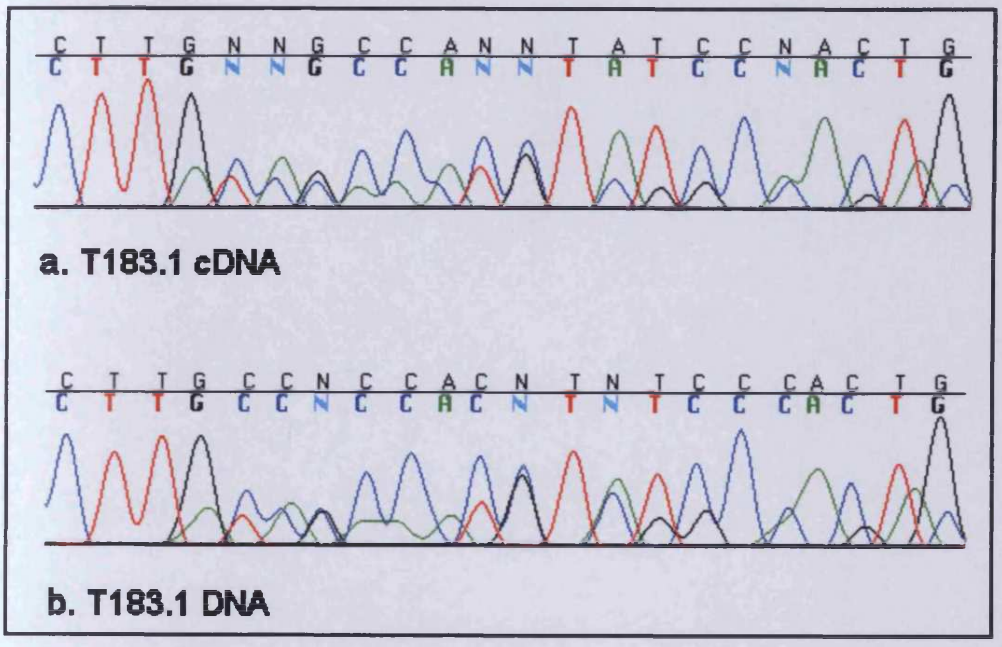

Figure 5.6: Chromatograms of somatic mutation 7448del10 from tumour T183.1.
a. Forward sequence of fragment 22 in T183.1 SC cDNA.
b. Forward sequence of exon 42 in T181.1 whole tumour DNA.

5.2.3/ Conclusion

In this panel, all germline mutations were identified (one was a germline deletion that was previously published, and the remaining two were identified in this study), and were located in different exons, and as different types of mutation for each patient. In addition 4 somatic mutations were also identified in this panel.

Approximately 70% of disease-causing alterations in NF1 are either frameshift or nonsense mutations which are predicted to cause a premature truncation in the protein (Shen *et al.*, 1996). In this study, each of the 6 disease causing mutations (germline and somatic) is predicted to result in the synthesis of a truncated*NF1* transcript. The evaluation of exonic

splicing enhancer (ESE) motifs by ESE-finder and RESCUE-ESE yielded mixed results, though it would not appear that ESEs are significantly modified by the mutations found in this panel. Only one mutation in the panel, C4084T (R1362X), occurred inside the GRD.
The somatic nature of a mutation was determined by its absence from either blood lymphocyte DNA or from cDNA from other tumours from the same patient. It was however not possible to determine whether the mutations were located on different alleles, as this analysis would have required cloning of each sample and this was not permitted due to the time constraints. No somatic mutation was identified in T181.2 cDNA; this could be an intronic change, such as a splice-related mutation, or a multi-exonic deletion, both of which would not have been identified by cDNA direct sequencing. The reduced amount of material from this tumour did not allow for an LOH assay.

Of the disease-causing and somatic mutations, 2/3 was pyrimidine-to-purine (Y>R) transversion and 1/3 was a pyrimidine-pyrimidine (Y>Y) transition at a CpG dinucleotide. Mutations C1939X and R1362X occurred at residues conserved in human, rat (D45201), *Fugu* (AF064564) and *Drosophila* (L26501) NF1 homologues, whereas mutation L1569X occurred in a residue that was conserved in human, rat (D45201) and *Fugu* (AF064564).

5.3 Discussion

5.3.1/ Schwann cell culture

The heterogeneous cellular content of benign neurofibromas has made the task of both understanding how the tumour arises in NF1 and finding a somatic mutation difficult. It is only recently that SC have been shown to play a critical role in tumour formation. In a heterogeneous background of $NF1^{+/-}$ cell types, which are likely to contribute to tumorigenesis process (Zhu *et al.*, 2002), homozygous $NF1^{-/-}$ SC harbour the somatic mutation necessary to tumour formation. Isolating the $NF1^{-/-}$ SC could therefore provide a tool to target the ever elusive *NF1* somatic mutation, which in turn would offer a better insight into neurofibroma tumorigenesis.
Culturing SC has however proved a challenging task because normal SC do not respond to standard culture conditions. Similarly, neurofibroma-derived SC have showed an altered response to normal SC culture conditions (Serra *et al.*, 2000), suggesting that some pathways involved in SC growth are connected to or interfere with neurofibromin, and these are still

poorly understood. Recently, a protocol for SC culture that allows isolation of *NF1*$^{+/-}$ and *NF1*$^{-/-}$ SC from a neurofibroma has been devised (Rosenbaum *et al.*, 2000; Serra *et al.*, 2000). This study endeavoured to establish this new culture technique in the NF1 lab and to use it as a means to isolate the SC population carrying the somatic mutation for later screening. Schwann cell culture proved to be a time-consuming process, where many different factors can hinder completion. Generally, there are different ways in which a cultured neurofibroma cell population can grow. These different population "types" have previously been reported (Muir *et al.*, 2001) and characterised following their growth and response to enrichment-targeted use of laminin and a soluble neuregulin. The cell lines developed in this screening study could be categorised as "type 2", where the SC are amenable to enrichment using laminin and heregulin, grow rapidly and stain positive for S100.

5.3.2/ cDNA screening and *NF1* mutation spectra

The *NF1* gene is a large gene, which can make the task of screening its sequence time-consuming. The use of the *NF1* cDNA sequence considerably reduced the size of the material that needed to be screened from 350kb genomic DNA to approximately 12kb of cDNA (Shen *et al.*, 1996). The small number of fragments (24 cDNA fragments compared to 60 genomic fragments) and the accuracy and increasing speed of direct sequencing has made this a fast and reliable technique.

One of the *caveats* inherent in using a RNA-based technique is that RNA is less stable than DNA and is easily degraded. Previous studies have reported an "aged blood" effect, which may occur when blood is left unprocessed for some period of time before RNA extraction (Wimmer *et al.*, 2000). The resulting RNA may exhibit sequencing artefacts, such as splicing aberrations, when screened for mutation. Similar outcomes have been observed when dealing with tumours that have not been frozen immediately after excision (Serra *et al.*, 2001; Section 6.2.1.5). By contrast, SC can be cultured from tumour received in medium after 2-3 days of transport.

Therefore, there are two advantages to extracting the RNA from cultured cells. Firstly, the RNA quality and quantity is higher than that obtained from whole tumours, probably due to the fact that RNA extraction is completed immediately after pelleting the cultured cells and that the chance for RNA degradation is considerably reduced. The cDNA screening

completed from SC RNA yielded no artefactual changes compared to cDNA screening from frozen whole tumours (section 6.2.1.5).

Secondly, and perhaps most importantly, targeting the cells sustaining the two hits permitted the identification of 4/5 somatic mutations. The 80% detection rate is the highest reported for somatic mutation. Prior to this study, a handful of *NF1* discrete somatic mutations have been found (Appendix) and the detection rate has ranged from 3% to 48% by use of SSCP/HA (John *et al.*, 2000; Serra *et al.*, 2001), an enzymatic mutation detection system (Weist *et al.*, 2004), DHPLC/cDNA-DHPLC (Upadhyaya *et al.*, 2004) and PTT (Eisenbarth *et al.*, 2000) on whole tumour DNA/RNA. In the somatic mutation analysis conducted on whole tumour DNA/cDNA in Chapter 6, the detection rate was 18%. As discussed in Section 6.3.3, there may be many explanations to account for the reduced identification of somatic mutations in NF1 tumours. In brief, and not withstanding limitations inherent to the detection methods, these include epigenetic inactivation, haploinsufficiency (with dominant-negative mutation or involvement of non-allelic alterations), or as yet unknown modifier genes that somehow influence somatic deletions over microlesions in some patients. However, the present results would also argue that the main limiting factor to somatic mutation detection remains cellular heterogeneity. With the removal of all cells except $NF1^{-/-}$ SC (or at the very least, a significant enrichment of the $NF1^{-/-}$ SC population), the somatic mutation detection rate rises to 80%, with only one detection method.

In the results from tumour T175.2, the point mutation was clearly identified in SC cDNA, but the peaks were much smaller in total DNA, probably due to the masking presence of other cell types. In patient P181, 2/3 somatic mutations were identified. By contrast, DHPLC screening of 3 neurofibromas from the same patient only identified one somatic mutation. It may still be possible that the "trend" observed by Weist and co-workers, where tumours from one patient harbour in majority somatic micro-lesions (possibly due to the input of modifier genes), is seen in the three patients from the present study (Weist *et al.*, 2003; Section 6.3.3). By contrast, a "trend" of LOH in tumours may be predominant in the patients described in Section 6.2.4.2.2 (discussed in Section 6.3.3). Nevertheless, the present results emphasize the need to overcome cellular heterogeneity in order to obtain a true estimation of microlesions in the *NF1* somatic mutational spectrum.

As the aim of this chapter was to consider on the use of SC culture and RNA isolation for successful mutation detection purposes, and the study panel is altogether very small,

observations on mutation distribution and comparisons to other studies cannot be undertaken here. Overall, the observations on both germline and somatic spectra, regarding the widely varying type and location, and the lack of correlation between somatic and germline mutations (in both nature and position on the gene) mirror those made in Section 6.3. Additionally, SC culturing did not seem to introduce artefactual mutations (at least in the *NF1* gene), as the mutations identified proved to be present in whole tumour DNA and/or blood DNA whenever it was possible to check.

5.3.3/ Conclusion

This study assessed the combined use of SC culture to isolate the somatic mutation-carrying SC and cDNA screening of the *NF1* gene. These techniques yielded the highest mutation detection rate thus far, with 2/2 germline mutations and 4/5 (80%) somatic mutations identified.

Whilst the use of SC culture should be considered as a useful tool for improved identification of the somatic mutation, this technique was both time-consuming and labour intensive. As such, SC culture is unlikely to be an appropriate tool for routine screening, but should nevertheless be considered in smaller research-based studies, or when a mutation cannot be identified by conventional means. Moreover, DNA obtained from $NF1^{-/-}$ SC could also be used in LOH screening, as it should greatly reduce the dosage effect observed in whole tumour DNA screen, by reducing the contaminating background cell population (Serra *et al.*, 2001).

Screening of cDNA provided a fast and reliable method to screen the coding sequence of the *NF1* gene. However, it will not identify a number of mutations: multiexonic changes are likely to be missed, and mutations close to the exon/intron splice site, if located in the intron, will not appear in the sequence

Combining SC cDNA screening with other mutation detection techniques, such as LOH, or DHPLC of genomic DNA to detect splice-related mutations in genomic DNA should be considered when investigating the NF1 somatic mutational spectrum.

CHAPTER 6: GERMLINE AND SOMATIC MUTATION DETECTION IN NF1-ASSOCIATED TUMOURS

6.1 Introduction

To date, over 730 mutations have been reported in the *NF1* gene (HGMD, http://www.hgmd.org; De Luca *et al.*, 2004; Mattocks *et al.*, 2004; Upadhyaya *et al.*, 2004; Kluwe *et al.*, 2005) and range from gross rearrangement to microlesions (Appendix). The majority (70%) of these mutations are predicted to result in a truncated protein (Shen *et al.*, 1996). Although mutations are spread along the *NF1* gene and no real mutational "hotspot" has been found thus far, the knowledge acquired from several mutational studies now underlines a number of recurrent mutations (Section 1.3.1.6). Determining the germline mutational spectrum revealed the presence of a putative second functional domain (Fahsold *et al.*, 2000), coinciding with a cysteine-serine rich domain (Izawa *et al.*, 1996); the increased clustering of missense alterations in exons 11-17 sets it apart from the rest of the gene.

Establishing the *NF1* mutational spectrum not only highlights putative functional regions, but also helps to elucidate the mutational mechanisms involved and identify the elusive genotype-phenotype relationship. It would be of particular interest, for example, to determine the spectrum of *NF1* mutations in the various tumours occurring in the condition. Just as neurofibromas are a hallmark of NF1, optic pathway gliomas are frequently seen in NF1 patients (Listernick *et al.*, 1999). These tumours are thought to arise from $NF1^{-/-}$ astrocytes cells, with the participation of the $NF1^{+/-}$ micro-environment (Bajenaru *et al.*, 2005), in a process similar to that seen in $NF1^{-/-}$ Schwann cells and $NF1^{+/-}$ surrounding cells in the formation of neurofibromas (Zhu *et al.*, 2002). Loss of *NF1* gene expression has been observed in NF1-related, but not sporadic, gliomas (Wimmer *et al.*, 2002), but there are only a handful of studies that attempt to classify the *NF1* mutational spectrum in these tumours (Table 6.1).

While the picture on germline *NF1* mutations has become much clearer, the somatic mutational spectrum of *NF1* has yet to be defined. Each neurofibroma arises from an independent somatic event and loss of heterozygosity of the *NF1* gene is often the first sign of a somatic hit (Colman *et al.*, 1995); however, only a handful of studies have assessed somatic micro-lesions with the various available techniques and varying degrees of success (Table 1.2). To date, fewer than 40 somatic microlesions have been identified and these seem so far

to be broadly similar to the germline mutations in both their nature and location. Interestingly, some alterations have been found both as somatic and germline mutations in different patients (Appendix).

For this analysis, a panel of 75 NF1-related tumours from 31 patients were available for mutation detection by a combination of techniques. Additionally, blood samples for 24 NF1 patients with gliomas were obtained from Professor Gareth Evans, Manchester and were screened for mutations along the entire length of the *NF1* gene.

The aims of this study were to

1) Identify somatic mutations from NF1-associated tumours

2) Compare somatic and germline mutational spectra

3) Ascertain whether specific germline mutations predispose to the development of MPNSTs

4) Ascertain whether certain germline mutations are associated with the development of optic gliomas.

Table 6.1: Germline mutations in patients with gliomas in the literature.

Mutations marked with an * have also been reported in other patients (whether these other patients harboured gliomas is unknown). The number of mutations summarized here is likely to be an underestimate, as relatively few studies report the clinical features of their patient panel. Nucleotide numbering is based on GeneBank accession no. M82814. The first base of the initiator methionine is taken as the start of the cDNA.

Germline mutation	Reference
x7, 1019insT	Ars *et al.*, 2003
x7, 989insC	Ars *et al.*, 2003
x7, 998insA	Ars *et al.*, 2003
x4b, 580delC	Ars *et al.*, 2000a
x4c, 590delC	Ars *et al.*, 2003
x10b, A1466G (Y489C)*	Ars *et al.*, 2003
IVS10a, 1393-9 T>A	Ars *et al.*, 2000a
x10c, 1541delAG*	Ars *et al.*, 2003
x12a, 1756delACTA*	Ars *et al.*, 2003
x13, 2173insT	Ars *et al.*, 2003
x24, A4267G (K1423E)*	Upadhyaya *et al.*, 2004
x29, 5351insC	Ars *et al.*, 2003
x29, G5546A (R1849Q)*	Ars *et al.*, 2000a
x40, 7204delCA	Ars *et al.*, 2003
x44, C7702T (Q2568X)*	Ars *et al.*, 2003

6.2 Results

Alterations were sought using denaturing high-performance liquid chromatography (DHPLC), direct sequencing and deletion junction PCR; loss of heterozygosity analysis (LOH) targeted specifically somatic hits and cDNA was also used for rapid screening of the coding region. The observations regarding the use of these techniques are presented in the following sections.

6.2.1/ Combined mutation detection techniques

6.2.1.1/Deletion junction-specific PCR assay

Initial attempts to reproduce the published conditions (Lopez-Correa *et al.*, 2001) were unsuccessful and a variety of optimisation trials still yielded a number of non-specific bands, as well as being generally irreproducible. The assay proved extremely sensitive to temperature differences and to the rapidity with which a thermocycler would go from one temperature to the other. Finally, the published conditions were used and the assay was conducted on one specific thermocycler (ABI). The assay yielded a very clear positive control, and although small non-specific band could be seen, they did not affect the results.

Although the two different positive controls amplified demonstrated a 3.4kb fragment indicative of a 1.5Mb deletion (Lopez-Correa *et al.*, 2001), no sample in the panel was found to present such a large deletion (Figure 6.1).

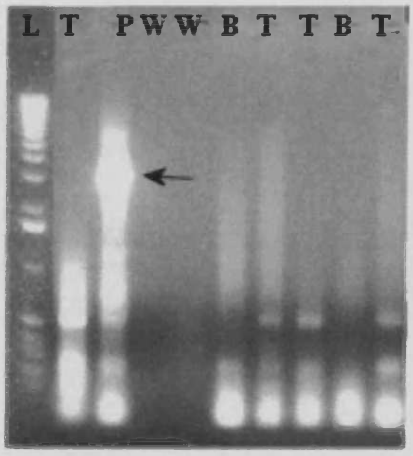

Figure 6.1: Representation of results obtained using the deletion-junction specific PCR assay.
No sample (blood or tumour) amplified the chimeric fragment characteristic of a large 1.5Mb deletion, in comparison to the positive control. The arrow points to the 3.4kb chimeric fragment in the positive control sample. PCR reactions usually had two positive controls (only one pictured here), from two different patients harbouring a large deletion. *L*, ladder; *P*, positive control, *B*, blood; *T*, tumour; *W*, water control.

6.2.1.2/ LOH assay

Intragenic microsatellite markers (I4b, I12b, J1J2, I27.13, EVI20 and I38) and RFLP (E5, I41 and C7CT), as well as four extragenic markers (HHH202 and UT172 located proximal to the gene; EW206 and EW207, distal of the gene) were used in this study as previously described (Upadhyaya *et al.*, 1998, 2003). Very little optimisation of the electrophoresis wattage, running time and silver staining process was required. The extragenic marker UT172 consistently failed to amplify with the DNA from the most recent tumours and was therefore not used in some samples. Similarly, several samples were refractory to PCR for some markers. The detailed results of the LOH assay are presented in Table 6.6. LOH was often observed as a dosage difference in the allele (Figures 6.6 and 6.7), compared with the blood (or other tumour) DNA pattern. This was more likely to be due to the mixed populations of cells found in neurofibromas, as well as surrounding non-neurofibroma tissue, which cannot always completely be excluded. Positive results were repeated at least twice to ensure accuracy.

6.2.1.3/ DHPLC analysis

The large majority of blood and tumour DNA samples were screened by DHPLC in an attempt to identify microlesions in the *NF1* gene. Any aberrant chromatogram profile was repeated to rule out false positive results, and upon confirmation, the fragment was screened by direct sequencing. When available, a positive DNA control with a known mutation was used.

DHPLC technology relies in part on the bond between base-pairs in a DNA strand, so that a mismatched pair resulting from a mutation will have a weaker bond, can be more easily denatured at a set temperature, and will elute earlier than its wild-type counterpart. With the aid of the WaveMaker software (Transgenomics, Crewe, UK), up to three oven temperature were determined for each fragment (Table 2.3). Of the 60 exons, the three alternatively spliced exons (9a, 23a and 48a) were excluded from the study, as they have never been found to harbour a mutation (Upadhyaya and Cooper, 1998; Fahsold *et al.*, 2000; Messiaen *et al.*, 2000; Han *et al.*, 2001). The DHPLC is suited to the analysis of DNA fragments ranging from 170-700 base-pairs (Xiao and Oefner, 2001), and the fragments designed for direct sequencing were also used for DHPLC analysis.

The chromatograms observed ranged from very distinct peaks to "shoulders" on the side of the main peak (Figures 6.2, 6.4 and 6.9). Different mutations in the same exon can yield

different chromatograms because the nature and location of the mutation may also influence the appearance of the chromatogram. Incidentally, an interesting profile was seen in blood DNA from 2227 and its matching tumour samples: although similar in shape to the other chromatograms, DHPLC peaks in these two samples appeared earlier than did the comparable peaks in the other samples tested (Figure 6.4). This result required the run to be repeated as the event could have been artefactual (caused for example by an air bubble). Following sequencing, sample 2227 exhibited homozygosity for an intronic polymorphism in IVS20 (3496+33 A), where the A allele has been reported to substitute the C allele in 33% of the population (De Luca *et al.*, 2003). Thus, sample 2227 harboured an A-T pair, sharing a weaker 2-hydrogen bond than the more common G-C 3-hydrogen bond, which caused an early profile to appear.

For the DHPLC analysis alone, approximately 6000 PCR reactions were required to complete the mutation screening.

6.2.1.4/ Direct sequencing

Direct sequencing was used to identify any alteration linked to an aberrant DHPLC profile and was used in the major part of the cDNA second screening, as the technique became more user-friendly. Over the course of this study, the set-up time for sequencing was considerably reduced by the introduction of plate purification systems (Montage by Millipore) for both PCR and sequencing product purification. The sequencing mix (Big Dye) was also adjusted to the new set-up and the reduced amount of Big Dye proved more cost-effective while retaining the same sequencing quality. The sequencing analysis was conducted on the automated ABI 3300, which allowed the analysis of 16 samples per hour.

Although it was re-designed twice in the lab, the set primer for exon 10b did not amplify the *NF1* gene exclusively and a number of alterations were seen at sequencing that were not reported further in the following mutation study: alterations 1393-9 T>G in intron10a, G1394C (S465T), A1467C (T486T), A1470G (R487R) and A1475C (Y489C) in exon 10b, and 1527+7 A>G in intron 10b. The sequence changes were verified using BLAST; all but A1475C were found to correspond to an *NF1* pseudogene on chromosome 2. It is not known whether all alterations were allelic. Although the primers used were verified when they were first designed, the *NF1* gene possess many pseudogenic sequences (Section 1.2.5). It may also be that pseudogene sequences vary between individuals. Consequently, all the alterations presented in the following sections were checked by BLAST to ensure they did not

correspond to pseudogene sequences. It should be noted that alteration A1475C (Y489C) has been reported in 8 NF1 patients as a missense mutation that created a new donor splice site and which resulted in the deletion of 62 nucleotides (Osborn and Upadhyaya, 1999; Ars *et al.*, 2000a; Fahsold *et al.*, 2000; Messiaen *et al.*, 2000; Han *et al.*, 2001; Mattocks *et al.*, 2004).

6.2.1.5/ cDNA screening

cDNA was used in this study in two contexts. Firstly, 7 tumour samples (T141.5, T141.6, T141.11, T142.3, T143.5, T149 and T156.3), with no identified somatic mutations in the genomic DNA, were screened using cDNA to assess the technique (for both DHPLC and direct sequencing), and to try to identify mutations that may have been missed. Secondly, the cDNA from four additional tumours' (T168, T177, T184 and T185) was screened for mutations. The use of 24 cDNA fragments instead of 57 genomic fragments reduced the screening time. Consequently, direct sequencing of the cDNA fragment was chosen over DHPLC screening, as it did not require multiple PCR reactions for the same fragment and proved to be much quicker. While the use of cDNA would not allow for identification of splice-related mutations and other intronic alterations, it would also prevent the amplification of *NF1* pseudogenes.

The main limitation of using cDNA fragments did not come from the technique itself but from the original RNA quality. Most of the tumours received were stored in tissue culture medium at room temperature, for the two or three days of transit to the NF1 laboratory, and RNA degradation could not be prevented during that time. As a consequence, sequencing artefacts were occasionally observed (skipping of exon and intron 37, skipping of exon and intron 40, partial intron 4a and exon 4b sequence overlapping normal exon 4b sequence). Sequencing for these samples had to be repeated, and confirmation at the genomic DNA level was always sought.

Previous studies have reported an "aged blood" effect, which occurs when blood is left unprocessed for a period of time (a 48 hours delay was bench-tested by Wimmer and co-workers) before RNA extraction (Wimmer *et al.*, 2000; Ars *et al.*, 2003). The resulting RNA can exhibit artefacts, such as apparent splicing aberrations, when screened for mutation, however such alterations are not seen in the corresponding genomic DNA. Reported abnormal transcripts include the insertion of cryptic exon (exon 4a-2; Ars *et al.*, 2000b; Wimmer *et al.*, 2000), the skipping of constitutional exon such as exon 20 (Thomson and Wallace, 2002) or

43 (Thomson and Wallace, 2002; Vandenbroucke *et al.*, 2002; Vandenbroucke *et al.*, 2001); in all cases, the DNA was extracted several days after drawing blood. Similar aberrant transcripts have also been reported when handling neurofibroma RNA (Serra *et al.*, 2001a).

It is possible that the same RNA 'aging' problem occurred with tumours used here. As mentioned in Section 5.2.2.1, this was not tested against tissues preserved immediately after excision, and therefore could not be verified here; however, cDNA derived from RNA from cultured Schwann cells did not harbour such alterations when screened for mutations. To preserve the RNA, tumours should be frozen immediately after excision. In this study, none of the tumours received were snap frozen for preservation, as they were used for different purposes and came from different sources, some of which may lack the appropriate facilities for freezing the tumours.

6.2.2/ *In silico* analysis

Recently, the effect of missense and nonsense *NF1* mutations on splicing has been revisited using both prediction programs and experimental assays (Zatkova *et al.*, 2004). This group demonstrated that alterations that were predicted to disrupt exonic splicing enhancer (ESE) elements also reduced their splicing enhancement capacity in a quantitative assay, thus revealing the mechanism underlying the exon skipping effect of these mutations. In the light of these observations, samples harbouring a base-pair substitution were assessed for the presence of ESE elements in the sequence surrounding the alteration (8 bases on either side). Two prediction programs were used: ESEfinder (www.exon.cshl.org/ESE; Cartegni *et al.*, 2003) and RESCUE-ESE (www.genes.mit.edu/burgelab/rescue-ese/; Fairbrother *et al.*, 2004). The disruption/loss of predicted ESE sites has been deemed to have a dominant effect over the increase in the score of another ESE (Zatkova *et al.*, 2004), and all the results presented here were analysed accordingly and discussed in Section 6.3.2.1. While the results may present an insight into the functional aspect of an alteration, full analysis would require the use of additional *in vitro* assays, which were not undertaken here.

The cDNA and amino acid sequences of *NF1* gene homologues in rat (D45201), *Fugu* (AF064564) and *Drosophila* (L26501) were retrieved from EntrezGenes (http://www.ncbi.nih.gov/entrez/) and aligned to the *NF1* cDNA and amino acid sequences using CLUSTALW (http://www.ebi.ac.uk/clustalw/). Genomic sequences of the *NF1* gene homologues in rat and *Drosophila* were retrieved from the UCSC genome browser

(http://www.ebi.ac.uk/clustalw/) and aligned to the *NF1* DNA sequence using CLUSTALW. Conservation of the *NF1* gene sequence at the nucleotide sequence level is estimated to be over 90% between human, rat and *Fugu*, and ~60% between human and *Drosophila* (Bernards, 1993; Kehrer-Sawatzki *et al.,* 1998). Thus, a mutation within a codon conserved between species is more likely to be pathogenic.

For intronic and exonic alterations, a splice site prediction program (http://www.fruitfly.org/seq_tools/splice.html) was used to determine if a new splice site was predicted to be formed by the mutation. Finally, in a number of cases, the complexity of a sequence of 10bp on either side of an alteration was sought using the Complexity program (http://wwwmgs.bionet.nsc.ru/mgs/programs/lzcomposer), to gain an insight into the mechanism underlying the mutation (Chuzhanova et *al.*, 2002).

6.2.3/ Germline mutations in NF1 patients with optic gliomas

A panel of 24 lymphocyte blood DNA samples from NF1 patients who had presented with a glioma was obtained from Prof. Gareth Evans in Manchester. The samples were screened for germline mutations by DHPLC and any changes in the chromatograms were analysed by direct sequencing. The sequence changes identified are summarised in Tables 6.2 and 6.3.

6.2.3.1/ Splice-related alterations

Similarly, patient 2248 had a C to A substitution 3 bases upstream of exon 12a, in the splice acceptor site. A mutation causing a frameshift and the addition of 14 amino acids in the protein has previously been reported at the same nucleotide: 1722-3 C>G (Ars *et al.*, 2000a). As the alteration occurred outside of the canonical AG dinucleotide, similar substitutions bearing a pathogenic significance were sought in the literature: in the *NF1* gene, a substitution to A at the normally conserved pyrimidine at position (–3) has been reported in intron 4b (Park and Pivnick, 1998), with the predicted skipping of the following exon and truncation of the protein. A total of 109 mutations at position (-3), including 19 C>A substitutions, have also been reported in other disease genes (*NF2*, *MLH1*; HGMD). However, a missense alteration has also been found in this patient (section 6.2.3.5) and the nucleotide substitution has previously been predicted to result in a truncated protein.

An A to G transition was found 3 bases into the splice donor site of intron 11 (1721+3A>G) in patient 2264. This mutation has previously been reported in the germlines of 6 NF1 patients to be associated with the skipping of exon 11 by inactivation of the 5' splice site and truncation of the protein (Purandare *et al.*, 1994; Fahsold *et al.*, 2000; Ars *et al.*, 2003; De Luca *et al.*, 2004). A similar transition at the relatively conserved (+3) purine of the 5' splice-site was identified in intron 36 (Side *et al.*, 1997; Ars *et al.*, 2003), with the predicted skipping of the following exon and/or truncation of the protein. A total of 157 mutations at position (+3), including 58 A>G substitutions, have also been reported in other disease genes (*RB1*, *BRCA1*, *APC*, *MLH1*; HGMD).

6.2.3.2/ Nonsense alterations

Patient 2254 presented a C>T transition at a CpG dinucleotide in exon 16 (C2246T, R816X). Following methylation at its 5 position, the cytosine of a CpG dinucleotide is prone to undergo spontaneous deamination to thymine and this mechanism, coupled with less than completely efficient DNA repair, is thought to underlie the C>T transition (Cooper and Youssoufian, 1988). The mutation has been reported in 9 NF1 patients, including 8 individuals with typical NF1 features and an NF1-Noonan family (Maynard *et al.*, 1997; Bahuau *et al.*, 1998; Fahsold *et al.*, 2000; De Luca *et al.*, 2004). This alteration created a stop at codon 816 and PTT confirmed the truncating nature of the mutation (Bahuau *et al.*, 1998). Additionally, ESEfinder predicted the creation of a new ESE element for SR protein SF2/ASF in the mutated sequence, suggesting a truncating effect for the mutation rather than interference with normal splicing.

Another C>T transition at a CG dinucleotide was found at nucleotide 574 (exon 4b) in patient 2263. The R192X mutation has previously been reported in 6 NF1 patients (Toliat *et al.*, 2000; Fahsold *et al.*, 2000; Messiaen *et al.*, 2000; De Luca *et al.*, 2004) and predicted to create a truncated protein. Nevertheless, ESEfinder predicted two ESE elements for protein SRp40, which were modified by the C>T transition: one motif was lost whereas the second motif score increased. RESCUE-ESE also predicted the destruction of one hexamer. The loss of predicted ESE elements may indicate an association between the nonsense mutation and aberrant splicing.

6.2.3.3/ Small insertions

Insertions of 1 and 2 base-pairs were found in patients 2255 and 2246 respectively.
In patient 2246, an unreported insertion was identified in exon 34. Mutation 6523insGA occurred in a sequence of 5 GA repeats, which is likely to account for the slippage in replication (Cooper and Krawczak, 1993). The 2-base pair insertion, while creating a frameshift, may also result in a truncated protein, due to the appearance of a stop codon in the downstream sequence (T2179X).

Another unreported alteration in exon 7 occurred in patient 2255. Mutation 1011insT resulted in a frameshift and the appearance of a stop codon (D338X), most likely creating a truncated protein. The insertion may have arisen due to the presence of imperfect inverted repeats (AAGANAA/TNTNTCNTT), where an unmatched A base may have provided a template for gap repair (Cooper and Krawczak, 1993).

6.2.3.4/ Small deletions

The most represented alteration in this panel, small deletions of 2 and 4 base-pairs were identified in 5 patients, two of which lesions have previously been reported.
Identified in exon 37 of patient 2258, alteration 6789delTTAC is a recurrent mutation found in 13 NF1 patients to date and has been predicted to result in a truncated neurofibromin (Robinson *et al.*, 1995; Boddrich *et al.*, 1997; Hoffmeyer *et al.*, 1998; Fahsold *et al.*, 2000; Messiaen *et al.*, 2000; De Luca *et al.*, 2004, Upadhyaya *et al.*, 2004). It has been proposed that the presence of direct (ACA) and inverted (ACTT...9bp...AAGT) repeats and symmetric elements (CTGACA/ACAGTC) makes that DNA sequence more prone to mutations (Robinson *et al.*, 1995; Boddrich *et al.*, 1997; Upadhyaya *et al.*, 2004). In particular, Robinson and co-workers proposed that the symmetric elements allowed for the formation of a Möbius-like-loop structure where only the middle four (of six) mismatched bases are deleted. The structure may also be subject to inadequate mismatch repair processes and nucleotides 6789-92 in exon 37 seem to represent a mutational "warmspot" in the *NF1* gene (Boddrich *et al.*, 1997). Indeed, five additional mutations have been found in these nucleotides: 6790insTT in 1 patient (Boddrich *et al.*, 1997), 6791insA in 8 patients (Upadhyaya *et al.*, 1996b; Abernathy *et al.*, 1997; Colman *et al.*, 1997; Osborn and Upadhyaya, 1999; Fahsold *et al.*, 2000; De Luca *et al.*, 2004; Upadhyaya *et al.*, 2004), C6792A in 19 patients (Robinson *et al.*, 1995; Messiaen *et al.*, 1997; Hoffmeyer *et al.*, 1998;

Ars *et al.*, 2000a; Fahsold *et al.*, 2000; Messiaen *et al.*, 2000; Serra *et al.*, 2001a; Kluwe *et al.*, 2003a; Ars *et al.*, 2003; De Luca *et al.*, 2004; Mattocks *et al.*, 2004), C6792G in 4 patients (Messiaen *et al.*, 1997; Fahsold *et al.*, 2000; Messiaen *et al.*, 2000; Mattocks *et al.*, 2004) and 6792insA in 3 patients (Fahsold *et al.*, 2000; Ars *et al.*, 2003).

Sample 2257 harboured a four base-pair alteration in exon 4b; mutation 495delTGTT has previously been reported in 6 NF1 patients (Fahsold *et al.*, 2000; Han *et al.*, 2001; De Luca *et al.*, 2004) and resulted in a frameshift, and the appearance of a stop at residue 176 (D176X). The deletion may have occurred through slipped mispairing due to the presence of TGTT direct repeats in the surrounding sequence (Cooper and Krawczak, 1993). Other deletions have also been reported in the immediate vicinity: 496delGT in 1 patient (Toliat *et al.*, 2000), 496delGTTT in 1 patient (De Luca *et al.*, 2003) and 499delTGTT in 12 patients (Toliat *et al.*, 2000; Fahsold *et al.*, 2000; Ars *et al.*, 2003; De Luca *et al.*, 2004; Mattocks *et al.*, 2004), suggesting that the presence of the direct repeats may present a mutational hotspot in codons 165-167.
Sequence:
5'-TTAACtgttTGTTCAGAAGACAATGTTGATGTTCATGATATAGAATTGTTACA-3'

A small deletion of 2 base-pairs, 1306delTC was identified in exon 10a of patient 2250. The resulting frameshift created a stop codon at position 437, and is thus likely to cause a truncation in the transcript.

Alteration 1542delGG was found in exon 10c of patient 2256. The subsequent frameshift is expected to create a stop codon further downstream in the cDNA sequence at residue 556 (D556X), in exon 11, assuming splicing of intron 10c is not impaired which would subsequently truncate the protein. This mutation occurred in a stretch of 4 G nucleotides and has not previously been reported.

Lastly, patient 2260 had a two base-pair deletion in exon 2, 97delAA, that produced a frameshift in the cDNA with the appearance of a stop codon at residue 36 (S36X). The alteration has not previously been reported. The presence of a CAGT sequence close to the deletion site may have provided a template for slipped mispairing and deletion of AA, to form a second CAGT sequence.
Sequence: 5'-CAGAACACACATACCaaAGTCAGTACTGAGCA-3'

6.2.3.5/ Missense and silent alterations

A silent sequence change was found in exon 8 of patient 2252. Interestingly, C379C (C1137T) was the only non-polymorphic mutation found in this patient. The nucleotide change may be of functional importance and can therefore not be ruled out as a disease-causing mutation. The C at position 1137 is conserved between human, *Fugu* and *Drosophila* gene sequences, but has been replaced by a T in the rat sequence. Additionally, ESEfinder predicted nucleotide 1137 to be located in an ESE motif and the C>T transition would reduce the affinity of protein SRp40 for the motif. These results indicate that this silent mutation may actually induce a defect in splicing.

Patient 2267 had a silent mutation in exon 2. The C to T transition at nucleotide 168 did not change the amino-acid serine encoded by codon 56. This putative polymorphism has previously been reported as present both in NF1 patients with disease-causing mutations and unaffected individuals (Mattocks *et al.*, 2004). The C allele is predominant (95.9%) to the T allele (4.1%) in the general population. The mutation occurred in a CG dinucleotide. Furthermore, the C allele is conserved between human, *Fugu* and *Drosophila* whereas a T allele is present in the rat *Nf1* sequence. The issue of variability, in human, of a nucleotide otherwise conserved through evolution has previously been raised by Krawczak *et al.* (1999). The author offered two hypotheses to explain this phenomenon. Firstly, it may be that the nucleotide was part of a motif (important sequence) conserved through evolution, but no longer used in human, and thus free to undergo substitution. Secondly, it s possible that the polymorphism originally existed in the other organisms, but were lost due to inbreeding as a consequence of the animals' commercial or industrial importance. Finally, in ESEfinder, the alteration resulted in the loss of an ESE element.

Patient 2253 showed an A>G transition at nucleotide 1013 in exon 7, resulting in the appearance of a glycine in the place of an asparagine residue at codon 338. This alteration has previously been reported in one NF1 family and found to segregate with the disease (Upadhyaya *et al.*, 1997). Interestingly, the A at position 1013 in the human *NF1* gene is conserved in the rat, *Fugu* and *Drosophila* homologues, as is the asparagine residue in the protein. The high degree of conservation through eukaryotic evolution (more than 500 Myrs) underlies the functional importance in the protein. The substitution to asparagine not only

introduces a polar group to the structure, but also a much larger side chain, which could both impair the capacity of amino acid 338 to interact with other groups. Additionally, and although ESEfinder did not predict any ESE elements, RESCUE-ESE identified two hexamers in the wild-type sequence that were lost in the mutated sequence, which may indicate a role for the mutation in splicing. Finally, and perhaps more importantly, the nucleotide substitution is predicted to create a new (cryptic) donor splice site. As functional assay were not conducted in the present study, it would be difficult to determine the part played by ESE disruption and apparition of a cryptic splice-site in the mutation's pathogenicity.

A leucine to isoleucine substitution was found at codon 575 in patient 2248, in addition to an intronic mutation (IVS11-3C>A), potentially splice-related. Interestingly, the C>A transversion occurred at base 1722, the first base of exon 12a, and may therefore have an effect on normal splicing by affecting the splice acceptor site. This mutation has recently been reported in a patient fulfilling the NF1 diagnostic criteria, and was predicted to result in a truncated protein (Mattocks *et al.*, 2004). The leucine to isoleucine substitution should not have a great impact on the protein structure itself, as it does not change the polarity and only slightly modifies the side-chain. As neither mutation (IVS11-3C>A and C1722A) has been functionally characterized, determining their contribution to pathogenicity is difficult and would require further investigation.

6.2.3.6/ Intronic alterations and known polymorphisms

6.2.2.6.1/ Polymorphisms

A reported polymorphism in exon 5 was found in 2/24 samples (8.3%). The G>A transition at nucleotide 702 is frequently encountered in NF1 samples and does not alter the leucine at residue 234. However, the alteration is used as the basis for an RFLP marker using enzyme *Rsa*I (Hoffmeyer and Assum, 1994). Mattocks *et al.* (2004) reported an heterozygosity of 53% for this polymorphism. The G allele is conserved in human and *Drosophila*, whereas the A allele is present in rat and *Fugu* homologues.

An intron 3 polymorphism, 288+41 G>A, was detected in 2/24 samples (8.3%). The reported heterozygosity for this alteration is 21% (De Luca *et al.*, 2003). An example of the chromatogram resulting from this alteration is depicted in Figure 6.2.

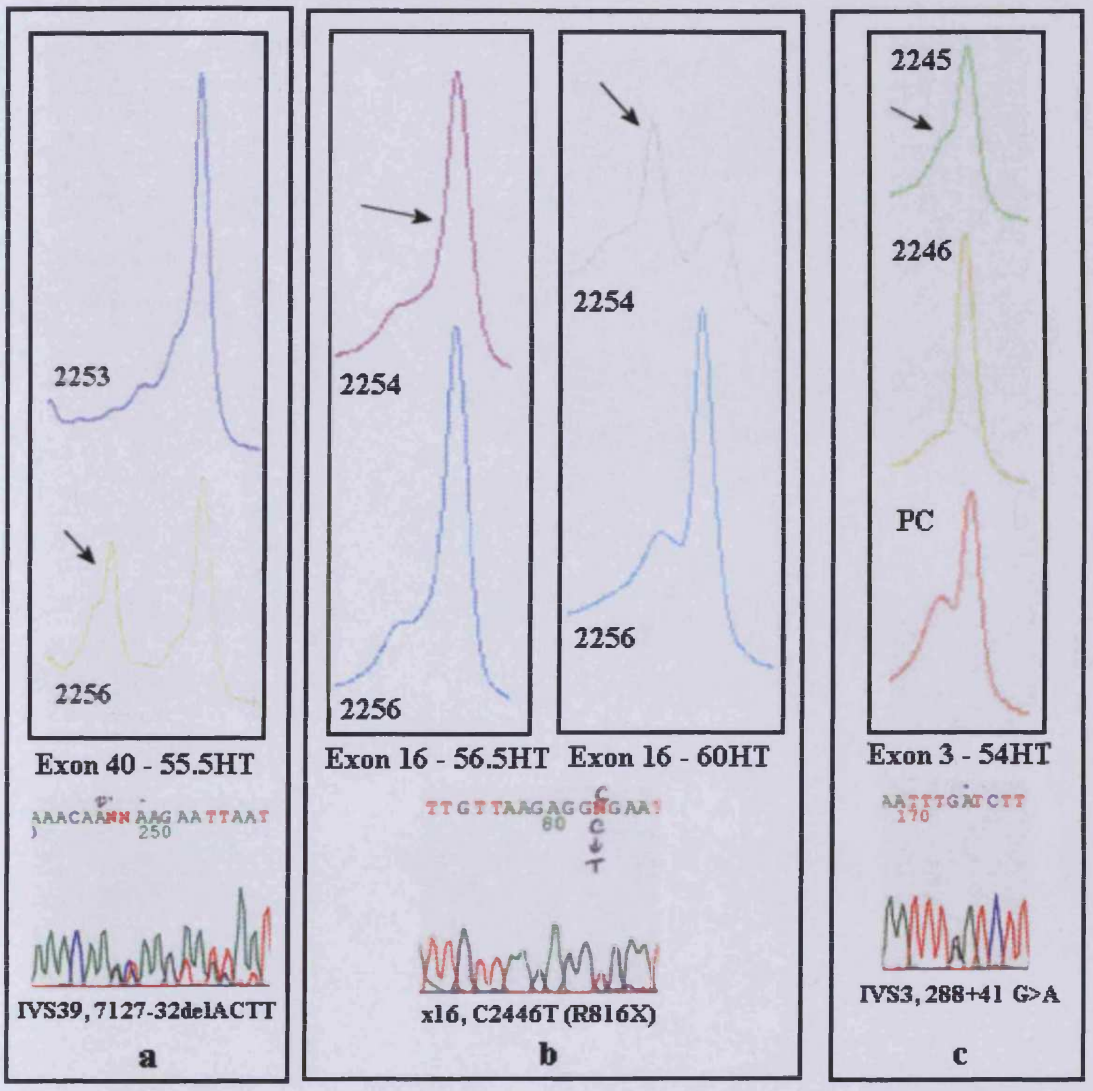

Figure 6.2: Chromatograms of germline alterations in patients affected with gliomas,

a. Profile of intronic alteration (IVS39) in sample 2256. The alteration is clearly seen on the chromatogram as an additional peak, compared to that of sample 2253. The deletion is located close to the beginning of the fragment (forward primer starts 82 base-pairs upstream of exon 40), which may explain the early peak on the chromatogram. The mutation type (frameshift) may also influence the resulting chromatogram.

b. Missense germline mutation in sample 2254 at two different denaturing temperatures. The first temperature shows a barely noticeable widening of the peak, compared to sample 2256. At a higher denaturing temperature, two peaks are visible. The denaturing temperatures for each fragment were determined using the WaveMaker software, so that two or three temperatures cover the whole fragment.

c. Intronic polymorphism (IVS3) in sample 2245. As is often the case, an alteration can be seen as "shoulder" on the side of the main peak, compared to a normal sample. The positive control is a nucleotide substitution in the exon (G278A).

Patient	Exon/Intron	Genomic mutation	Amino acid mutation	Type/effect	Reference
2246	**x34**	**6523insGA**	**T2179X**	frameshift / truncating?	This report
	IVS3	289-86delCT		deletion / ?	This report
2248	**x12a**	**C1722A**	**L5751I**	missense / truncating?	Mattocks *et al.*, 2004
	IVS11	1722-3 C>A		splicing?	This report
2250	**x10a**	**1306delTC**	**V437X**	frameshift / truncating?	This report
	IVS4a	479+55 T>A		Intronic / ?	This report
2252	x8	C1137T	C379C	silent	This report
2253	**x7**	**A1013G**	**D338G**	missense (splice)	Upadhyaya *et al.*, 1997
2254	**x16**	**C2446T**	**R816X**	nonsense / truncating	Maynard *et al.*, 1997
2255	**x7**	**1011insT**	**D338X**	frameshift / truncating?	This report
	IVS25	4368-44insAG		Intronic / ?	This report
2256	**x10c**	**1542delGG**	**D556X (x11)**	frameshift / truncating?	This report
	IVS39	7127-32delACTT		Intronic / ?	This report
2257	**x4a**	**495delTGTT**	**frameshift / D176X**	deletion/ truncation	Fahsold *et al.*, 2000
	IVS17	2851+32insAC		Intronic / ?	This report
2258	**x37**	**6789delTTAC**	**frameshift / V2268X**	deletion / truncation	Robinson *et al.*, 1995
2260	**x2**	**97delAA**	**S36X**	frameshift / truncating?	This report
2263	**x4b**	**C574T**	**R192X**	nonsense / truncating	Fahsold *et al.*, 2000
2264	**IVS11**	**1721+3 A>G**	**skip of exon 11**	splice / truncating	Purandare *et al.*, 1994
2267	x2	C168T	S56S	polymorphism	Mattocks *et al.*, 2004

Table 6.2: Pathogenic alterations and rare polymorphisms identified in the germlines of patients with gliomas. Putative disease-causing mutations are in bold.

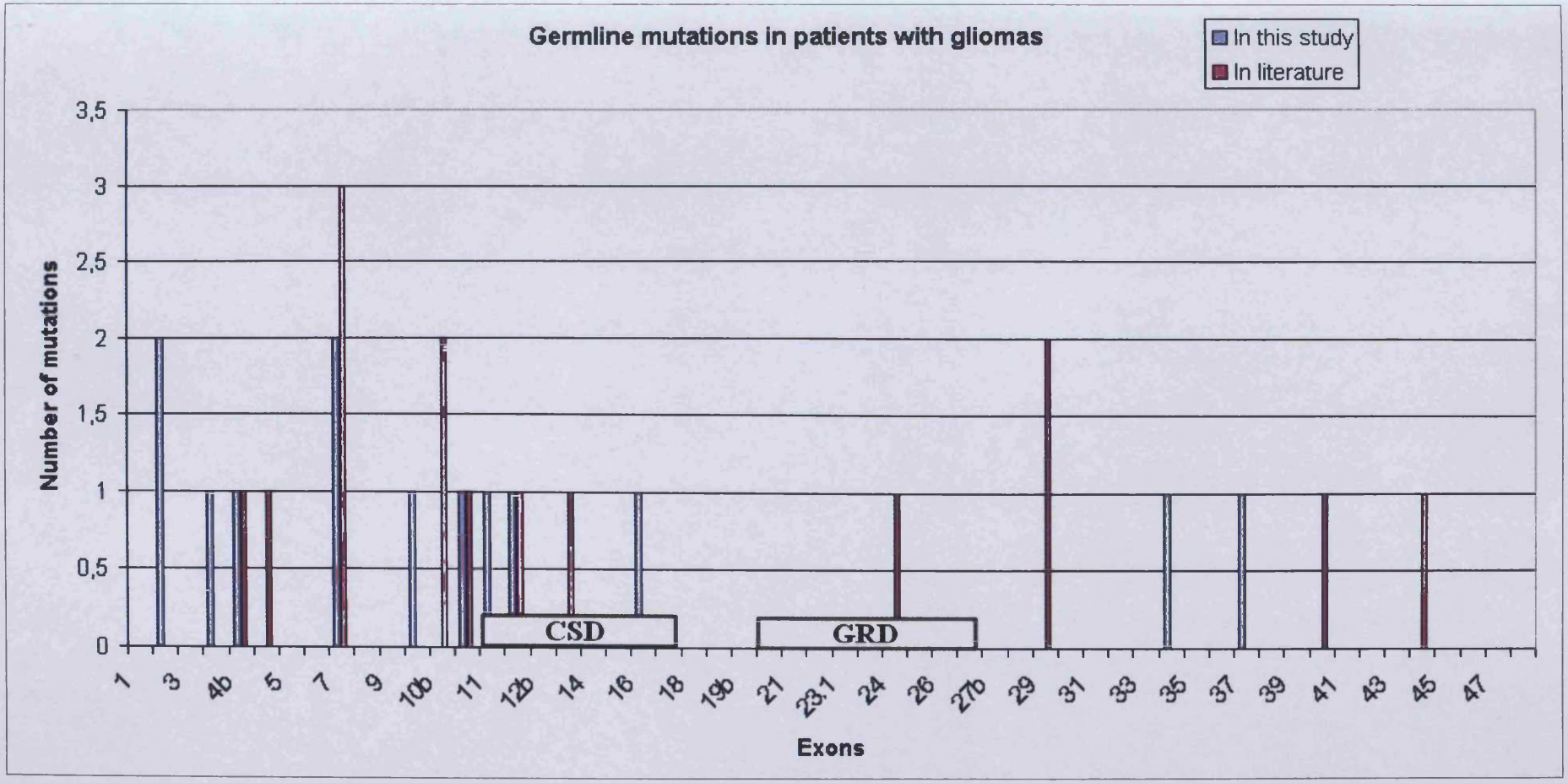

Figure 6.3 : Distribution of the germline mutation in patients with glioma.

The pathogenic mutations appear to be distributed mainly on or before the cysteine/serine-rich domain (CSD). The results in this study are in line with the data available in the literature. *GRD*, GAP-related domain.

Patients	Exon/Intron	Genomic mutation	Amino acid mutation	Het	Reference
2245, 2249	IVS3	288+41 G>A		21%	De Luca *et al.*, 2003
2245, 2262	Exon 5	G702A	L234L	53%	Mattocks *et al.*, 2004
2245, 2249	IVS10c	1641+39 C>T			Fahsold *et al.*, 2000
2248, 2250, 2252, 2259	IVS29	5546+19 T>A		7-41%	Han *et al.*, 2001; De Luca *et al.*, 2003
2251, 2260, 2266	IVS39	7126+37 G>C			Rodenhiser and Hovland, 1995
				5-24%	Han *et al.*, 2001; De Luca *et al.*, 2003

Table 6.3: Polymorphisms identified in the germlines of patients with gliomas.
Fahsold *et al.* (2000) did not indicate a definite heterozygosity (Het), but each allele is estimated to be represented with a frequency of at least 30%.

A transition in intron 10c, 1641+39 T>C, was observed in 2/24 samples (8.3%). This alteration has been reported as a polymorphism by Fahsold *et al.* (2000). The exact reported heterozygosity is not known. However, the frequency of both alleles was estimated to be higher than 30%.

As the most represented lesion in this sample panel, 5546+19 T>A was found in 4/24 patients (16.6%). The intron 29 polymorphism has previously been reported and heterozygosity was estimated at between 7% and 41% (Han *et al.*, 2001; De Luca *et al.*, 2003).

The last of the reported polymorphisms, 7126+37 G>C in intron 39, was found in 3/24 patients (12.5%). This transversion has previously been found in NF1 patients with known disease-causing mutations, as well as unaffected individuals, and the heterozygosity was reported to be up to 24% (Han *et al.*, 2001; De Luca *et al.*, 2003; Mattocks *et al.*, 2004).

6.2.2.6.2/Other intronic alterations

The alterations reported here were found in only one sample of the panel. While they were located far within the intron and away from the donor/acceptor splice sites and might indeed represent previously unreported intronic polymorphisms, a functional role for these alterations cannot be ruled out in the complex mRNA processing machinery.

Sample 2246, with an identified small insertion in exon 34, showed a 2 base-pair deletion in intron 3, 289-86delCT. A transversion, 479+55 T>A, was found in intron 4a of sample 2250 (known deletion in exon 10a); the nucleotide (T) was conserved in rat and *Drosophila*. A small insertion, 2990+32insAC was found in intron 17 of sample 2257, which also harbours a deletion in exon 37. Alteration 4368-44insAG was identified in intron 25 of patient 2255 (known insertion in exon 7). While this alteration has not previously been reported, Fashold *et al.* (2000) identified a closely located alteration, 4368-46 G>C. Finally, a 4-base deletion was found in intron 39 of sample 2256 (with a germline deletion in exon 10c). Alteration 7127-35delACTT has not previously been reported.

6.2.3.7/ Conclusion

In this panel, 22/24 samples were found to harbour at least one alteration in the *NF1* gene. A pathogenic mutation was identified in a total of 12/24 (50%) samples; this rises to 54% if the silent mutation in 2252 is considered pathogenic (by disruption of normal splicing). Five of the twelve mutations are novel (41%). Deletions represent the largest group of mutations. Transitions were found in 3/12 samples, two being pyrimidine to pyrimidine (Y>Y) at CpG dinucleotides, and one a purine to purine (R>R). Transversions were also found in 3/12 samples, all of which were pyrimidine to purine (Y>R).

Of the twelve mutations identified, eleven are expected to result in a truncated protein. This is in keeping with a trend reported by Ars and co-workers where all patients with gliomas harbour mutations resulting in a putative truncated neurofibromin (Ars *et al.*, 2000a, 2003). Interestingly, the pathogenic mutations appear to cluster at the beginning of the *NF1* gene, with 2 mutations each in exon 2 and 7. This observation appears to be in keeping with the mutations published so far in patient with gliomas (Figure 6.3).

6.2.4/ NF1-related tumour panel

6.2.4.1/ Germline mutations

Of the 31 patients in this study, three had known large germline deletions (P150, P176 and P186). Blood lymphocyte DNA was available for one of these three patients only, and was not screened by DHPLC, but used as a control. Additionally, blood lymphocyte DNA was not available for 4 of the 31 patients. In these cases, the screening of multiple tumours from the same patient made it possible to distinguish germline and somatic mutations. For all the other

individuals in the panel, germline alterations were sought by screening the coding region (and the intronic region close to the exon) of the *NF1* gene in 57 fragments optimised for DHPLC. Samples yielding an unusual profile on the chromatogram were repeated and, if similar results were observed, followed by direct sequencing of the fragment. Two patients, P169 and P178, were brothers, whereas the other 29 patients were unrelated.

6.2.4.1.1/ Splice-related sequence alteration

A single base-pair modification was observed in the acceptor site of intron 6 of patient P148. Alteration 889-2A>G has previously been reported as a mutation affecting the splice acceptor site and was predicted to result in a truncated protein in 2 NF1 patients (Klose *et al.*, 1999; Mattocks *et al.*, 2004).

Patient P151 showed a nucleotide substitution at the obligate GT nucleotide of the intron 35 splice donor site; 6641 +1G>A has previously been reported to result in splicing defect and a frameshift in the cDNA (De Luca *et al.*, 2004). A G>T transversion had also been found at the same nucleotide and predicted to result in a truncated protein (Park and Pivnick, 1998). Additionally, Mattocks *et al.* (2004) identified a deletion at the same nucleotide in the splice donor site (IVS35, 6641+1delG), which was predicted to create a truncated protein.

Similarly, patient P160 harboured a splice-related transition at position (+1) of intron 3. The mutation has not previously been reported. As the mutation is located at a conserved G of the splicing donor site, it is likely to impair normal splicing, as confirmed by the "Splice Site Prediction" program. Comparable G>A transitions at (+1) of the donor splice site have been reported in nearly all *NF1* introns and are predicted to result in the skipping of the exon (IVS2, IVS7, Ars *et al.*, 2003; IVS4b, IVS26, Fahsold *et al.*, 2000; IVS8, Hoffmeyer *et al.*, 1995; IVS10a, IVS32, De Luca *et al.*, 2004; IVS10c, Wiest *et al.*, 2003; IVS18, Purandare *et al.*, 1995a; IVS19a, IVS28, Ars *et al.*, 2000a) and/or protein truncation (IVS6, Klose *et al.*, 1999; IVS9, Eisenbarth *et al.*, 2000; IVS10b, IVS25, Ars *et al.*, 2000a; IVS16, Maynard *et al.*, 1997; IVS29, IVS31, IVS36, IVS45, Fahsold *et al.*, 2000).

Although no blood lymphocyte DNA was available for patient P157, unusual chromatograms in both tumours from the patient indicated a germline alteration (figure 6.4). A transversion, 7907+3 A>T, was identified in intron 45 of patient P157. The generally conserved motif of

the splice donor site has a purine at position (+3). Therefore, the appearance of a pyrimidine at this position may impair the splicing mechanism. Alterations at the (+3) position have been commented upon in section 6.2.3.1. Furthermore, another single base-pair change has been reported to occur in position (+5) of the same splice donor site (7907+5 G>A; De Luca *et al.*, 2004).

6.2.4.1.2/ Nonsense alterations

Nonsense germline mutations were observed in 7 patients. The mutations are located in exons 5, 11, 16, 22, 37 and 42. Four mutations are reported here for the first time.

Mutation W571X in exon 11 was observed in two brothers, patients P169 and P178. The transition at nucleotide 1714 has not previously been reported. Both nucleotide 1714 (G) and amino acid 571 (W) are conserved in human, rat and *Drosophila*, which may indicate a functional importance for this amino acid located in the cysteine/serine-rich domain. Additionally, Rescue-ESE identified the loss of an hexamer following the mutation, so that the alteration should be tested for a functional effect on normal splicing.

Patient P133 possesses the recurrent mutation R816X previously described in 9 NF1 patients, and an additional NF1 patient affected with glioma from this study, and was fully described in section 6.2.3.2.

Patient P143 harboured a germline alteration in exon 37 that resulted in the appearance of a stop codon at residue 2264. The C>G transversion has previously been reported in 4 NF1 patients and is located in the exon 37 mutational hotspot (Messiaen *et al.*, 1997; Hoffmeyer *et al.*, 1998; Fahsold *et al.*, 2000; Mattocks *et al.*, 2004; section 6.2.2.4).
Messiaen *et al.* (1997) identified the mutation by sequencing, after a shorter fragment was seen by RT-PCR assay: the predominant effect of the nonsense mutation was to induce the skipping of exon 37. This was the first report of such an effect for a nonsense mutation in the *NF1* gene. More recently, the role of mutation C6792G in splicing was investigated using ESE prediction software's and a minigene assay. The mutation was found to decrease the score of an SRp40 element, and the reduction in splicing enhancement capacity was confirmed by the minigene assay (Zatkova *et al.*, 2004). Similar results were observed for

another C>A transversion at the same nucleotide (6794), which also results in the appearance of a stop codon.

Patient P147 harboured a C>T transition at nucleotide 7519 in exon 42, resulting in the appearance of a stop codon (Q2507X). This alteration has not previously been reported.

A nonsense mutation (G663A, W221X) was identified in exon 5 of patient P168, who also harbours a common polymorphism in the same exon. The transition has not previously been reported. The search for ESE motifs yielded conflicting results; however, two hexamers were lost following mutation, which may indicate a disruption of normal splicing by the mutation.

Finally, a nonsense mutation (C3826T, R1276X) was found in exon 22 of patient P167. The mutation has previously been reported in 8 NF1 patients (Heim *et al.*, 1995; Side *et al.*, 1997; Upadhyaya *et al.*, 1997b; Osborn and Upadhyaya, 1999; Fahsold *et al.*, 2000; Messiaen *et al.*, 2000) and is predicted to result in a truncated protein. The C is located in a CG dinucleotide, which may account for the hypermutability. Additionally, a C>G transversion has been reported at the same nucleotide and gives rise to a missence mutation (Mattocks *et al.*, 2004). Nucleotide 3826 is conserved in rat, *Fugu* and *Drosophila* homologues, as is amino acid 1276 and is located within the GRD.

6.2.4.1.3/ Small insertions

Small insertions of 1 or 2 base-pairs were identified in 2 patients and neither has previously been reported. The two alterations are likely to produce a truncated transcript as they induce the appearance of a stop codon.

Patient P145 harboured a two-nucleotide insertion in exon 28 (5001insTG), which ultimately resulted in the appearance of a stop codon at residue 1677 (T1677X). The insertion gives rise to perfect inverted repeats (GtgT/ACAC), so that the imperfect complementarity of the original sequence may have mediated the insertion by forming a secondary structure (Cooper and Krawczak, 1993).

Sequence: 5'- TCCTGGGTCAGGGAGtgTACACCAAGTATCAT – 3'

Alteration 6291insA in exon 33 of patient P155 generated a stop codon in the new reading frame, N2107X. The alteration also produced a perfect inverted repeat (CTT/aAG), which may have mediated the mutation (Cooper and Krawczak, 1993).
Sequence: 5'- GGTCCGCTCTCCCTTaAGAGCTTCCACACAT – 3'

6.2.4.1.4/ Small deletions

The most frequently identified micro-deletions encountered in this panel were small deletions of 1 to 6 base pairs and were found in 8 patients.
An unusual 3-peaks profile on the chromatogram revealed an in-frame deletion in exon 28 of patient P135 (Figure 6.4). Alteration 4967delTCTATA results in the deletion of 2 amino acids, tyrosine and isoleucine (codons 1657 and 1658) while still retaining the valine at the newly formed codon 1656. The resulting protein is predicted to lack two amino acids that may have functional or structural importance in the protein, although located just outside the GRD. This alteration has previously been reported (Wu *et al.*, 1999). Additionally, a similar in-frame 6 base-pair deletion (4973del6) has also been identified in exon 28 (Mattocks *et al.*, 2004). In both cases, the deletion of 6 bases (TCTATA) was located in a repeated motif (TCTATA TCTATA) and can be explained by slipped mispairing (Cooper and Krawczak, 1993).
Sequence:
5'-CAGtctataTCTATAACTGTAACTCCTGGGTCAGGGAGTACA-3'

Patient P130 harboured a 1 base-pair deletion in exon 33. Deletion 6117delG is expected to create a frameshift expected to generate a stop codon in the downstream sequence, thus truncating the protein. This alteration in this patient has also been confirmed through cDNA-DHPLC of lymphocyte RNA in an separate study (Upadhyaya *et al.*, 2004)

A similar deletion (2233delA) was identified in exon 13 of patient P141 and is expected to truncate the protein at codon 747. The alteration has not previously been reported.

Patient P185 harboured a deletion (773delA) in exon 6 that is predicted to give rise to a truncated protein. This mutation occurred in a run of 3 As and has not previously been reported.

Deletion 434delTC occurred in 3 TC repeats in exon 4a of patient P139 and is predicted to truncate the protein at codon 154. The alteration has not previously been reported. However, a missense mutation has been found at nucleotide 434 (T434C, L145P; Mattocks *et al.*, 2004).

Mutation 3731delT, found in patient P140, has previously been reported by cDNA-DHPLC in this and in another NF1 patient (Upadhyaya *et al.*, 1997; Upadhyaya *et al.*, 2004). The alteration is predicted to result in a truncated protein. Other deletions have been reported in the nearby sequence [3737delT (Upadhyaya *et al.*, 1997), 3737delTGTT (Fahsold *et al.*, 2000) and 3739delTTTG (Ars et al., 2003)] suggesting that the sequence may prone to mutations.

Finally, mutation 5272delC was identified in exon 29 of patient P152. The deletion has previously been reported and predicted to result in a truncated protein (John *et al.*, 2000).

6.2.4.1.5/ Complex insertion-deletions

A rare type of mutation, an indel, 6512delATGAGAGAinsC, was observed in exon 34 of patient P149. This mutation has not previously been reported and is expected to result in a truncation of the neurofibromin at codon 2166 (F2166X). The alteration may have arisen from the initial insertion of the C followed by slipped mispairing within a direct repeat (5'-CCTGGCTCCTATGAGAGAcGAGACTTTTG-3') followed by a failed repair attempt that subsequently deleted 7 bases (5'-CCTGGCTCCTcGAGACTTTTGCTTTG-3'). To support this model, complexity analysis indicates an involvement for direct repeats, giving rise first to an insertion that increases complexity, then to a deletion that decreases complexity. The alterations might perhaps be better described as 6512insCdelATGAGAGA.

6.2.4.1.6/ Silent and intronic alterations

Patient P152 presented both a silent (synonymous) mutation in exon 28 (G4866A, V1622V) and homozygosity for the rare C allele of the intron 28 polymorphism (5205+23C). A 1bp deletion in exon 29 was identified as a disease-causing mutation in this patient. The 4866G nucleotide is conserved in rat and *Fugu*, but not *Drosophila* homologues. Additionally, ESEfinder identified a SC35 motif that is lost following alteration. Furthermore, a

polymorphism at the same nucleotide, G4866C (V1622), has been reported with 3% heterozygosity (De Luca *et al.*, 2004).

Interestingly, patient P136 appeared to have no disease-causing mutation that could be identified, only a silent (synonymous) mutation, T1810C (L604L), present in exon 12a, in the cysteine/serine-rich domain. This alteration has previously been reported as a polymorphism (Mattocks *et al.*, 2004), as it occurred in a patient in whom a disease causing mutation had already been identified. The T allele is not conserved through evolution, but the C allele is found in rat, *Fugu* and *Drosophila*; the amino acid leucine is however conserved in all species. A missense mutation has also been found at the same nucleotide (T1810G, L604V; Upadhyaya *et al.*, 1998).

Polymorphism G702A (L234L) in exon 5 was found to be heterozygous in 11/31 (35%) and homozygous (for the A allele) in 4/31 (13%) of samples and is the most recurrent polymorphism in this panel. The alteration was described in section 6.2.2.6.

Of the 12 different intronic alterations identified, three have been described in section 6.2.2.6. The reported polymorphism in intron 3, 288+41 G>A, was found in 3/31 samples (10%), and in IVS10c, 1641+39 C>T was seen in 4/31 samples (13%); the intronic transversion in intron 39 (7126+37 G>C) was identified in 6/ 31 patients (19%).

Additionally, three previously reported polymorphisms could be confirmed.. A transition in intron 17 (2851-16 T>C) was found in one sample (3%), in keeping with the reported heterozygosity of 4.5% (Mattocks *et al.*, 2004). The sample harbouring the IVS17 alteration also had a disease-causing deletion in exon 13. Three samples (10%) harboured a polymorphism in intron 20 (3496+33 C>A), whose heterozygosity was determined to be 33% (De Luca *et al.*, 2004). Only one of the three samples exhibited a disease-causing mutation. In intron 28, the transition 5205+23T>C was seen in 11 patients (35%) in heterozygous form. Moreover, the C allele was homozygous in 2 patients (6.5%, P152 and P155). The frequency of this polymorphism has been estimated to be over 30% for both alleles (Fahsold *et al.*, 2000; Han *et al.*, 2001).

Finally, four intronic alterations are reported here for the first time, three of which were only identified in one sample each: 1845+46 G>T (IVS12a); 5943+88 A>G (IVS31) and 8097+55

T>C (IVS47). Each of these alterations was found in a sample also harbouring a disease-causing mutation, indicating they are likely to be rare variants.

A transversion 1845+46 G>T in IVS12a was found in sample 2177, which also has a 1-base insertion in exon 33 as the disease-causing mutation. Transition 5943+88 A>G in IVS31 was found in sample 2318, where a one-base deletion has also been identified in exon 6. Neither nucleotide was found conserved in rat and *Drosophila*. An alteration in intron 47, 8097+55 T>C, was found in sample 2126, which also harbours an in-frame deletion of 2 amino acids, the protein (x28, 4967del6). The nucleotide at this position is conserved in both rat and *Drosophila*.

A transversion in intron 43 (7675+78 A>C) was seen in 2 patient samples, both of which harbour a disease-causing mutation in other exons. The A nucleotide is conserved in both rat and *Drosophila* homologues.

6.2.4.1.7/ Conclusion

Putative disease-causing germline mutations were found in 21/28 patients (75%), twelve of which were novel (57%). Nucleotide substitutions account for 50% of the disease-causing mutations; of the six nonsense alterations described here, there were 3 Y>Y transitions (2 at a CpG dinucleotide), 2 R>R transitions and 1 Y>R transversion. Of the novel missense mutations, W221X and W571X, the affected residues were conserved in human, rat, *Fugu* and *Drosophila*, whereas Q2507X occurred at an amino acid residue that is conserved in human, rat and *Fugu*. Of the 21 pathogenic mutations, 12 (57%) are expected to result in a premature truncation of the protein.

The alterations identified are summarized in Tables 6.4 and 6.5. The mutations were distributed across the *NF1* gene, with no apparent mutational hotspot (Figure 6.5). Similarly, the mutations ranged from small insertion or deletions to nonsense mutations, the last two being the most represented.

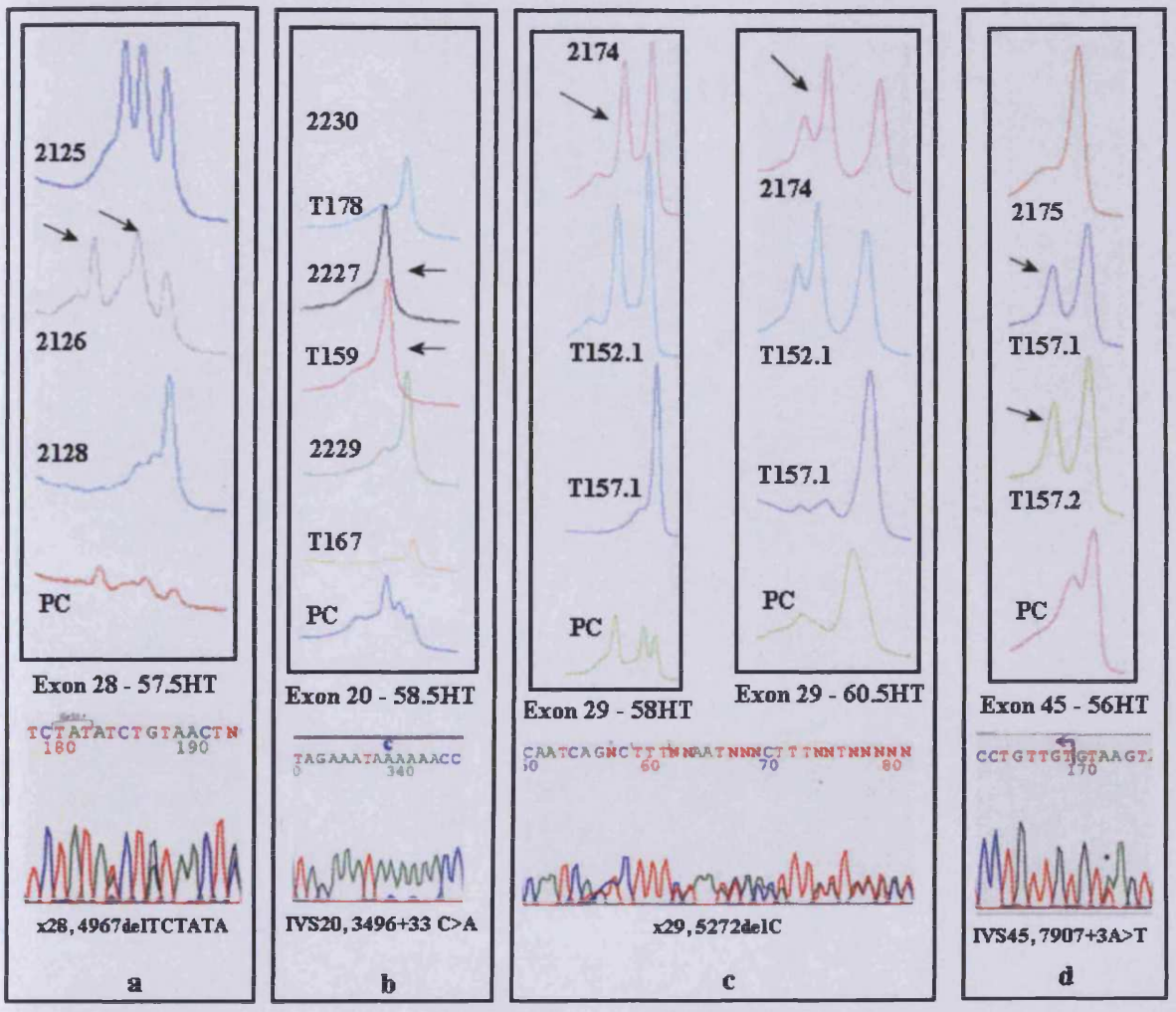

Figure 6.4: Chromatograms of germline alterations in NF1 patients

a. Chromatogram of a 6 base-pair deletion in sample 2126. The mutation is seen as 3 peaks, similar to those in the positive control (with mutation 4967ATCTAT). Sample 2125 is heterozygous for the common intron 28 polymorphism (5205+23T>C) whereas sample 2128 does not harbour any alteration.

b. Sample 2227 and corresponding tumour T159 show an early peak compared to the other sample, due to their homozygosity for an intron 20 polymorphism (3496+33 C>A). The other samples harbour no alteration in this exon. The positive control is a four base-pair exonic deletion.

c. The germline mutation (5272delC) is clearly identified in blood (2174) and corresponding tumour (T152.1) at both denaturing temperatures. By contrast, the positive control (C5224T) is more marked at the lowest temperature.

d. No blood DNA sample was available for patient P157. However, the germline mutation (7907+3 A>T) is clearly identified by DHPLC in both tumours. The positive control is a nucleotide substitution in exon 45 (A7828G).

Table 6.4: Pathogenic alterations and rare polymorphisms identified in the germline of a panel of NF1 patients.

The pathogenic mutation is indicated in bold. *as*, acceptor site; *ds*, donor site. In patients where a blood DNA sample was not available, the use of multiple tumours is indicated by (Txx).

Patients	DNA	Exon	Genomic mutation	Amino acid mutation	Type / effect	Reference
P130	2124	**x33**	**6117delG**	**L2048X**	frameshift / truncating	Upadhyaya *et al.*, 2004
P133	2125/ 2142	**x16**	**C2446T**	**R816X**	nonsense / truncating	Maynard *et al.*, 1997
P135	2126	**x28**	**4967delTCTATA**	**DVYI1656-8>V1656**	in frame deletion / -2 aa	Wu *et al.*, 1999
		IVS47	8097+55 T>C		intronic / ?	This report
P136	2127	x12a	T1810C	L604L	silent / ?	Mattocks *et al.*, 2004
P139	2128	**x4a**	**434delTC**	**F154X**	frameshift / truncating?	This report
P140	(T140)	**x22**	**3731delT**	**V1265X**	frameshift / truncating	Upadhyaya *et al.*, 2004
P141	2141	**x13**	**2233delA**	**M747X**	frameshift / truncating?	This report
P143	2279	**x37**	**C6792G**	**Y2264X**	nonsense / splicing	Messiaen *et al.*, 1997
P145	2155	**x28**	**5001insTG**	**T1677X**	frameshift / truncating?	This report
P147	2156	**x42**	**C7519T**	**Q2507X**	nonsense / truncating?	This report
P148	2158	**IVS7**	**889-2A>G**		as / truncating	Klose *et al.*, 1999
P149	2157	**x34**	**6512insCdelATGAGAGA**	**F2166X**	frameshift / truncating?	This report
P150	(T150)		**90kb 5' end deletion**			Upadhyaya *et al.*, 1990
P151	2159	**IVS35**	**6641+1G>A**		ds / ?	This report
P152	2174	**x29**	**5272delC**	**L1758X**	frameshift / truncating	Osborn *et al.*, 1999
		x28	G4866A	V1622V	silent	This report
P155	2177	**x33**	**6291insA**	**N2107X**	frameshift / truncating?	This report
		IVS43	7675+78 A>C		intronic / ?	This report
		IVS12a	1845+46 G>T		intronic / ?	This report
P157	(T157)	**IVS45**	**7907+3A>T**		ds / ?	This report
P160	2205	**IVS3**	**288+1G>A**		ds / ?	This report
P167	2229	**x22**	**C3826T**	**R1276X**	nonsense / truncating?	Heim *et al.*, 1995
P168	B168	**x5**	**G663A**	**W221X**	nonsense / truncating?	This report

Table 6.4: continued

Patients	DNA	Exon	Genomic mutation	Amino acid mutation	Type / effect	Reference
P169	2207	**x11**	**G1713A**	**W571X**	nonsense / truncating?	This report
P178	2230	**x11**	**G1713A**	**W571X**	nonsense / truncating?	This report
P185	2318	**x6**	**773delA**	**I280X**	frameshift / truncating?	This report
		IVS31	5943+88 A>G		intronic / ?	This report
		IVS43	7675+78 A>C		intronic / ?	This report

Table 6.5: Germline polymorphisms in a panel of NF1 patients.

Samples with a * denote homozygosity for the rare allele. *Het*, reported heterozygosity.

Patients DNA	Exon/Intron	Genomic mutation	Het	Reference
2141, 2175, 2157	IVS3	288+41 G>A	21%	De Luca *et al.*, 2003
2124, 2125, 2141, 2157, 2175, (T140) B168, 2156, 2174,2176, 2205 2126*, 2155*, 2170*, 2227*	x5	A702G	53%	Mattocks *et al.*, 2004
2127, 2156, 2157, 2205	IVS10c	1641+39 C>T		Fahsold *et al.*, 2000
2141	IVS17	2851-16 T>C	4.5%	Mattocks *et al.*, 2004
2227, 2318, 2316	IVS20	3496+33C>A	33%	De Luca *et al.*, 2003
2124,2125, 2127, (T140), 2279, (T144), (T143) 2157, 2158, 2175, 2176, 2174*, 2177*	IVS28	5205+23T>C	7%	Fahsold *et al.*, 2000 Han *et al.*, 2001
2141, 2279, (T144), 2155, 2156,2158	IVS39	7126+37 G>C	5-24%	Rodenhiser and Hovland, 1995 Han *et al.*, 2001 De Luca *et al.*, 2004 Mattocks *et al.*, 2004

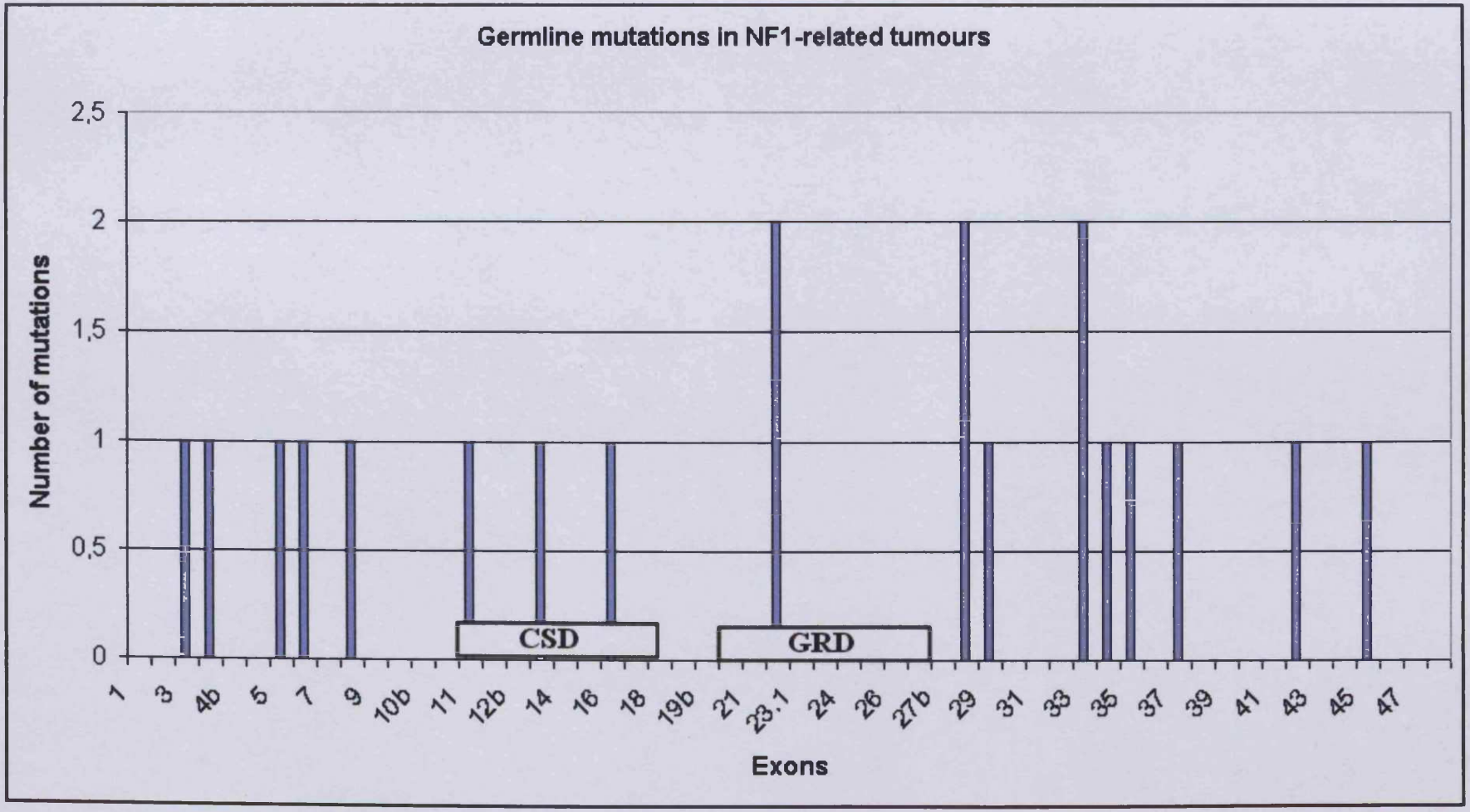

Figure 6.5 : Distribution of the germline mutation in NF1 patients.

GRD, GAP-related domain; *CSD*, cysteine/serine-rich domain.

6.2.4.2/ Somatic lesions

6.2.4.2.1/ LOH in NF1 tumours

The tumours in this panel were donated by 29 of the 31 patients from section 6.2.4.1; tumours from patients P176 and P186 were excluded due to the presence of a large germline deletion, which would result in the homozygosity of all markers used. The LOH panel was composed of 56 dermal neurofibromas, 3 plexiform neurofibromas and 12 malignant tumours [10 MPNSTs, 1 rhabdomyosarcoma, 1 medullary thyroid carcinoma (MTC)]. LOH was identified in 10 tumours (14%) whilst one benign tumour harboured instability with two markers. A summary of the LOH assay is shown in Table 6.6, and representations of the different LOH are shown in Figures 6.6 and 6.7.

Tumour T140.3 exhibited LOH on the basis of dosage estimation using microsatellite markers I4b to EVI20. Since markers on either side of the deleted markers (HHH202 at 5' and I38 at 3') were heterozygous, the deletion may extend upstream of the start of the *NF1* gene and end before intron 38. The fact that marker J1/J2, in the middle of the deletion, appeared to be heterozygous is not unusual, considering the heterogeneous cellular nature of neurofibromas.

A deletion potentially encompassing the *NF1* gene and surrounding sequences was observed in MPNST T145: microsatellite markers Ib4, EVI20 and J1J2 demonstrated a clear LOH on the polyacrylamide gel. All markers except EW207 were homozygous in blood lymphocyte DNAs, rendering them uninformative with respect to the upstream boundary of the deletion. EW207, but not EW206, was heterozygous in both blood and tumour DNAs, suggesting that the downstream boundary of the deletion is located 3' to the *NF1* gene. The use of an additional 17q telomeric maker, D17S802, confirmed that the deletion did not extend to the whole long arm, as the marker was heterozygous. Recently, a parallel study using a newly designed NF1 array-CGH defined the deletion to be of 1.61-1.75Mb (between 25690kb and 27490kb of 17q; Mantripragada *et al.*, 2005). Thus the deletion extended outside the *NF1*-REPS elements, and would not be identified by the deletion junction-specific PCR assay (Dorschner *et al.*, 2000; Lopez-Correa *et al.*, 2001; Serra *et al.*, 2001b).

Another MPNST, tumour T151, manifested a deletion potentially encompassing the first half of the *NF1* gene, up to intron 38. Clear LOH was evident in HHH202, EVI20 and J1J2, whilst

the other markers, up to I38, displayed an apparent homozygous profile in both blood and tumour DNA. As marker HHH202 is located at 17q11, approximately 0.6cM upstream of the *NF1* gene, the deletion may start close to the centromere and end before *NF1* intron 38, and thus be another example of uncommon large deletion.
From the same patient, tumour T165 (MPNST) exhibited LOH in 5 markers (HHH202, I4b, J1J2, EVI20 and I38), suggesting a deletion which spanned upstream of the *NF1* gene to intron 41. Moreover, markers I41, C7CT, EW206 and EW207 showed apparent homozygosity in blood and tumour DNA, suggesting the boundary of the deletion could extend past the 3' UTR region of the *NF1* gene. Marker D17S802 at the telomeric end showed an heterozygous profile for both T151 and T165. Tumour T165 was known to be an MPNST metastasis, but it was not known whether T151 was the original tumour. According to the present results, it would appear as though MPNSTs T151 and T165 harbour two different types of LOH, and thus would arise from two independent somatic events. Both tumours however appear to show extensive LOH, in keeping with the observation that tumours from the same patient may have a tendency to harbour similar somatic mutations (Serra *et al.*, 2001b). Additionally, tumour T165 was also assayed with array-CGH, and the LOH proved to extend outside the span of the array (25590bp-27890bp on 17q) , and thus be over 2.2Mb (Mantripragada *et al.*, 2005).
As marker HHH202 is estimated to be at locus D17S33 (23149bp on 17q), the deletion could actually be over 4.7Mb, but would not however extend to the entire long arm, as maker D17S802 at 17q25.3 showed heterozygosity. It is also of note that in study by Serra and co-workers, LOH in NF1 tumours has been found to extend to HHH202, but never to centromeric markers (Serra *et al.*, 2001b). Therefore it is possible that the deletion may extend further 5'to HHH202, but not to the centromere.

MPNST T169 exhibited a dosage difference for microsatellite markers J1J2 and I12b. Additionally, markers from exon 5 to EW206 were uninformative (homozygous) for this tumour, suggesting a deletion larger than that identified by these two markers. Telomeric marker D17S802 appeared heterozygous for the tumour, whereas an additional marker D17S1824 (located at 17q11.2, approximately 370kbp downstream of HHH202) showed LOH, suggesting that, despite the apparent heterozygosity for intragenic marker I4b, the deletion may extend upstream of the *NF1* gene, but does not encompass the entire long arm.

In five tumours (four benign neurofibromas and one malignant tumour), LOH was identified with only one marker. Whilst this could be attributed to the cellular heterogeneity of

neurofibromas (Peltonen *et al.*, 1988) and/or the sensitivity of the technique used, microsatellite instability cannot be excluded.
Benign neurofibroma T147 exhibited LOH at microsatellite marker I12b, however, the two flanking markers (E5 at 5' and J1/J2 at 3') are heterozygous, so the deletion is likely to be confined between exon 5 and intron 27b.

Both neurofibromas T149 and T152.1 harboured LOH in marker J1/J2 exclusively, with heterozygous markers on either side for T149 (I12b in 5' and I27.13 in 3') and T152.1 (E5 in 5' and I27.13 in 3'). Marker J1/J2 is the first of three markers in the very large intron 27b and located approximately 22370 base pairs upstream of the next marker, I27.13. The utmost boundaries of these deletions can therefore be between intron 12b and intron 27b for T149 and between exon 5 and intron 27b for T152.1.

Tumour T159 was a large palmar neurofibroma and interestingly, the only NF1 feature in this patient. The tumour DNA exhibited LOH at marker I38 only; however, markers from exon 5 to EW207 were uninformative for this tumour, and may therefore not be able to reveal a much larger deletion. However, markers D17S1824 and D17S802 were both heterozygous in the tumour.

The only MTC in the study, T185, showed LOH at marker I38, with a deletion seemingly confined between intron 27b (J1/J2 heterozygous) and intron 41. Additionally, many tumours had stretches of uninformative markers that cannot be ruled out as possible regions of LOH.

Finally, tumour T142.2 represents a unique and interesting case in this panel. The neurofibroma did not exhibit LOH with any of the *NF1* intragenic makers. However, the benign tumour demonstrated microsatellite instability at two markers, EVI20 and J1J2. Marker J1J2 presents the 2 bands found in blood lymphocyte DNA and an additional band (Figure 6.7), whereas marker EVI20 showed a compression (Figure 6.6). Although instability is to be expected of microsatellite markers, this was the only case observed in the NF1 intragenic markers in this panel.

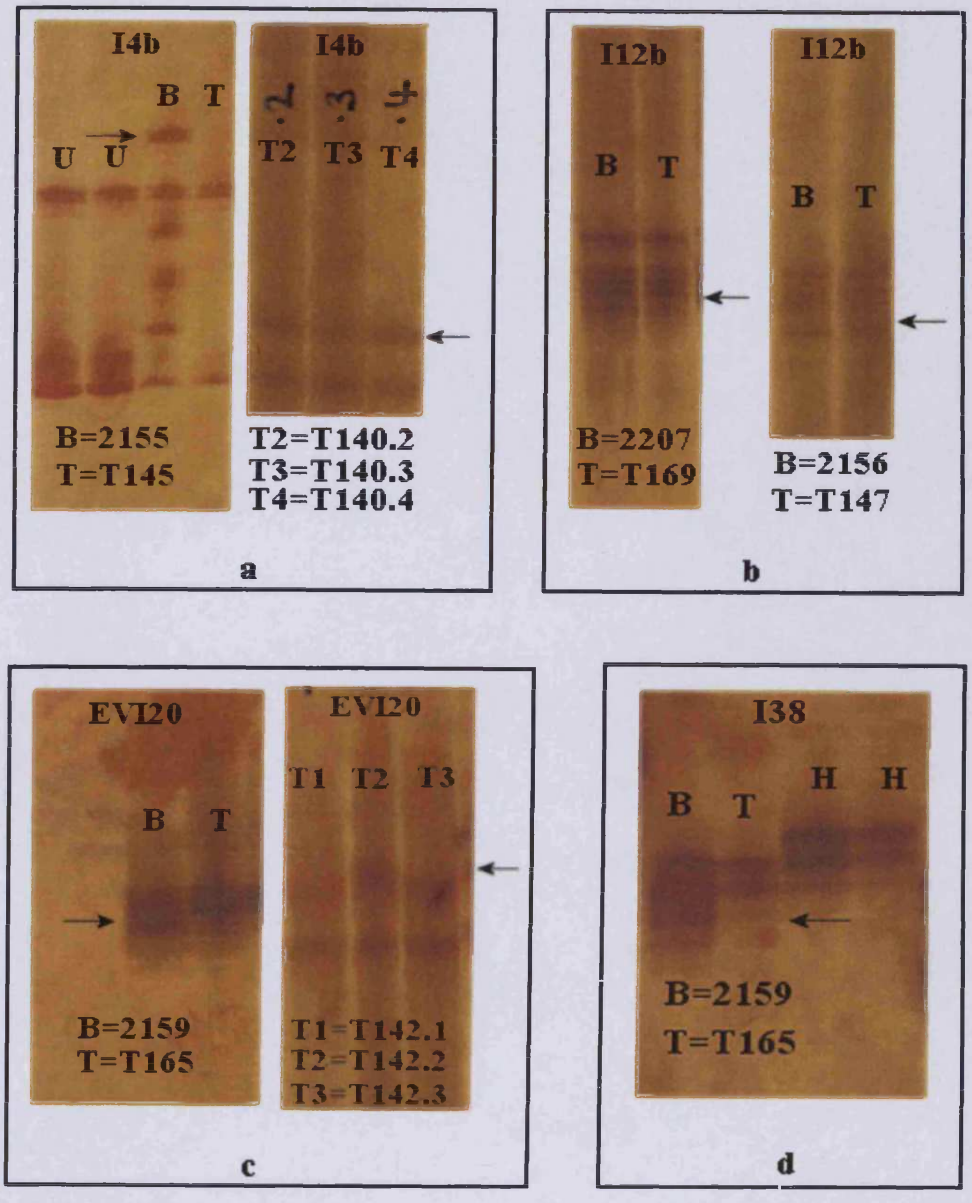

Figure 6.6: Representation of LOH using *NF1* intragenic markers.

a. Marker I4b (inside intron 4b): the arrow points to the loss of the upper allele in MPNST T145 as compared to the corresponding blood sample, and to a dosage difference in the neurofibroma T140.3 as compared to other tumours from the same patient. *U*, uninformative, unrelated sample.

b. Marker I12b (inside intron 12b): the arrow points to a dosage difference in both tumours compared to the corresponding blood.

c. EVI20 (inside intron 27b): A dosage difference is visible in MPNST T165 as compared to the corresponding blood sample. In neurofibroma T142.2, the arrow points to an expansion of the upper allele, indicative of microsatellite instability rather than LOH.

d. Marker I38 (inside intron 38): The arrow points to the allelic loss in MPNST T165 as compared to the corresponding blood. *H*, heterozygous, unrelated samples.

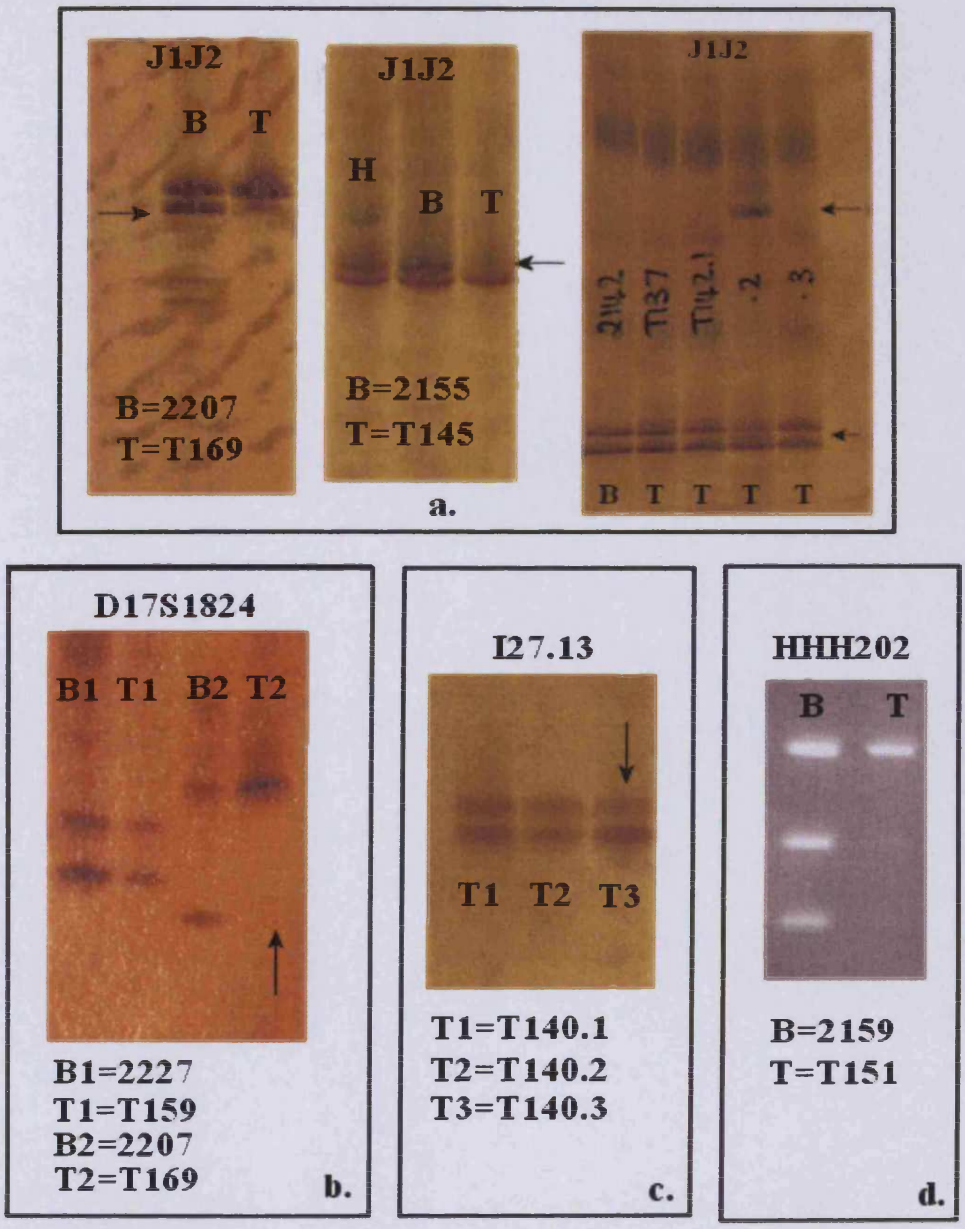

Figure 6.7: Representation of LOH using *NF1* intragenic and extragenic markers.

a. Marker J1J2 (inside intron 27b): the arrow points to the loss of the upper allele in MPNST T145 and to a loss of the lower in tumour T169, as compared to the corresponding blood. In T142.2, instability was seen as a shift in the upper band.

b. Marker D17S1824 (17q11.2): the arrow points to a clear LOH in T169 compared to the corresponding blood. Tumour T159 acts as an heterozygous control.

c. I27.13 (inside intron 27b): A dosage difference is visible in T140.3 as compared to other tumours from the same patient.

d. Marker HHH202 (0.6cM centromeric): This is representative of the RFLP assays. The blood sample is heterozygous for the restriction site, whereas only one allele is seen in the tumour.

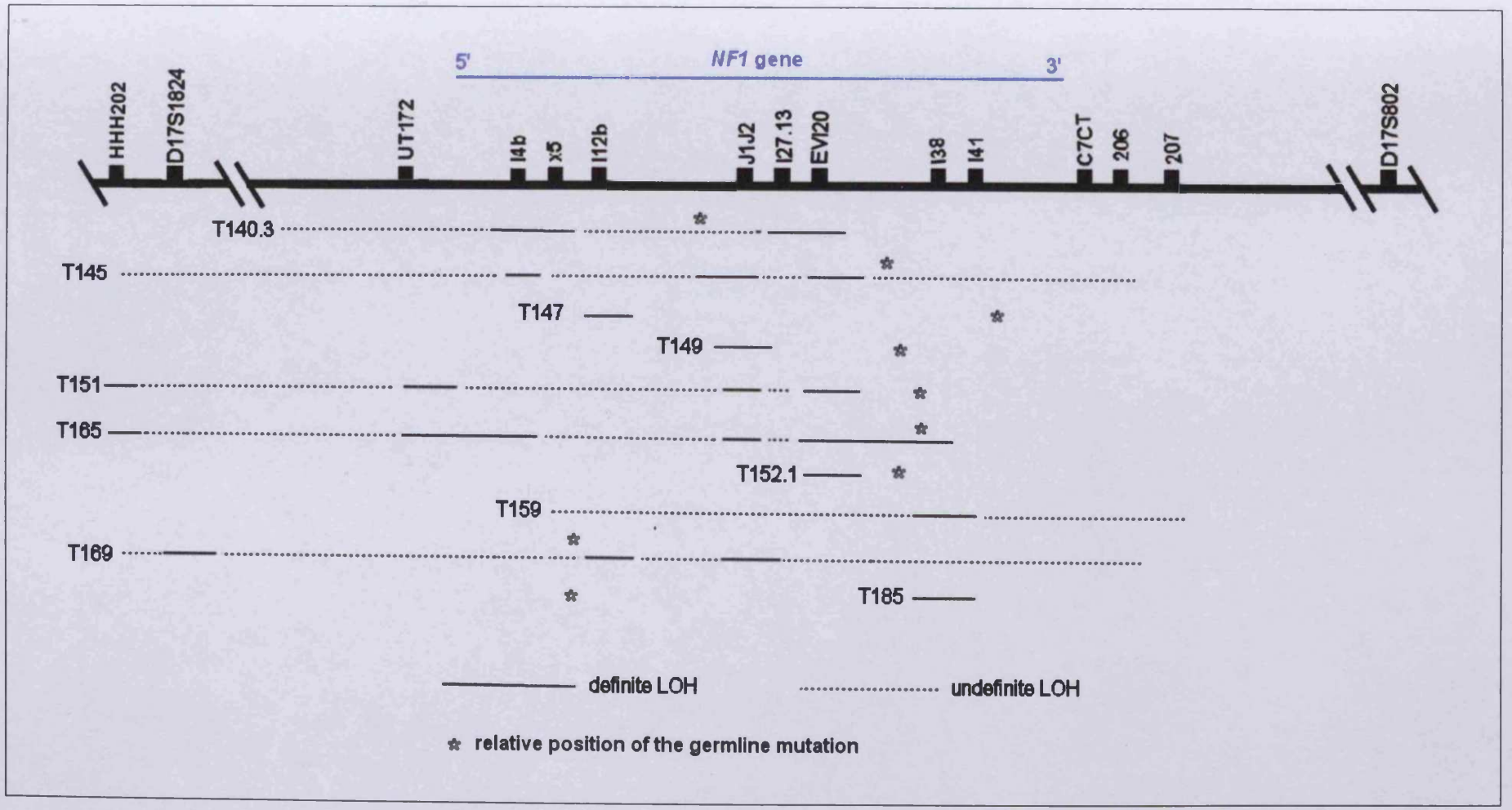

Figure 6.8: Distribution of LOH in NF1 tumours.

Due to the homozygosity of some markers in both blood and tumour, the exact extent of the LOH could not be defined. The dotted lines correspond to these regions of uncertainty. The orientation is from centromere to telomere.

Table 6.6: Results from the LOH study on 71 NF1-related tumours from 29 patients.
Informative markers are represented with a ■, uninformative markers (homozygosity) with a □; definite LOH is represented with a ● with an orange background; when the boundaries of LOH could not be defined due to marker homozygosity, a fainter orange background was extended to encompass the possible LOH. In sample T142.2, markers showing instability are depicted with a ◊ on green background. nd: repeated PCR for these samples was unsuccessful.

		Markers												
Patients	Blood / Tumours	HHH202	UT172	I4b	E5	I12b	J1J2	I27.13	EVI20	I38	I41	C7CT	EW206	EW207
P133	2125	■	■	■	■	■	■	■	■	■	■	■	■	□
	T133.1	■	■	■	■	■	■	■	■	■	■	■	■	□
	T133.2	■	■	■	■	■	■	■	■	■	■	■	■	□
	T133.3	■	■	■	■	■	■	■	■	■	■	■	■	□
	T133.4	■	■	■	■	■	■	■	■	■	■	■	■	□
	T133.5	■	■	■	■	■	■	■	■	■	■	■	■	□
	T133.6	■	■	■	■	■	■	■	■	■	■	■	■	□
	T137	■	■	■	■	■	■	■	■	■	■	■	■	□
	T142.1	■	■	■	■	■	■	■	■	■	■	■	■	□
	T142.2	■	■	■	■	■	◊	■	◊	■	■	■	■	□
	T142.3	■	■	■	■	■	■	■	■	■	■	■	■	□
P135	2126	■	□	■	□	■	■	■	■	■	□	■	□	■
	T135.1	■	□	■	□	■	■	■	■	■	□	■	□	■
	T135.2	■	□	■	□	■	■	■	■	■	□	■	□	□
P136	2127	■	■	■	□	■	■	□	■	■	■	■	□	□
	T136.1	■	■	■	□	■	■	□	■	■	■	■	□	□
	T136.2	■	■	■	□	■	■	□	■	■	■	■	□	□
P139	2128	□	nd	■	□	■	■	□	■	□	■	□	□	■
	T139	□	nd	■	□	■	■	□	■	□	■	□	□	■
P140	T140.1	■	□	■	■	□	■	■	■	■	■	■	□	■
	T140.2	■	□	■	■	□	■	■	■	■	■	■	□	■
	T140.3	■	□	●	●	□	■	●	●	■	■	■	□	■
	T140.4	■	□	■	■	□	■	■	■	■	■	■	□	■
	T140.5	■	□	■	■	□	■	■	■	■	■	■	□	■
P141	2141	□	□	■	■	■	■	□	□	■	□	□	■	□
	T141.1	□	□	■	■	■	■	□	□	■	□	□	■	□
	T141.2	□	□	■	■	■	■	□	□	■	□	□	■	□
	T141.3	□	□	■	■	■	■	□	□	■	□	□	■	□
	T141.4	□	□	■	■	■	■	□	□	■	□	□	■	□
	T141.5	□	□	■	■	■	■	□	□	■	□	□	■	□
	T141.6	□	□	■	■	■	■	□	□	■	□	□	■	□
	T141.7	□	□	■	■	■	■	□	□	■	□	□	■	□
	T141.8	□	□	■	■	■	■	□	□	■	□	□	■	□
	T141.9	□	□	■	■	■	■	□	□	■	□	□	■	□
	T141.10	□	□	■	■	■	■	□	□	■	□	□	■	□
	T141.11	□	□	■	■	■	■	□	□	■	□	□	■	□
	T141.12	□	□	■	■	■	■	□	□	■	□	□	■	□

Table 6.6: continued

		Markers												
Patients	Blood Tumours	HHH202	UT172	I4b	E5	I12b	J1J2	I27.13	EVI20	I38	I41	C7CT	EW206	EW207
	T141.13	□	□	■	■	■	■	□	□	■	□	□	■	□
	T141.14	□	□	■	■	■	■	□	□	■	□	□	■	□
	T141.15	□	□	■	■	■	■	□	□	■	□	□	■	□
	T141.16	□	□	■	■	■	■	□	□	■	□	□	■	□
P143	T143.1	□	□	□	□	■	□	■	■	■	■	■	■	□
	T143.2	□	□	□	□	■	□	■	■	■	■	■	■	□
	T143.3	□	□	□	□	■	□	■	■	■	■	■	■	□
	T143.4	□	□	□	□	■	□	■	■	■	■	■	■	□
	T143.5	□	nd	□	□	■	□	■	■	■	■	■	■	□
	T143.6	□	□	□	□	■	□	■	■	■	■	■	■	□
	T143.7	□	□	□	□	■	□	■	■	■	■	■	■	□
P147	2156	□	□	■	■	■	■	□	□	■	□	□	□	□
	T147	□	□	■	■	●	■	□	□	■	□	□	□	□
P144	T144.2	□	□	□	□	■	□	■	■	■	■	■	□	□
	T144.3	□	□	□	□	■	□	■	■	■	■	■	□	□
P149	2157	■	□	■	■	■	■	■	■	■	■	■	□	□
	T149	■	□	■	■	■	●	■	■	■	■	■	□	□
P150	T150.1	□	nd	□	□	□	□	□	□	■	□	□	■	□
	T150.2	□	nd	□	□	□	□	□	□	■	□	□	■	□
	T150.4	□	nd	□	□	□	□	□	□	■	□	□	■	□
P152	2174	□	□	■	■	□	■	■	□	■	■	□	■	□
	T152.1	□	□	■	■	□	●	■	□	■	■	□	■	□
	T152.2	□	□	■	■	□	■	■	□	■	■	□	■	□
P153	2175	□	□	■	■	■	■	■	■	■	■	■	■	□
	T153	□	□	■	■	■	■	■	■	■	■	■	■	□
P155	2177	nd	□	□	□	□	□	nd	□	□	□	□	□	□
	T155	nd	□	□	nd	nd	□	nd	□	□	□	nd	□	□
P157	T157.1	■	□	□	□	■	■	□	□	□	■	□	□	□
	T157.2	■	□	□	□	■	■	□	□	□	■	□	□	□
P159	2227	■	nd	■	□	□	□	□	□	■	□	□	□	□
	T159	■	nd	■	□	□	□	□	□	●	□	□	□	□
P156	2170	□	□	□	□	□	□	■	□	□	□	□	□	□
	T156	□	□	□	□	□	□	■	□	□	□	□	□	□
P167	2229	■	nd	■	□	■	□	□	□	■	□	□	□	□
	T167	■	nd	■	□	■	□	□	□	■	□	□	□	□
P130	2124	□	■	■	■	■	□	■	■	■	■	■	■	□
	T130	□	■	■	■	■	□	■	■	■	■	■	■	□
P145	2155	□	□	■	□	□	■	□	■	□	□	□	□	■
	T145	□	□	●	□	□	●	□	●	□	□	□	□	■
P148	2158	□	■	■	□	■	■	■	■	■	■	□	□	□
	T148	□	■	■	□	■	■	■	■	■	■	□	□	□
P151	2159	■	□	□	□	□	■	□	■	■	□	□	□	□
	T151	●	□	□	□	□	●	□	●	■	□	□	□	□
	T165	●	nd	●	□	□	●	□	●	●	□	□	□	□
P154	2176	■	□	■	■	■	■	■	■	■	■	■	□	□
	T154	■	□	■	■	■	■	■	■	■	■	■	□	□

Table 6.6: continued

		Markers												
	Blood	HHH202	UT172	I4b	E5	I12b	J1J2	I27.13	EVI20	I38	I41	C7CT	EW206	EW207
Patients	Tumours													
P160	2205	□	nd	■	■	□	■	■	■	□	■	■	■	□
	T160	□	nd	■	■	□	■	■	■	□	■	■	■	□
P168	B168	■	nd	■	■	■	■	■	■	■	■	□	□	■
	T168	■	nd	■	■	■	■	■	■	■	■	□	□	■
P169	2207	□	nd	■	□	■	■	□	□	□	□	□	□	■
	T169	□	nd	■	□	●	●	□	□	□	□	□	□	■
P178	2230	□	nd	□	□	■	■	□	□	□	■	□	□	■
	T178	□	nd	□	□	■	■	□	□	□	■	□	□	■
P184	2316	■	nd	■	□	□	□	□	□	■	□	□	□	□
	T184	■	nd	■	□	□	□	□	□	■	□	□	□	□
P185	2318	■	nd	□	□	□	■	□	□	■	■	□	□	■
	T185	■	nd	□	□	□	■	□	□	●	■	□	□	■

6.2.4.2.2/ Microlesions in NF1 tumours

The tumours in this panel were donated by the 31 patients previously described in section 6.2.4.1 and comprised 60 dermal neurofibromas, 3 plexiform neurofibromas and 12 malignant tumours (10 MPNSTs, 1 rhabdomyosarcoma, 1 MTC). The study was mainly conducted using DHPLC analysis, although, some fragments were screened by direct sequencing. Additionally, three MPNSTs (T168, T184 and T185) were screened only by direct sequencing of the tumour cDNA; mutations were then confirmed in genomic DNA.

Unfortunately, no new mutation was identified by analysing the cDNA of the 8 tumours previously screened by DHPLC. Although the germline mutation was evident in all cases, no additional somatic mutation was identified, consistent with the DHPLC results. Nonsense-mediated mRNA decay (NMD) has been proposed as a mechanism to explain the under-representation of a mutated transcript (Hoffmeyer *et al.*, 1995; Messiaen *et al.*, 2000). In an earlier study, allelic exclusion has been demonstrated at *NF1* locus in approximately 10% of the samples (Osborn and Upadhyaya, 1999). While this event may indeed have hampered the search for a somatic mutation, the cellular heterogeneity of the tumour is likely to be the main limiting factor.

6.2.4.2.2.1/Splice-related alterations

Neurofibroma T176.3 presented a G to C transversion at the canonical first base of the splice donor site of intron 23. The 4110+1 G>C mutation has previously been reported as a germline mutation inducing the skipping of intron 23.2 and creating a truncated protein (Fahsold *et al.*, 2000). Additionally, a G>A transition has also been found at this nucleotide (Upadhyaya *et al.*, 1997).

Tumour T186 showed a splice-related mutation in intron 17 (2990+1 G>A). As the mutation occurs at a conserved nucleotide in the splice donor site, it might be expected to impair normal splicing, inducing skipping of the exon and/or protein truncation. Disruption of the normal splice site by the mutation was supported by data derived from the splice site prediction program. Similar IVS+1 G>A alterations at other introns in the *NF1* gene have been reviewed in section 6.2.4.1.1.

6.2.4.2.2.2/ Nonsense alterations

A nonsense mutation was found in 3 neurofibromas, in exons 27a, 28 and 31. Owing to the appearance of a stop codon, these mutations are expected to result in a truncated protein. However, a role in aberrant splicing cannot be ruled out.

Neurofibroma T139 harboured a C>G transversion at nucleotide 4637 of exon 27a. The S1546X mutation was not found in blood lymphocyte DNA from patient P139 (germline mutation in exon 4a) and has not previously been reported. The search for ESEs by ESEfinder revealed the loss of a motif for SC35 after mutation.

Nonsense mutation C7285T (R2429X) was identified in exon 41 of benign tumour T140.4. The mutation was also confirmed in a parallel study using cDNA-DHPLC analysis (Upadhyaya *et al.*, 2004) and occurred in a CpG dinucleotide. Both ESEfinder and RESCUE-ESE predicted the appearance of a new ESE motif, suggesting that the mutation would result in a truncation of the protein, rather than having an effect on splicing.

An unusual chromatogram for neurofibroma tumour T176.1 (but not T176.2 or T176.3) by DHPLC revealed an alteration in exon 28 (figure 6.9). Mutation C4812G (Y1604X) has not been reported.

Similarly, a different chromatogram profile for tumour T176.2, as compared to the other tumours from patient P176 (Figure 6.9), revealed that neurofibroma T176.2 harboured a nonsense mutation in exon 31. The G>A transition at nucleotide 5927 has not been reported.

6.2.4.2.2.3/ Small insertions

Neurofibroma T137 harboured a 2 base-pair insertion in exon 31. Mutation 5894insAC is expected to create a frameshift, ultimately truncating the protein with the appearance of a stop codon (D1991X) and is reported here for the first time. A number of mutations have been reported in codons 1963-1967: 5887delA (Ars *et al.*, 2000), A5893T (De Luca *et al.*, 2003), C5896T (Messiaen *et al.*, 2000) and 5898delGA (Mattocks *et al.*, 2004), suggesting that this sequence may be prone to mutations.

The previously unreported alteration 2271insG was found in exon 14 of neurofibroma T150.2 and is expected to truncate the protein shortly downstream of the insertion (E767X). Patient P150 has a 90kb 5' end deletion as a germline mutation (Upadhyaya *et al.*, 1990). The insertion may have occurred by slipped mispairing due to the presence of AG direct repeats in the surrounding sequence (Cooper and Krawczak, 1993). A number of mutations have been reported in the adjacent sequence: 2269delAA (De Luca *et al.*, 2004), 2271delAA (De Luca *et al.*, 2003) and 2272delAG (Fahsold *et al.*, 2000), which may indicate a propensity for mutations in the sequence. The insertion reported here also disrupts a symmetric element (AGAA/AAGA).

Sequence: 5'-GCAGCACTTCAGAAAgAGAGTGATGGCACTG-3'

6.2.4.2.2.4/ Small deletions

The most frequent micro-deletions observed in this study were small deletions of 1 and 11 base-pairs that were found in two neurofibromas and two MPNSTs.

In patient P141, neurofibroma T141.13 was found to harbour a deletion (5729delT) in exon 30. The resulting frameshift is expected to truncate the protein in exon 31 (L1920X) assuming splicing is not impaired. The deletion occurred in a run of 3 T's and has not previously been reported.

Although no lymphocyte blood DNA was available for comparison, neurofibroma T157.1 demonstrated a clear 2-peaks profile in exon 20 compared to the single peak of T157.2, another neurofibroma from the same patient (Figure 6.9). The previously unreported mutation 3491delC is expected to result in a truncated protein. The deletion may have been mediated by slipped mispairing using a nearby CTCAT as template (5'-GTCTCATGCACTcCATA-3'). The splicing-related germline mutation (IVS45+3 A>T) in patient P157 was seen in both tumours.

Direct sequencing of malignant tumour T168 cDNA revealed a deletion in a run of 3 As in exon 34. Mutation 6442delA is predicted to truncate the protein and is reported here for the first time. Patient P168 had a germline truncating mutation in exon 5 (W571X), which was first identified in the tumour.

Similarly, an 11 base-pair deletion in exon 27a of MPNST T184 was identified by direct sequencing of the tumour cDNA and confirmed in genomic DNA. The unreported mutation is expected to create a frameshift, ultimately leading to the appearance of a stop codon in the downstream sequence (W1538X). A germline mutation could not be identified in patient P184 either through DHPLC screening of the blood DNA 2316 or, indirectly, in the cDNA sequencing of tumour T184. The deletion found in the tumour cDNA and genomic DNA was not observed in the matched blood lymphocyte DNA.

6.2.4.2.2.5/ Complex insertion-deletions

An unusual chromatogram profile, compared to that of other benign tumours from the same patient, revealed a complex mutation (3124insATdelGTAG) in exon 19a of neurofibroma T143.2 (Figure 6.9). The mutation is predicted to result in a truncated protein and has not previously been reported. The germline mutation in patient P143 was a nonsense mutation in exon 37. The presence of a symmetric element (ATAAGATG/GTAGAATA) may have mediated the formation of the mutation (Cooper and Krawczak, 1993), as was indicated by the complexity analysis. It is unclear whether the micro-deletion or micro-insertion occurred first.

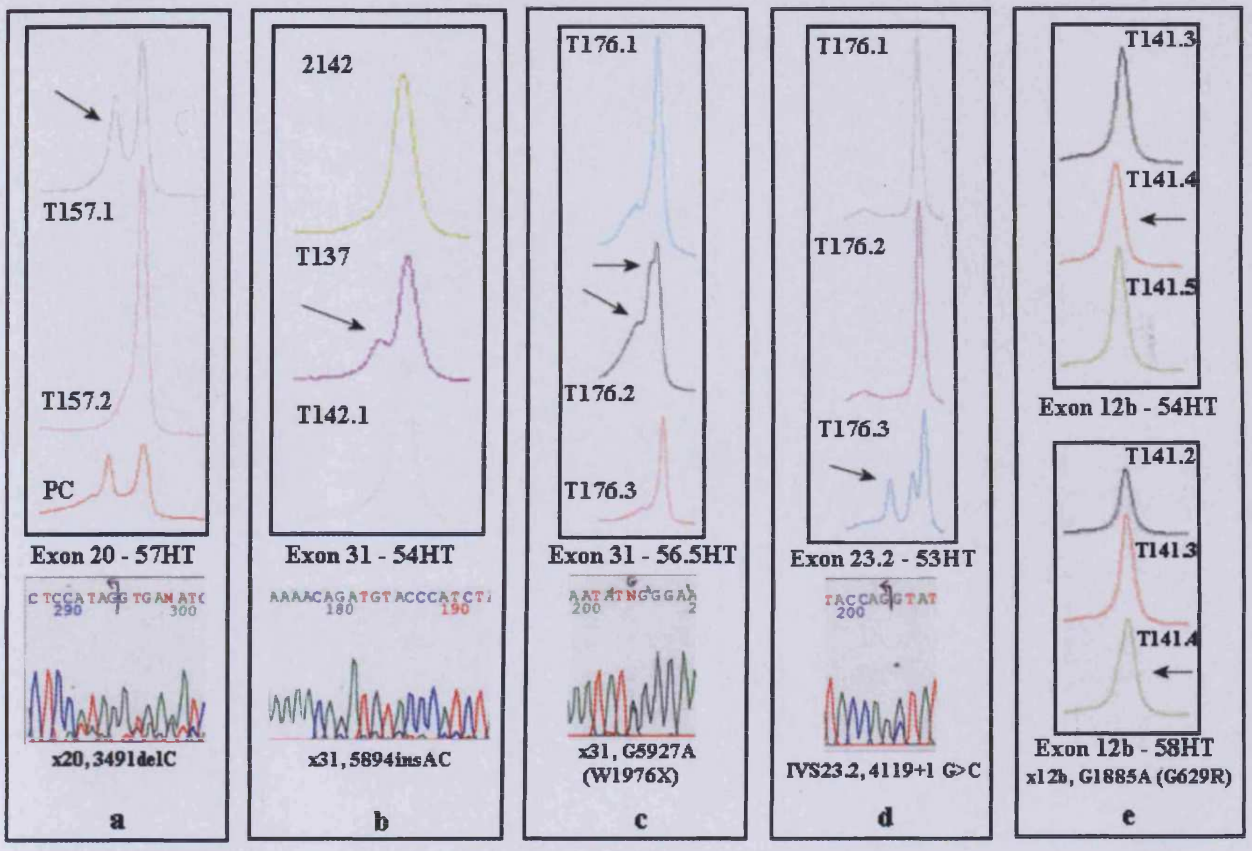

Figure 6.9: Chromatograms of somatic alterations in NF1 tumours

a. The two peaks indicative of a somatic deletion in exon 20 of tumour T157.1 are contrasted with the single peak of another tumour (T157.2) from the same patient. In the absence of blood lymphocyte DNA from patient P157, the somatic nature of the mutation was confirmed by sequencing of exon 20 in both tumours.

b. As is often seen in somatic mutation detection, the insertion in neurofibroma T137 is seen as a "shoulder" on the main peak, compared to the profiles of another tumour and corresponding blood DNAs from the same patient.

c. and d. Chromatograms of somatic mutation in two different tumours in a set of three. In both cases, the somatic nature of the nucleotide substitution is indicated by its absence in the chromatograms from the other two tumours.

e. Close examination of the chromatograms of tumour T141.4 reveals a widening of the peak at both denaturing temperatures, compared to the profiles of the other tumours. Sequencing of exon 12b revealed a missense mutation.

Table 6.7: Results of the *NF1* mutations screen by DHPLC

Neurofibromas

	P133											P135	
Exon	2125	T133.1	T133.2	T133.3	T133.4	T133.5	T133.6	T137	T142.1	T142.2	T142.3	2126	T135.1
x1	*	*	*	*	*	*	*	*	*	*	*	*	*
x2	*	*	*	*	*	*	*	*	*	*	*	*	*
x3	*	*	*	*	*	*	*	*	*	*	*	*	*
x4a	*	*	*	*	*	*	*	*	*	*	*	*	*
x4b	*	*	*	*	*	*	*	*	*	*	*	*	*
x4c	*	*	*	*	*	*	*	*	*	*	*	*	*
x5	P	*	*	*	*	*	*	*	*	*	*	P	*
x6	*	*	*	*	*	*	*	*	*	*	*	*	*
x7	*	*	*	*	*	*	ND	*	*	*	*	*	*
x8	*	*	*	*	*	*	*	*	*	*	*	*	*
x9	*	*	*	*	*	*	*	*	*	*	*	*	*
x10a	*	*	*	*	*	*	*	*	*	*	*	*	*
x10b	*	*	*	*	*	*	ND	*	*	*	*	*	*
x10c	*	*	*	*	*	*	*	*	*	*	*	*	*
x11	*	*	*	*	*	*	*	*	*	*	*	*	*
x12a	*	*	*	*	*	*	*	*	*	*	*	*	*
x12b	*	*	*	*	*	*	*	*	*	*	*	*	*
x13	*	*	*	*	*	*	*	*	*	*	*	*	*
x14	*	*	*	*	*	*	*	*	*	*	*	*	*
x15	*	*	*	*	*	*	*	*	*	*	*	*	*
x16	GM	*	*	*	*	*	*	*	*	*	*	*	*
x17	*	*	*	*	*	*	*	*	*	*	*	*	*
x18	*	*	*	*	*	*	*	*	*	*	*	*	*
x19a	*	*	*	*	*	*	*	*	*	*	*	*	*
x19b	*	*	*	*	*	*	*	*	*	*	*	*	*
x20	*	*	*	*	*	*	*	*	*	*	*	*	*
x21	*	*	*	*	*	*	*	*	*	*	*	*	*
x22	*	*	*	*	*	*	*	*	*	*	*	*	*
x23.1	*	*	*	*	*	*	*	*	*	*	*	*	*
x23.2	*	*	*	*	*	*	*	*	*	*	*	*	*
x24	*	*	*	*	*	*	ND	*	*	*	*	*	*
x25	*	*	*	*	*	*	*	*	*	*	*	*	*
x26	*	*	*	*	*	*	*	*	*	*	*	*	*
x27a	*	*	*	*	*	*	*	*	*	*	*	*	*
x27b	*	*	*	*	*	*	*	*	*	*	*	*	*
x28	P	*	*	*	*	*	*	*	*	*	*	GM	*
x29	*	*	*	*	*	*	*	*	*	*	*	*	*
x30	*	*	*	*	*	*	*	*	*	*	*	*	*
x31	*	*	*	*	*	*	*	SM	*	*	*	*	*
x32	*	*	*	*	*	*	*	*	*	*	*	*	*
x33	*	*	*	*	*	*	*	*	*	*	*	*	*
x34	*	*	*	*	*	*	*	*	*	*	*	*	*
x35	*	*	*	*	*	*	*	*	*	*	*	*	*
x36	*	*	*	*	*	*	*	*	*	*	*	*	*
x37	*	*	*	*	*	*	*	*	*	*	*	*	*
x38	*	*	*	*	*	*	*	*	*	*	*	*	*
x39	*	*	*	*	*	*	*	*	*	*	*	*	*
x40	*	*	*	*	*	*	*	*	*	*	*	*	*
x41	*	*	*	*	*	*	*	*	*	*	*	*	*
x42	*	*	*	*	*	*	ND	*	*	*	*	*	*
x43	*	*	*	*	*	*	*	*	*	*	*	*	*
x44	*	*	*	*	*	*	*	*	*	*	*	*	*
x45	*	*	*	*	*	*	*	*	*	*	*	*	*
x46	*	*	*	*	*	*	*	*	*	*	*	*	*
x47	*	*	*	*	*	*	*	*	*	*	*	P	*
x48	*	*	*	*	*	*	*	*	*	*	*	*	*
x49	*	*	*	*	*	*	ND	*	*	*	*	*	*

SM somatic mutation **GM** germline mutation **S** silent **I** intronic alteration
ND not done **P** polymorphism * completed

Table 6.7: continued

	Neurofibromas												
		P136			P139		P140					P141	
Exon	T135.2	2127	T136.1	T136.2	2128	T139	T140.1	T140.2	T140.3	T140.4	T140.5	2141	T141.1
x1	*	*	*	*	*	*	*	*	*	*	*	*	*
x2	*	*	*	*	*	*	*	*	*	*	*	*	*
x3	*	*	*	*	*	*	*	*	*	*	*	P	*
x4a	*	*	*	*	GM	*	*	*	*	*	*	*	*
x4b	*	*	*	*	*	*	*	*	*	*	*	*	*
x4c	*	*	*	*	*	*	*	*	*	*	*	*	*
x5	*	*	*	*	*	*	P	P	P	P	P	P	*
x6	*	*	*	*	*	*	*	*	*	*	*	*	*
x7	*	*	*	*	*	*	*	*	*	*	*	*	*
x8	*	*	*	*	*	*	*	*	*	*	*	*	*
x9	*	*	*	*	*	*	*	*	*	*	*	*	*
x10a	*	*	*	*	*	*	*	*	*	*	*	*	*
x10b	*	*	*	*	*	*	*	*	*	*	*	*	*
x10c	*	P	*	*	*	*	*	*	*	*	*	*	*
x11	*	*	*	*	*	*	*	*	*	*	*	*	*
x12a	*	S	*	*	*	*	*	*	*	*	*	*	*
x12b	*	*	*	*	*	*	*	*	*	*	*	*	*
x13	*	*	*	*	*	*	*	*	*	*	*	GM	*
x14	*	*	*	*	*	*	*	*	*	*	*	*	*
x15	*	*	*	*	*	*	*	*	*	*	*	*	*
x16	*	*	*	*	*	*	*	*	*	*	*	*	*
x17	*	*	*	*	*	*	*	*	*	*	*	P	*
x18	*	*	*	*	*	*	*	*	*	*	*	*	*
x19a	*	*	*	*	*	*	*	*	*	*	*	*	*
x19b	*	*	*	*	*	*	*	*	*	*	*	*	*
x20	*	*	*	*	*	*	*	*	*	*	*	*	*
x21	*	*	*	*	*	*	*	*	*	*	*	*	*
x22	*	*	*	*	*	*	GM	GM	GM	GM	GM	*	*
x23.1	*	*	*	*	*	*	*	*	*	*	*	*	*
x23.2	*	*	*	*	*	*	*	*	*	*	*	*	*
x24	*	*	*	*	*	*	*	*	*	*	*	*	*
x25	*	*	*	*	*	*	*	*	*	*	*	*	*
x26	*	*	*	*	*	*	*	*	*	*	ND	*	*
x27a	*	*	*	*	*	SM	*	*	*	*	*	*	*
x27b	*	*	*	*	*	*	*	*	*	*	*	*	*
x28	*	*	*	*	*	*	P	P	P	P	P	*	*
x29	*	*	*	*	*	*	*	*	*	*	*	*	*
x30	*	*	*	*	*	*	*	*	*	*	*	*	*
x31	*	*	*	*	*	*	*	*	*	*	*	*	*
x32	*	*	*	*	*	*	*	*	*	*	*	*	*
x33	*	*	*	*	*	*	*	*	*	*	*	*	*
x34	*	*	*	*	*	*	*	*	*	*	*	*	*
x35	*	*	*	*	*	*	*	*	*	*	*	*	*
x36	*	*	*	*	*	*	*	*	*	*	*	*	*
x37	*	*	*	*	*	*	*	*	*	*	*	*	*
x38	*	*	*	*	*	*	*	*	*	*	*	*	*
x39	*	*	*	*	*	*	*	*	*	*	*	P	*
x40	*	*	*	*	*	*	*	*	*	*	*	*	*
x41	*	*	*	*	*	*	*	*	*	SM	*	*	*
x42	*	*	*	*	*	*	*	*	*	*	*	*	*
x43	*	*	*	*	*	*	*	*	*	*	*	*	*
x44	*	*	*	*	*	*	*	*	*	*	*	*	*
x45	*	*	*	*	*	*	*	*	*	*	*	*	*
x46	*	*	*	*	*	*	*	*	*	*	*	*	*
x47	*	*	*	*	*	*	*	*	*	*	*	*	*
x48	*	*	*	*	*	*	*	*	*	*	*	*	*
x49	*	*	*	*	*	*	*	*	*	*	*	*	*

Table 6.7: continued

Neurofibromas

Exon	T141.2	T141.3	T141.4	T141.5	T141.6	T141.7	T141.8	T141.9	T141.10	T141.11	T141.12	T141.13	T141.14
x1	*	*	*	*	*	*	*	*	*	*	*	*	*
x2	*	*	*	*	*	*	*	*	*	*	*	*	*
x3	*	*	*	*	*	*	*	*	*	*	*	*	*
x4a	*	*	*	*	*	*	*	*	*	*	*	*	*
x4b	*	*	*	*	*	*	*	*	*	*	*	*	*
x4c	*	*	*	*	*	*	*	*	*	*	*	*	*
x5	*	*	*	*	*	*	*	*	*	*	*	*	*
x6	*	*	*	*	*	*	*	*	*	*	*	*	*
x7	*	*	*	*	*	*	*	*	*	*	*	*	*
x8	*	*	*	*	*	*	*	*	*	*	*	*	*
x9	*	*	*	*	*	*	*	*	*	*	*	*	*
x10a	*	*	*	*	*	*	*	*	*	*	*	*	*
x10b	*	*	*	*	*	*	*	*	*	*	*	*	*
x10c	*	*	*	*	*	*	*	*	*	*	*	*	*
x11	*	*	*	*	*	*	*	*	*	*	*	*	*
x12a	*	*	*	*	*	*	*	*	*	*	*	*	*
x12b	*	*	SM	*	*	*	*	*	*	*	*	*	*
x13	*	*	*	*	*	*	*	*	*	*	*	*	*
x14	*	*	*	*	*	*	*	*	*	*	*	*	*
x15	*	*	*	*	*	*	*	*	*	*	*	*	*
x16	*	*	*	*	*	*	*	*	*	*	*	*	*
x17	*	*	*	*	*	*	*	*	*	*	*	*	*
x18	*	*	*	*	*	*	*	*	*	*	*	*	*
x19a	*	*	*	*	*	*	*	*	*	*	*	*	*
x19b	*	*	*	*	*	*	*	*	*	*	*	*	*
x20	*	*	*	*	*	*	*	*	*	*	*	*	*
x21	*	*	*	*	*	*	*	*	*	*	*	*	*
x22	*	*	*	*	*	*	*	*	*	*	*	*	*
x23.1	*	*	*	*	*	*	*	*	*	*	*	*	*
x23.2	*	*	*	*	*	*	*	*	*	*	*	*	*
x24	*	*	*	*	*	*	*	*	*	*	*	*	*
x25	*	*	*	*	*	*	*	*	*	*	*	*	*
x26	*	*	*	*	*	*	*	*	*	*	*	*	*
x27a	*	*	*	*	*	*	*	*	*	*	*	*	*
x27b	*	*	*	*	*	*	*	*	*	*	*	*	*
x28	*	*	*	*	*	*	*	*	*	*	*	*	*
x29	*	*	*	*	*	*	*	*	*	*	*	*	*
x30	*	*	*	*	*	*	*	*	*	*	*	SM	*
x31	*	*	*	*	*	*	*	*	*	*	*	*	*
x32	*	*	*	*	*	*	*	*	*	*	*	*	*
x33	*	*	*	*	*	*	*	*	*	*	*	*	*
x34	*	*	*	*	*	*	*	*	*	*	*	*	*
x35	*	*	*	*	*	*	*	*	*	*	*	*	*
x36	*	*	*	*	*	*	*	*	*	*	*	*	*
x37	*	*	*	*	*	*	*	*	*	*	*	*	*
x38	*	*	*	*	*	*	*	*	*	*	*	*	*
x39	*	*	*	*	*	*	*	*	*	*	*	*	*
x40	*	*	*	*	*	*	*	*	*	*	*	*	*
x41	*	*	*	*	*	*	*	*	*	*	*	*	*
x42	*	*	*	*	*	*	*	*	*	*	*	*	*
x43	*	*	*	*	*	*	*	*	*	*	*	*	*
x44	*	*	*	*	*	*	*	*	*	*	*	*	*
x45	*	*	*	*	*	*	*	*	*	*	*	*	*
x46	*	*	*	*	*	*	*	*	*	*	*	*	*
x47	*	*	*	*	*	*	*	*	*	*	*	*	*
x48	*	*	*	*	*	*	*	*	*	*	*	*	*
x49	*	*	*	*	*	*	*	*	*	*	*	*	*

Table 6.7: continued

Neurofibromas

	P143								P144			P147	
Exon	T141.15	T141.16	T143.1	T143.2	T143.3	T143.4	T143.5	T143.6	T143.7	T144.2	T144.3	2156	T147
x1	*	*	*	*	*	*	*	*	*	*	*	*	*
x2	*	*	*	*	*	*	*	*	*	*	*	*	*
x3	*	*	*	*	*	*	*	*	*	*	*	*	*
x4a	*	*	*	*	*	*	*	*	*	*	*	*	*
x4b	*	*	*	*	*	*	*	*	*	*	*	*	*
x4c	*	*	*	*	*	*	*	*	*	*	*	*	*
x5	*	*	*	*	*	*	*	*	*	*	*	P	*
x6	*	*	*	*	*	*	*	*	*	*	*	*	*
x7	*	*	*	*	*	*	*	*	*	*	*	*	*
x8	*	*	*	*	*	*	*	*	*	*	*	*	*
x9	*	*	*	*	*	*	*	*	*	*	*	*	*
x10a	*	*	*	*	*	*	*	*	*	*	*	*	*
x10b	*	*	*	*	*	*	*	*	*	*	*	*	*
x10c	*	*	*	*	*	*	*	*	*	*	*	P	*
x11	*	*	*	*	*	*	*	*	*	*	*	*	*
x12a	*	*	*	*	*	*	*	*	*	*	*	*	*
x12b	*	*	*	*	*	*	*	*	*	*	*	*	*
x13	*	*	*	*	*	*	*	*	*	*	*	*	*
x14	*	*	*	*	*	*	*	*	*	*	*	*	*
x15	*	*	*	*	*	*	*	*	*	*	*	*	*
x16	*	*	*	*	*	*	*	*	*	*	*	*	*
x17	*	*	*	*	*	*	*	*	*	*	*	*	*
x18	*	*	*	*	*	*	*	*	*	*	*	*	*
x19a	*	*	*	SM	*	*	*	*	*	*	*	*	*
x19b	*	*	*	*	*	*	*	*	*	*	*	*	*
x20	*	*	*	*	*	*	*	*	*	*	*	*	*
x21	*	*	*	*	*	*	*	*	*	*	*	*	*
x22	*	*	*	*	*	*	*	*	*	*	*	*	*
x23.1	*	*	*	*	*	*	*	*	*	*	*	*	*
x23.2	*	*	*	*	*	*	*	*	*	*	*	*	*
x24	*	*	*	*	*	*	*	*	*	*	*	*	*
x25	*	*	*	*	*	*	*	*	*	*	*	*	*
x26	*	*	*	*	*	*	*	*	*	*	*	*	*
x27a	*	*	*	*	*	*	*	*	*	*	*	*	*
x27b	*	*	*	*	*	*	*	*	*	*	*	*	*
x28	*	*	P	P	P	P	P	P	P	P	P	*	*
x29	*	*	*	*	*	*	*	*	*	*	*	*	*
x30	*	*	*	*	*	*	*	*	*	*	*	*	*
x31	*	*	*	*	*	*	*	*	*	*	*	*	*
x32	*	*	*	*	*	*	*	*	*	*	*	*	*
x33	*	*	*	*	*	*	*	*	*	*	*	*	*
x34	*	*	*	*	*	*	*	*	*	*	*	*	*
x35	*	*	*	*	*	*	*	*	*	*	*	*	*
x36	*	*	*	*	*	*	*	*	*	*	*	*	*
x37	*	*	GM	GM	GM	GM	GM	GM	GM	*	*	*	*
x38	*	*	*	*	*	*	*	*	*	*	*	*	*
x39	*	*	P	P	P	P	P	P	P	*	*	P	*
x40	*	*	*	*	*	*	*	*	*	*	*	*	*
x41	*	*	*	*	*	*	*	*	*	*	*	*	*
x42	*	*	*	*	*	*	*	*	*	*	*	GM	*
x43	*	*	*	*	*	*	*	*	*	*	*	*	*
x44	*	*	*	*	*	*	*	*	*	*	*	*	*
x45	*	*	*	*	*	*	*	*	*	*	*	*	*
x46	*	*	*	*	*	*	*	*	*	*	*	*	*
x47	*	*	*	*	*	*	*	*	*	*	*	*	*
x48	*	*	*	*	*	*	*	*	*	*	*	*	*
x49	*	*	*	*	*	*	*	*	*	*	*	*	*

Table 6.7: continued

Neurofibromas

	P149		P150			P152			P153		P155		P157
Exon	2157	T149	T150.1	T150.2	T150.4	2174	T152.1	T152.2	2175	T153	2177	T155	T157.1
x1	*	*	*	*	*	*	*	*	*	*	*	ND	*
x2	*	*	*	*	*	*	*	*	*	*	*	*	*
x3	P	*	*	*	*	*	*	*	P	*	*	*	*
x4a	*	*	*	*	*	*	*	*	*	*	*	*	*
x4b	*	*	*	*	*	*	*	*	*	*	*	*	*
x4c	*	*	*	*	*	*	*	*	*	*	*	*	*
x5	P	*	*	*	*	P	*	*	P	*	*	*	*
x6	*	*	*	*	*	*	*	*	*	*	*	*	*
x7	*	*	*	*	*	*	*	*	*	*	*	*	*
x8	*	*	*	*	*	*	*	*	*	*	*	*	*
x9	*	*	*	*	*	*	*	*	*	*	*	*	*
x10a	*	*	*	*	*	*	*	*	*	*	*	*	*
x10b	*	*	*	*	*	*	*	*	*	*	*	ND	*
x10c	P	*	*	*	*	*	*	*	*	*	*	*	*
x11	*	*	*	*	*	*	*	*	*	*	*	*	*
x12a	*	*	*	*	*	*	*	*	*	*	I	*	*
x12b	*	*	*	*	*	*	*	*	*	*	*	*	*
x13	*	*	*	*	*	*	*	*	*	*	*	*	*
x14	*	*	*	SM	*	*	*	*	*	*	*	ND	*
x15	*	*	*	*	*	*	*	*	*	*	*	*	*
x16	*	*	*	*	*	*	*	*	*	*	*	ND	*
x17	*	*	*	*	*	*	*	*	*	*	*	*	*
x18	*	*	*	*	*	*	*	*	*	*	*	*	*
x19a	*	*	*	*	*	*	*	*	*	*	*	*	*
x19b	*	*	*	*	*	*	*	*	*	*	*	ND	*
x20	*	*	*	*	*	*	*	*	*	*	*	*	SM
x21	*	*	*	*	*	*	*	*	*	*	*	*	*
x22	*	*	*	*	*	*	*	*	*	*	*	*	*
x23.1	*	*	*	*	*	*	*	*	*	*	*	*	*
x23.2	*	*	*	*	*	*	*	*	*	*	*	*	*
x24	*	*	*	*	*	*	*	*	*	*	*	*	*
x25	*	*	*	*	*	*	*	*	*	*	*	*	*
x26	*	*	*	*	*	*	*	*	*	*	*	ND	*
x27a	*	*	*	*	*	*	*	*	*	*	*	ND	*
x27b	*	*	*	*	*	*	*	*	*	*	*	*	*
x28	*	*	*	*	*	*	*	*	*	*	*	*	*
x29	*	*	*	*	*	GM	*	*	*	*	*	*	*
x30	*	*	*	*	*	*	*	*	*	*	*	*	*
x31	*	*	*	*	*	*	*	*	*	*	*	*	*
x32	*	*	*	*	*	*	*	*	*	*	*	*	*
x33	*	*	*	*	*	*	*	*	*	*	GM	*	*
x34	GM	*	*	*	*	*	*	*	*	*	*	*	*
x35	*	*	*	*	*	*	*	*	*	*	*	*	*
x36	*	*	*	*	*	*	*	*	*	*	*	*	*
x37	*	*	*	*	*	*	*	*	*	*	*	*	*
x38	*	*	*	*	*	*	*	*	*	*	*	*	*
x39	*	*	*	*	*	*	*	*	*	*	*	*	*
x40	*	*	*	*	*	*	*	*	*	*	*	*	*
x41	*	*	*	*	*	*	*	*	*	*	*	*	*
x42	*	*	*	*	*	*	*	*	*	*	*	*	*
x43	*	*	*	*	*	*	*	*	*	*	I	*	*
x44	*	*	*	*	*	*	*	*	*	*	*	*	*
x45	*	*	*	*	*	*	*	*	*	*	*	*	GM
x46	*	*	*	*	*	*	*	*	*	*	*	*	*
x47	*	*	*	*	*	*	*	*	*	*	*	*	*
x48	*	*	*	*	*	*	*	*	*	*	*	ND	*
x49	*	*	*	*	*	*	*	*	*	*	*	*	*

Table 6.7: continued

	Neurofibromas								PNF			
	P159			P176			P185	P186	P156		P167	
Exon	T157.2	2227	T159	T176.1	T176.2	T176.3	2318	T186	2170	T156	2229	T167
x1	*	*	*	*	*	*	*	*	*	*	*	*
x2	*	*	*	*	*	*	*	*	*	*	*	*
x3	*	*	*	*	*	*	*	*	*	*	*	*
x4a	*	*	*	*	*	*	*	*	*	*	*	*
x4b	*	*	*	*	*	*	*	*	*	*	*	*
x4c	*	*	*	*	*	*	*	*	*	*	*	*
x5	*	P	*	*	*	*	*	*	P	*	*	*
x6	*	*	*	*	*	*	GM	*	*	*	*	*
x7	*	*	*	*	*	*	*	*	*	*	*	*
x8	*	*	*	*	*	*	*	*	*	*	*	*
x9	*	*	*	*	*	*	*	*	*	*	*	*
x10a	*	*	*	*	*	*	*	*	*	*	*	*
x10b	*	*	*	*	*	*	*	*	*	*	*	*
x10c	*	*	*	*	*	*	*	*	*	*	*	*
x11	*	*	*	*	*	*	*	*	*	*	*	*
x12a	*	*	*	*	*	*	*	*	*	*	*	*
x12b	*	*	*	*	*	*	*	*	*	*	*	*
x13	*	*	*	*	*	*	*	*	*	*	*	*
x14	*	*	*	*	*	*	*	*	*	*	*	*
x15	*	*	*	*	*	*	*	*	*	*	*	*
x16	*	*	*	*	*	*	*	*	*	*	*	*
x17	*	*	*	*	*	*	*	SM	*	*	*	*
x18	*	*	*	*	*	*	*	*	*	*	*	*
x19a	*	*	*	*	*	*	*	*	*	*	*	*
x19b	*	*	*	*	*	*	*	*	*	*	*	*
x20	*	P	*	*	*	*	P	*	*	*	*	*
x21	*	*	*	*	*	*	*	*	*	*	*	*
x22	*	*	*	*	*	*	*	*	*	*	GM	*
x23.1	*	*	*	*	*	*	*	*	*	*	*	*
x23.2	*	*	*	*	*	SM	*	*	*	*	*	*
x24	*	*	*	*	*	*	*	*	*	*	*	*
x25	*	*	*	*	*	*	*	*	*	*	*	*
x26	*	*	*	*	*	*	*	*	*	*	*	*
x27a	*	*	*	*	*	*	*	*	*	*	*	*
x27b	*	*	*	*	*	*	*	*	*	*	*	*
x28	*	*	*	SM	*	*	*	*	*	*	*	*
x29	*	*	*	*	*	*	*	*	*	*	*	*
x30	*	*	*	*	*	*	*	*	*	*	*	*
x31	*	*	*	*	SM	*	I	*	*	*	*	*
x32	*	*	*	*	*	*	*	*	*	*	*	*
x33	*	*	*	*	*	*	*	*	*	*	*	*
x34	*	*	*	*	*	*	*	*	*	*	*	*
x35	*	*	*	*	*	*	*	*	*	*	*	*
x36	*	*	*	*	*	*	*	*	*	*	*	*
x37	*	*	*	*	*	*	*	*	*	*	*	*
x38	*	*	*	*	*	*	*	*	*	*	*	*
x39	*	*	*	*	*	*	*	*	*	*	*	*
x40	*	*	*	*	*	*	*	*	*	*	*	*
x41	*	*	*	*	*	*	*	*	*	*	*	*
x42	*	*	*	*	*	*	*	*	*	*	*	*
x43	*	*	*	*	*	*	I	*	*	*	*	*
x44	*	*	*	*	*	*	*	*	*	*	*	*
x45	GM	*	*	*	*	*	*	*	*	*	*	*
x46	*	*	*	*	*	*	*	*	*	*	*	*
x47	*	*	*	*	*	*	*	*	*	*	*	*
x48	*	*	*	*	*	*	*	*	*	*	*	*
x49	*	*	*	*	*	*	*	*	*	*	*	*

Table 6.7: continued

Malignant tumours

	P130		P145		P148		P151			P154		P160	
Exon	2124	T130	2155	T145	2158	T148	2159	T151	T165	2176	T154	2205	T160
x1	*	*	*	*	*	*	*	*	*	*	*	*	*
x2	*	*	*	*	*	*	*	*	*	*	*	*	*
x3	*	*	*	*	*	*	*	*	*	*	*	GM	*
x4a	*	*	*	*	*	*	*	*	*	*	*	*	*
x4b	*	*	*	*	*	*	*	*	*	*	*	*	*
x4c	*	*	*	*	*	*	*	*	*	*	*	*	*
x5	P	*	P	*	*	*	*	*	*	P	*	P	*
x6	*	*	*	*	*	*	*	*	*	*	*	*	*
x7	*	*	*	*	GM	*	*	*	*	*	*	*	*
x8	*	*	*	*	*	*	*	*	*	*	*	*	*
x9	*	*	*	*	*	*	*	*	*	*	*	*	*
x10a	*	*	*	*	*	*	*	*	*	*	*	*	*
x10b	*	*	*	*	*	*	*	*	*	*	*	*	*
x10c	*	*	*	*	*	*	*	*	*	*	*	P	*
x11	*	*	*	*	*	*	*	*	*	*	*	*	*
x12a	*	*	*	*	*	*	*	*	*	*	*	*	*
x12b	*	*	*	*	*	*	*	*	*	*	*	*	*
x13	*	*	*	*	*	*	*	*	*	*	*	*	S
x14	*	*	*	*	*	*	*	*	*	*	*	*	*
x15	*	*	*	*	*	*	*	*	*	*	*	*	*
x16	*	*	*	*	*	*	*	*	*	*	*	*	*
x17	*	*	*	*	*	*	*	*	*	*	*	*	*
x18	*	*	*	*	*	*	*	*	*	*	*	*	*
x19a	*	*	*	*	*	*	*	*	*	*	*	*	*
x19b	*	*	*	*	*	*	*	*	*	*	*	*	*
x20	*	*	*	*	*	*	*	*	*	*	*	*	*
x21	*	*	*	*	*	*	*	*	*	*	*	*	*
x22	*	*	*	*	*	*	*	*	*	*	*	*	*
x23.1	*	*	*	*	*	*	*	*	*	*	*	*	*
x23.2	*	*	*	*	*	*	*	*	*	*	*	*	*
x24	*	*	*	*	*	*	*	*	*	*	*	*	*
x25	*	*	*	*	*	*	*	*	*	*	*	*	*
x26	*	*	*	*	*	*	*	*	*	*	*	*	*
x27a	*	*	*	*	*	*	*	*	*	*	*	*	*
x27b	*	*	*	*	*	*	*	*	*	*	*	*	*
x28	P	*	GM	*	P	*	*	*	*	*	*	*	*
x29	*	*	*	*	*	*	*	*	*	*	*	*	*
x30	*	*	*	*	*	*	*	*	*	*	*	*	*
x31	*	*	*	*	*	*	*	*	*	*	*	*	*
x32	*	*	*	*	*	*	*	*	*	*	*	*	*
x33	GM	*	*	*	*	*	*	*	*	*	*	*	*
x34	*	*	*	*	*	*	*	*	*	*	*	*	*
x35	*	*	*	*	*	*	GM	*	*	*	*	*	*
x36	*	*	*	*	*	*	*	*	*	*	*	*	*
x37	*	*	*	*	*	*	*	*	*	*	*	*	*
x38	*	*	*	*	*	*	*	*	*	*	*	*	*
x39	*	*	P	*	P	*	*	*	*	*	*	*	*
x40	*	*	*	*	*	*	*	*	*	*	*	*	*
x41	*	*	*	*	*	*	*	*	*	*	*	*	*
x42	*	*	*	*	*	*	*	*	*	*	*	*	*
x43	*	*	*	*	*	*	*	*	*	*	*	*	*
x44	*	*	*	*	*	*	*	*	*	*	*	*	*
x45	*	*	*	*	*	*	*	*	*	*	*	*	*
x46	*	*	*	*	*	*	*	*	*	*	*	*	*
x47	*	*	*	*	*	*	*	*	*	*	*	*	*
x48	*	*	*	*	*	*	*	*	*	*	*	*	*
x49	*	*	*	*	*	*	*	*	*	*	*	*	*

Table 6.7: continued

Malignant tumours

	P169		P178		P184
Exon	2207	T169	2230	T178	2316
x1	*	*	*	*	*
x2	*	*	*	*	*
x3	*	*	*	*	*
x4a	*	*	*	*	*
x4b	*	*	*	*	*
x4c	*	*	*	*	*
x5	*	*	*	*	*
x6	*	*	*	*	*
x7	*	*	*	*	*
x8	*	*	*	*	*
x9	*	*	*	*	*
x10a	*	*	*	*	*
x10b	*	*	*	*	*
x10c	*	*	*	*	*
x11	GM	*	GM	*	*
x12a	*	*	*	*	*
x12b	*	*	*	*	*
x13	*	*	*	*	*
x14	*	*	*	*	*
x15	*	*	*	*	*
x16	*	*	*	*	*
x17	*	*	*	*	*
x18	*	*	*	*	*
x19a	*	*	*	*	*
x19b	*	*	*	*	*
x20	*	*	*	*	P
x21	*	*	*	*	*
x22	*	*	*	*	*
x23.1	*	*	*	*	*
x23.2	*	*	*	*	*
x24	*	*	*	*	*
x25	*	*	*	*	*
x26	*	*	*	*	*
x27a	*	*	*	*	*
x27b	*	*	*	*	*
x28	*	*	*	*	*
x29	*	*	*	*	*
x30	*	*	*	*	*
x31	*	*	*	*	*
x32	*	*	*	*	*
x33	*	*	*	*	*
x34	*	*	*	*	*
x35	*	*	*	*	*
x36	*	*	*	*	*
x37	*	*	*	*	*
x38	*	*	*	*	*
x39	*	*	*	*	*
x40	*	*	*	*	*
x41	*	*	*	*	*
x42	*	*	*	*	*
x43	*	*	*	*	*
x44	*	*	*	*	*
x45	*	*	*	*	*
x46	*	*	*	*	*
x47	*	*	*	*	*
x48	*	*	*	*	*
x49	*	*	*	*	*

Patient	Germline mutation	Tumour sample	Exon/Intron	Genomic mutation	Aminoacid mutation	Type/effect	Reference
P133	x16, C2446T (R816X)	T137	**x31**	**5894insAC**	**D1991X**	frameshift / truncating?	This report
P139	x4a, 434delTC	T139	**x27a**	**C4637G**	**S1546X**	nonsense / truncating?	This report
P140	x22, 3731delT	T140.4	**x41**	**C7285T**	**R2429X**	nonsense / truncating	Upadhyaya *et al.*, 2004
P141	x13, 2233delA	T141.4	**x12b**	**G1885A**	**G629R**	missense / truncating	Gasparini *et al.*, 1996
		T141.13	**x30**	**5729delT**	**L1920X (x31)**	frameshift / truncating?	This report
P143	x37, C6792G (Y2264X)	T143.2	**x19a**	**3124insATdelGTAG**	**D1058X**	frameshift / truncating?	This report
P150	90kb 5' end deletion	T150.2	**x14**	**2271insG**	**E767X**	frameshift / truncating?	This report
P157	IVS45, 7907+3A>T	T157.1	**x20**	**3491delC**	**H1170X**	frameshift / truncating?	This report
P160	IVS3, 288+1G>A	T160	x13	A2034G	P678P	polymorphism	Fahsold *et al.*, 2000
P168	x5, G663A (W221X)	T168	**x34**	**6442delA**	**L2178X**	frameshift / truncating?	This report
P176	large deletion	T176.1	**x28**	**C4812G**	**Y1604X**	nonsense / truncating?	This report
		T176.2	**x31**	**G5927A**	**W1976X**	nonsense / truncating?	This report
		T176.3	**IVS23.2**	**4119+1 G>C**	skip exon 23.2	ds / truncating	Fahsold *et al.*, 2000
P184	NI	T184	**x27a**	**4580delCTCCAGAGCAC**	**W1538X**	frameshift / truncating?	This report
P186	large deletion	T186	**IVS17**	**2990+1 G>A**		ds / ?	This report

Table 6.8: Somatic mutations identified in a panel of NF1-related tumours. *NI*, not identified; *ds*, donor site.

6.2.4.2.2.6/ Missense and silent alterations

A slight change in DHPLC profile revealed a G>A transition at nucleotide 1885 in exon 12b of neurofibroma T141.4 (Figure 6.9). Missense alteration G629R has been identified as a germline mutation in 9 NF1 patients to date (Gasparini *et al.*, 1996; Ars *et al.*, 2003; De Luca *et al.*, 2004; Mattocks *et al.*, 2004) and predicted to create a cryptic 3' splice site and truncate the protein to a 616 amino acid neurofibromin product (Ars *et al.*, 2003). Residue 629 is conserved in rat, *Fugu* and *Drosophila* homologues.

Malignant tumour T160 harboured an A>G transition at nucleotide 2034 that did not modify the proline residue. The exon 13 alteration has previously been reported as a polymorphism with each allele having over 30% representation (Fahsold *et al.*, 2000).

6.2.4.2.3/ Conclusion

In the panel of 75 NF1-related tumours, pathogenic microlesions were found in 14 tumours (18%) whereas LOH accounted for 14% (10/71) of the samples. Since the majority (80%) of tumours in this panel are neurofibromas, it is likely that LOH has been underestimated in this population, due to the probable cellular heterogeneity exhibited by these tumours. Half of the LOH observed was found in malignant tumours, so that the LOH distribution was 45% in malignant tumours (5 out of 10 MPNST and 1 MTC) and 8.5% of benign tumours. The apparent homozygosity of markers, uninformative in some tumours, may also mask an actual deletion.

A microlesion accounting for the "second hit" necessary to tumour initiation was identified in 14 tumours (18%). In one tumour, T160, a somatic 'polymorphism' (not seen in the germline) was the only alteration identified. Of the 14 mutations, 11 were novel (75%) whereas 3 have previously been reported as germline mutations, two of which occurred at hypermutable CpG dinucleotides.

Nucleotide substitutions account for 50% of the mutation, five of which were transitions and three transversions. Furthermore, two nucleotide substitutions occurred at the canonical G of a splice donor site. Of the novel nonsense mutations, both Y1604X and W1976X occurred at a residue conserved in human, rat, *Fugu* and *Drosophila*. Mutation S1546X altered an amino acid conserved in human, rat, and *Fugu*.

Finally, all of these mutations are predicted to result in a prematurely truncated transcript.

6.3 Discussion

6.3.1/ Combined mutation detection techniques

6.3.1.1/ Deletion junction-specific PCR assay

This assay was designed to detect large deletions encompassing the *NF1* gene, which were not readily detectable by FISH (thus these were also termed "microdeletions"). However, the assay relies on the amplification of a 3.4 kb fragment characteristic of a recombination event at a *NF1*-REPs hotspot that Lopez-Correa and co-workers estimated to account for approximately 5% of *NF1* mutations (Lopez-Correa *et al.*, 2001). Other REP-mediated deletions (at breakpoints other than the one assessed here), as well as deletion occurring at rare deletion breakpoints cannot be identified by this assay (Dorschner *et al.*, 2000; Jenne *et al.*, 2001; Kehrer-Sawatzki et al., 2003; Petek *et al.*, 2003; Venturin *et al.*, 2004)., The LOH study also performed on the same samples may help to rule out large germline deletions. In theory, these samples should exhibit homozygosity for all the microsatellite and RFLP markers used, as only one allele would be amplified. This was not the case in the present panel: samples in which no disease-causing mutation was identified exhibited heterozygosity for either LOH markers or polymorphisms, belying the existence of a large deletion. A recent study on 31 NF1 patients revealed such a microdeletion in only 1 case (3%; Upadhyaya *et al.*, 2004).

6.3.1.2/ LOH assay

Gel-based LOH assay is a time consuming and labour-intensive technique, but is non-radioactive and cost-effective, and suited to the screening of large sample numbers at a time, with the possibility of double-loading the gel. The technique itself required very little optimisation. Its main drawback lies in the successful determination of the LOH status: in *NF1* benign tumours, LOH is indicated by a decrease in signal intensity of one allele rather than a complete allele loss. This is due to the mixed population of cells found in neurofibromas; thus the possibility of false-negative samples must always be considered (Colman *et al.*, 1995). In general, MPNSTS displayed larger deletions, but this could be due to the homogeneity of the tumour allowing more evident LOH to show. The LOH reported in this study (14%) is lower than that observed in other studies (Coleman *et al.*, 1995 ; Serra *et al.*,1997; Eisenbarth *et al.*, 2000) but in keeping with the results of John *et al.* (2000). It is interesting that the LOH detection in neurofibromas (9%, 5/56) reported is also in line with

results obtained using a fluorescent approach and a similar number of markers (9%, 3/33; Weist *et al.*, 2004). Although the gel-based LOH assay could be improved by prior Schwann cell enrichment (Serra *et al.*, 2001), LOH detection has now moved to more automated and quantitative techniques, using fluorescence and detection via a DNA sequencer (Dietmaier *et al.*, 1999; Faulkner *et al.*, 2004; Cai *et al.*, 2004). The use of fluorescence represents an improvement over the original technique in that it is less cumbersome and may resolve ambiguous cases more easily, while avoiding the "human error" associated with reading a gel. Gel-based LOH was the most efficient technique available at the time of this study. The fact that the results reported here are in keeping with those obtained using fluorescence (Weist *et al.*, 2004) is a testament to the efficacy and robustness of the technique.

A clear definition of the exact deletion boundary was often difficult to assess, and would require the use of a more closely spaced and more extensive set of *NF1* intragenic and extragenic markers. Due to time constraints, this avenue was not fully explored. Instead, the markers used here were spaced across the gene, and had proven informative in previous studies (John *et al.*, 2000; Upadhyaya *et al.*, 1998, 2004). Designing new markers would require a suitable DNA sequence (microsatellite or RFLP for inexpensive enzyme) as well as an assessment of the heterozygosity of the sequence (through screening of at least 20 normal and NF1 patient samples). For example, the common polymorphism in exon 28 was not suitable for RFLP assay. The *NF1* SNPs database on EntrezSNP (http://www.ncbi.nlm.nih.gov/entrez) was consulted for the most commonly represented SNPs, but few were amenable to inexpensive RFLP assays. A number of recurrent mutations and polymorphisms have been identified in the *NF1* sequence (Appendix) and could potentially be assessed rapidly using the real-time technology or DHPLC (Xiao and Oefner, 2001; Abbas *et al.*, 2004). Alternatively, a more expensive approach could take advantage of microarray chips for the rapid, simultaneous detection of the most commonly found mutations.

6.3.1.3/ Microlesion detection

Taking the two germline studies (sections 6.2.3 and 6.2.4.1) together, the detection rate (63%) reported here is slightly lower, but nevertheless in line with the findings of similar studies on NF1 patients using DHPLC (66-68%, Han *et al.*, 2001; De Luca *et al.*, 2004). It remains one of the highest mutation detection rates for a single technique in NF1.

To date, Messiaen et al. (2000) reported the highest mutation detection rate (95.5%) using an array of techniques (PTT, HA, FISH, Southern blot, cytogenetic analysis), which can be rather cumbersome and time consuming, especially for routine screening. Mutation detection by PTT has been widely used in NF1 studies, with a reported detection rate ranging between 39% and 80% (Heim *et al.*, 1995; Park and Pivnick, 1998; Osborn and Upadhyaya, 1999; Fahsold *et al.*, 2000; Messiaen *et al.*, 2000; Origone *et al.*, 2002; De Luca *et al.*, 2004).
Although the detection rates by DHPLC and PTT may be comparable in some studies, the use of DHPLC presents significant advantages in that it is DNA-based and not so labour intensive. Additionally, PTT fails to detect either small in-frame deletion/insertion or missense mutations. There are also pitfalls inherent in the use of an RNA-based study, such as nonsense-mediated mRNA decay (Osborn and Upadhyaya, 1999) and aberrant transcript ("aged blood" effect, Messiaen *et al.*, 2000, Wimmer *et al.*, 2000; Vandenbroucke *et al.*, 2001, 2002; Thomson and Wallace, 2002; Ars *et al.*, 2003), and any truncating mutation should be fully characterised. Alternatively, PTT and DHPLC analyses have been combined for a higher mutation detection rate (66% DHPLC vs. 68% DHPLC/PTT; De Luca *et al.*, 2004)

There are alterations beyond the reach of the DHPLC: for example, the technique does not detect large deletions (part of or whole of the *NF1* gene), the deletion of multiple exons, and large duplications or inversions, which taken together may represent 15-25% of all *NF1* gene mutations (Cowley *et al.*, 1998; Riva *et al.*, 2000; Han *et al.*, 2001; De Luca *et al.*, 2004). Mutations in the 3' or 5'untranslated regions and in the alternatively spliced exons were also not investigated in this study. Some intronic changes and regulatory sequences deep within the intron may also have been beyond the scope of the fragments. Additionally, mutations in the seven remaining patients may not have been detected at the denaturing temperatures used here, possibly due to the position of the alteration in the fragment or the nature of the surrounding sequence. In a retrospective study conducted by Han and co-workers, two additional mutations were identified in the panel by increasing the denaturing temperature to 2°C over the highest recommended temperature, as performed by others (Jones *et al.*, 1999; Han *et al.*, 2001). Alterations located at the very extremities of the fragment, or in a region of high GC content can also be overlooked. These may therefore account for some of the remaining 37%.

The mutation detection rate was substantially lowered when DHPLC screening was applied to somatic mutations in NF1 tumours. As the majority of the panel were neurofibromas, the heterogeneous nature of the tumours could explain the lower detection rate. The fact that somatic mutations were detected in 4/4 tumours from patients with large deletion in the *NF1* gene (P176 and P186) supports this observation.

DHPLC has been reported to be more sensitive than direct sequencing for mutation detection, with detection of allele frequency as low as 5% (Jones *et al.*, 1999; Xiao and Oefner, 2001). Nevertheless, direct sequencing of tumour cDNA uncovered a somatic mutation in 2/2 MPNSTs (the third tumour harboured LOH), and should be considered a reliable alternative mutation detection method.

The newly developed ACSA (automated comparative sequence analysis) obtained a reported 89% detection rate (Mattocks *et al.*, 2004), although close data examination by others suggested a number of misclassifications in the mutations identified and thus, in reality, a lower detection rate (Messiaen and Wimmer, 2005). The technique basically relies on the analysis of a fluorescently-labelled sequenced fragment, separated in its four component electrophoregrams (A, T, G, C), each aligned to a control. ACSA could present an attractive alternative to DHPLC screening, and it remains to be determined how this new technique would fare in the detection of somatic mutations.

In this study, mutation detection screening by DHPLC proved to have many advantages: it is a semi-automated and rapid technique whose mutation detection rate surpassed that of previous techniques used in *NF1* mutation screening. The technique can be further improved by use of cDNA, which not only reduces the screening time but also prevents the amplification of pseudogenes. Direct sequencing also proved effective and sensitive, and improvements in both the assay and the detection system can greatly increase rapidity.

6.3.2/ Germline mutations

6.3.2.1/ Germline mutational spectrum

Taking together the two studies in sections 6.2.3 and 6.2.4.1, a potentially pathogenic germline mutation was identified in approximately 63% of patients by DHPLC alone.

Altogether, 51% of the mutations described here are novel. This is in line with the most recent *NF1* mutations studies on large sample panels, where novel mutations range from 55-74% (Ars *et al.*, 2003; Mattocks *et al.*, 2004, De Luca *et al.*, 2004). These results underscore the need for continuing reassessment of the *NF1* mutational spectrum; with just under 700 microlesions identified, the percentage of novel mutations remains high.

No particular mutational hotspot was identified. A maximum of two mutations was observed in each exon (and closely flanking intronic sequences): 2, 4b, 7, 11, 16, 22, 28, 33, 34 and 37. Of these ten exons, four (4b, 7, 16 and 37) most commonly harboured reported mutations (based on the latest review of the literature on *NF1* mutations; Appendix) in NF1 patients (exons represented in over 40 patients, Figure 1.1). The same mutation in exon 16 (R816X) was represented twice, in a patient from each panel. The mutation occurred within a CpG dinucleotide, which would explain its over-representation.
From the numerous studies on the *NF1* gene, a number of recurrent mutations (Section 1.3.1.6), as well as "most-mutated" exons, are starting to emerge. Even though the number of novel mutations identified in the latest and present studies is still high, these mutational "warm" spots should be taken in consideration when investigating for *NF1* mutations.
Interestingly and as previously observed (Mattocks *et al.*, 2004), the GRD did not appear as a particular hot spot in this study, with only two mutations (6%) falling between exons 20-27a, and even came across as rather devoid of mutations as compared with the rest of the gene (Figure 6.5). The region between exons 11-17 harboured more mutations (19%), although the numbers are too small to describe it as a cluster. As Mattocks *et al.* (2004) found, more mutations were detected in 5' exons (40% of mutations occurred before exon 11).

Two groups of variations were almost equally represented: single base-pair substitutions accounted for 47% of alterations whereas frameshift mutations totalled 53%. This distribution differs with the one reported by Mattocks *et al.* (2004), where single nucleotide base-pair substitutions were by far the most represented, in particular missense mutations, although the latter may in part be misclassified and actually affect splicing, as pointed out by others (Messiaen and Wimmer, 2005). In the present study, missense mutations account for only 13% of the alterations. The discrepancy may lie with the differences in mutation detection technique used; DHPLC relies on the partial denaturation of heteroduplexes, and the position and nature of the alteration in the fragment may influence denaturation and thus detection. By contrast, comparative sequence analysis (CSA), as used by Mattocks and co-workers, relies

on the automated detection of differences in sequencing traces aligned to a control sequence, and may be more sensitive to single base-pair substitutions. The present results are also in keeping with those of De Luca *et al.* (2004) who reported a similar distribution of frameshift (48%) and single base-pair substitutions (52%), including missense mutations (13%), using DHPLC as a method of screening.

Of the single base-pair substitution, 80% were transitions, of which 33% occurred at CpG dinucleotides, and 20% were transversions. These proportions are in keeping with those reported by others (Ars *et al.*, 2000; Mattocks *et al.*, 2004). The single most represented alteration was small deletions (generally 1 or 2 and up to 6 nucleotides), accounting for 37.5% of the pathogenic mutations. It is also the most represented mutation in the *NF1* spectrum, in general (Section 1.3.1.4).

Overall, 69% of the mutations are expected to result in the premature truncation of neurofibromin. This high percentage of mutations resulting in a stop codon, directly or indirectly, is echoed by others, and ranges from 50% to 80% (Fahsold *et al.*, 2000; Messiaen et al., 2000; Han *et al.*, 2001; Mattocks *et al.*, 2004; De Luca *et al.*, 2004.) Furthermore, 57% of the truncating mutations identified here are predicted to terminate the protein upstream of, or within, the *NF1*-GRD.

These results should however be verified in the cDNA or by functional assay, as several studies have shown that nonsense, as well as missense and intronic mutations can affect normal splicing (Ars *et al.*, 2000; Mattocks *et al.*, 2004; Zatkova *et al.*, 2004).

In the present study, at least eight (24%) of the pathogenic mutations identified may affect the correct splicing, including two missense (L5751I, D338G) and one nonsense mutations (Y2264X). This is in line with the observations made by others, where mutations affecting splicing accounted for 12-28% (Messiaen *et al.*, 2000; Fahsold *et al.*, 2000; De Luca *et al.*, 2004). It was not possible to assess the functional impact at the mRNA level of the mutations identified in the present study. The three (missense and nonsense) mutations had however previously been reported with such effects. Ideally, all missense, nonsense and silent mutations should be assessed for effects in splicing and not only considered at the genomic level. Recently, a minigene assay has successfully been used to confirm the effect of missense or nonsense mutations on splicing alteration, also investigated *in silico* (Zatkova *et al.*, 2004). The same ESE prediction programs were also used on all the base-pair substitutions identified in the present study. However, the results yielded by the two programs can be ambiguous, and would certainly require *in vitro* investigations. As previously observed (Zatkova *et al.*, 2004),

there were instances where the two programs would predict the disruption of an ESE motif (or decrease in score) concomitantly with the creation of a novel ESE element or increase in score for a predicted motif. For example, mutation C574T in patient 2263 (Section 6.2.3.2) was predicted by ESEfinder to decrease the score of a SRp40 motif below threshold value and simultaneously increase a second SRp40 motif; RESCUE-ESE predicted the loss of one of two hexamers after mutation. Mutation G663A in patient P168 (Section 6.2.4.1.2) was predicted by ESEfinder to gain a SRp40 motif, whereas the mutation resulted in the loss of two hexamers in RESCUE-ESE. In such cases, Zatkova and co-workers estimated that the loss or decrease in score of an ESE element had a dominant effect over gain or increase in score. Furthermore, such cases also showed residual enhancement activity in *in-vitro* assays, which may suggest that mutations showing dual *in silico* results may have a milder effect on splicing. Other mutations were only predicted to create a novel ESE element, or to increase a score, and are not expected to alter splicing. Nonsense mutation C3826T in patient (Section 6.2.4.1.2) was one such mutation and was predicted by ESEfinder to increase a SF2/ASF element score and create a cluster of motifs for SRp40, SRp55 and SC35. In this particular case, the effect of the mutation is likely to be truncating rather than affecting the splicing. Finally, cases were also found where the mutation resulted only in the loss of an ESE element or reduction in score. The most interesting case is perhaps silent mutation C1137T in patient 2252 (Section 6.2.3.5). The mutation was predicted by ESEfinder to decrease the score of an SRp40 motif, and was also the only non-polymorphic mutation found in the patient. Thus an effect on splicing may account for the pathogenic effect of the mutation. Splicing appears to be a complex mechanism relying on a multitude of interaction (Zatkova *et al.*, 2004), and the results obtained here would warrant further investigation.

Thus far, the large size of the *NF1* gene has been an obstacle to devising functional assays. This type of assay has only been conducted on the *NF1*-GRD, which represent only a fraction of the entire protein, and the only region with a known biological function. For example, missense mutations in the *NF1*-GRD have been assessed for the impact of the altered conformation on the protein function (Li *et al.*, 1992; Gutmann *et al.*, 1993; Poullet *et al.*, 1994; Upadhyaya *et al.*, 1997). *In silico*, the NF1-GRD is also the only region of the protein available for study (Swiss-Pdb Viewer and PDB-Brookhaven crystal structure of the *NF1*-GRD; Guex *et al.*, 1997; Scheffzek *et al.*, 1998).

A number of polymorphic, non-pathogenic alterations were identified. The sequence variants were considered polymorphic when they had been reported in published studies (in unaffected individuals and/or in patients with a pathogenic mutation), or when they were found in several patients, some of whom already had a pathogenic mutation. There were discrepancies between the frequencies of polymorphisms published and those identified in this panel. However, these frequencies also vary between publications, and it is likely that the representation of a polymorphism is very much dependent upon the population studied. For example, polymorphism 4368-46G>C, in intron 25, was found to have a heterozygosity of 8% in the Italian population (De Luca *et al.*, 2004). By contrast, Fahsold *et al.* (2000) estimated a representation of over 30% for each allele. It remains to be determined how the presence of several polymorphisms in the same patient may affect the function of the protein.

Apart from the distribution of mutations in patients with gliomas and MPNSTs (discussed in the following sections 6.3.2.2 and 6.2.2.3), and as in previous studies (Origone *et al.*, 2002; Ars *et al.*, 2003; Castle *et al.*, 2003; De Luca *et al.*, 2004), it was not possible to correlate mutations with the rather limited information collected on disease features. This was not unexpected given the clinical variability of the disease, even in family members. As discussed in Section 1.1.5.1, owing to the enormous phenotypic variability manifest in NF1, it has been proposed that modifier genes (Easton *et al.*, 1993, Szudek *et al.*, 2000; Serra *et al.* 2001b; Weist *et al.*, 2003), as well as variable levels of mutated *NF1* transcript (Ars *et al.*, 2000) may account for the differences in clinical manifestation. Other theories include the importance of the location and type of the germline mutation on the *NF1* gene (Ars *et al.*, 2000) as well as other environmental and stochastic factors (Riccardi, 1993).

6.3.2.2/ Constitutional large *NF1* gene deletions may not be commonly associated with the development of MPNSTs

It has been reported that MPNSTs occurs at elevated incidence in patients with a large germline deletion (Dorshner *et al.*, 2000; De Raedt *et al.*, 2003). In the present panel, the germline mutation that was identified in seven of the nine patients harbouring an MPNST was a small insertion, deletion or nucleotide substitution. In the remaining two patients, germline heterozygosity for at least two *NF1* intragenic markers would suggest that no large deletion occurred in these samples. The deletion junction-specific also did not detect the most common 1.5 Mb deletion (López-Correa *et al.*, 2000; Dorshner *et al.*, 2000) in these samples. The

correlation between large deletions and MPNSTs is interesting not only because of the 3 embedded genes within the *NF1* gene, but also ~15 other genes of unknown function, are lost as a consequence of the most common 1.5 Mb deletion (De Raedt *et al.*, 2003). It has yet to be determined whether any of these genes influences the progression to malignancy. It may also represent a clinical signpost, since the lifetime risk of developing MPNSTs in these patients is estimated to be 16-26%, about twice that reported for NF1 patients (Evans *et al.*, 2002; De Raedt *et al.*, 2003). Of note, a number of patients harbouring large deletions involving the *NF1* gene did not exhibit the same distinctive phenotype (Rasmussen *et al.*, 1998; Upadhyaya *et al.*, 1998; Frahm *et al.*, 2004a; Mantripragada *et al.*, 2005), suggesting a role for additional events.

Little is yet known of the malignant progression in NF1 tumours, and it would be of interest to identify which factors intervene in the evolution of MPNSTs from both deletion and non-deletion patients.

6.3.2.3/ Comparison between germline mutational spectra in NF1 patients with and without gliomas

When compared, the germline mutations in patients with and without gliomas appeared to be slightly different, with a suggestion of clustering of pathogenic mutations in the 5' half of the gene (prior to exon 17) in those patients with optic gliomas.. When the mutations are divided in two arbitrary groups of "prior to exon 17" and "after exon 17", the distribution of mutations in NF1 patients with gliomas shows a statistical significance (Chi-square test, $\chi^2= 5.0111$, $p\leq0.05$, 1 degree of freedom).

The two patient panels are of relatively small size, and there have been few studies assessing germline mutations in patient with gliomas. An investigation on a larger scale would be required to determine whether the distribution observed here holds true. Strikingly, however, Ars *et al.* (2003) observed that most mutations in patients with optic gliomas were located in the first exons, and three were found in exon 7.

Of the sample panel investigated in section 6.2.4, patient clinical details were not always available. Only one patient in this panel (P168) is known to exhibit an optic pathway glioma, and the germline mutation identified in that patient was a truncating alteration located in exon 5, in keeping with the observations made on the glioma panel.. The mechanism underlying gliomagenesis in NF1 is still unclear. Loss of the *NF1* gene has been observed in NF1-related, but not sporadic gliomas (Jensen *et al.*, 1995; von Deimling *et al.*, 1993; Gutmann *et al.*,

2000; Kluwe *et al.*, 2001), and it has been suggested that astrocytes bear the two hits in the *NF1* gene (Bajenaru *et al.*, 2005). It would be of interest to determine whether *NF1* germline mutations are distributed differently in patients harbouring gliomas, and if this has a particular functional significance. A putative functional domain has previously been identified around exons 11-17, and the area was termed a hotspot for mutations (Fahsold *et al.*, 2000). However, approximately half of the mutations in this study and in the literature are located upstream of exon 11.

Finally, and in keeping with the observations by Ars *et al.* (2000, 2003), the majority (91%) of the pathogenic mutations in glioma patients are expected to truncate the protein. By contrast, only 57% of the germline mutations in the second panel are truncating.

6.3.3/ Somatic mutations

In this panel, LOH was identified in 14% of the samples. If analysed by tumour type, LOH accounts for only 8.5% of benign neurofibromas. The reported LOH in NF1 tumours varies widely between studies, ranging from 3% to 57% (Coleman *et al.*, 1995; Daschner *et al.*, 1997; Serra *et al.*, 1997; Kluwe *et al.*, 1999a, b; Eisenbarth *et al.*, 2000; John *et al.*, 2000; Rasmussen *et al.*, 2000; Serra *et al.*, 2001a; Upadhyaya *et al.*, 2004). LOH studies with a number of markers and tumours similar to that presented here report an LOH between 12-18% (John *et al.*, 2000; Upadhyaya *et al.*, 2004), somewhat higher than the present observation. This may be due to the heterogeneous cellular population in neurofibromas (Peltonen *et al.*, 1988), which represent the large majority (80%) of the present panel. A study by Serra *et al.* (2001a) demonstrated that enrichment of the Schwann cell population allowed for a better determination of LOH. Thus LOH may have been underestimated in these tumours.

Alternatively, and as discussed in this section, it may be of importance that more than half of the neurofibroma panel was obtained from three patients (three large series of 7, 10 and 16 tumours). If there is indeed a trend for tumours from the same patient to harbour similar types of mutations, and assuming that, in these three patients, microlesions are more widely represented (as seen by Weist *et al.*, 2003), this may reflect on the reduced LOH observed here.

Additionally, LOH in neurofibromas was generally confined to the *NF1* gene (and sometimes to one marker). Tumour T159 may be the only exception and the distal boundary of the deletion could not be determined, as markers were uninformative.

It is also interesting that 40% of the malignant tumours (4 MPNSTs and 1 MTC) exhibited LOH. This is in keeping with other reports, where LOH was found in up to 50% of MPNSTs (Frahm *et al.*, 2004a; Upadhyaya et al., 2004).

Even though the markers used did not allow determination of the boundaries of the deletions, it would appear that all four MPNSTs harbour deletions that extend up to and probably beyond the *NF1* gene. In tumours T145 and T165, an independent array-CGH assay confirmed the large deletions of 1.61-1.75Mb and over 2.2Mb, respectively (Mantripragada *et al.,* 2005). Tumour T151, from the same patient as T165, also appear to harbour a large LOH, from peri-centromeric marker to a breaking point before the marker in *NF1* intron 37. Tumour T169 would also appear to harbour a large deletion (estimated to be of 1.5 Mb by MLPA), although the boundaries could not be determined by LOH assay. None of the four deletion extended to the most telomeric marker. All four mutations extend beyond the *NF1*-REPS involved in the most common microdeletion (Dorschner *et al.*, 2000; Lopez-Correa *et al.*, 2000), and would belong to the large group of atypical large deletions previously reported (Kayes *et al.*, 1992, 1994; Wu *et al.*, 1995,1997; Cnossen *et al.*, 1997; Leppig *et al.*, 1997; Tonsgard *et al.*, 1997; Upadhyaya *et al.*, 1998; Dorschner *et al.*, 2000; Riva *et al.*, 2000; Jenne *et al.*, 2003; Kehrer-Sawazki *et al.*, 2003; Petek *et al.,* 2003; Kehrer-Sawazki *et al.*, 2004; Venturin *et al.*, 2004a, 2004b; Gervasini *et al.*, 2005; Kehrer-Sawazki *et al.*, 2005). Mitotic recombination (MR) has been suggested as an explanation for the large LOH identified in NF1 tumours (Serra *et al.*,2001b). The group conducted a LOH assay on a series of neurofibromas using markers spanning the whole of 17q and the *NF1* gene. Through culture and isolation of $NF1^{-/-}$ Schwann cells from these neurofibromas, and FISH analysis, the group demonstrated that the LOH observed resulted from mitotic recombination (MR) events between the centromere and *NF1*. As evidenced by the presence of both *NF1* alleles by FISH, a reduction to homozygosity, and not a deletion, accounted for the observed LOH. Furthermore, none of the 25 neurofibromas exhibiting LOH outside the *NF1* gene displayed LOH at the two centromeric markers.

As MR-mediated LOH has been evidenced in neurofibromas (Serra *et al.*, 2001b), and variable deletion size have been reported in NF1 tumours (Serra *et al.*, 1997, 2001b; John *et al.*, 2000; Upadhyaya *et al.*, 2004), the significance of the tumour type/deletion size correlation seen here should be reassessed on a larger MPNST panel.

A somatic microlesion was identified in 14 tumours, with 75% of novel mutations. The distribution and nature of the microlesions reported here appear to be similar to that of the germline spectrum. Indeed, three mutations identified in this panel have been reported as germline alterations, two of which occurred at hypermutable CpG dinucleotides. This is very much in keeping with what is known of *NF1* somatic mutations so far. A total of 38 somatic mutations have been reported and approximately 30% of these mutations have also been found in the germline of NF1 patients. Of the mutations that were found in both germline and somatic investigations, over 60% occur at CpG nucleotides. This would explain why some of these mutations have been reported in the germline of up to 18 patients.

In the present study, deletions were the single most represented type of mutation, and nucleotide substitution accounted for approximately 50% of all mutations. This distribution is close to that seen not only in the reported somatic, but also the germline spectrum (Sections 1.3.1 and 1.3.2). These results would suggest that the distribution of *NF1* somatic mutations parallels that of the germline mutations. Even in recent germline mutation studies, the proportion of novel mutations remains high (Ars *et al.*, 2003; Mattocks *et al.*, 2004, De Luca *et al.*, 2004), so the same would be expected of somatic mutation detection. The overlapping of the two mutational spectra seems to belie the possibility of a separate and distinct somatic micro-lesion spectrum. The somatic mutation detection rate remains however much lower than in the germline, and this may indicate the occurrence of yet-unidentified somatic mutational mechanism; The only noticeable difference, thus far, seem to be the involvement of CpG dinucleotides as they account for twice as many transitions in the somatic spectrum as compared to the germline one. The mechanism underlying this is unclear, but may implicate a more frequent occurrence of aberrant methylation (the cause of C:T transitions at CpG dinucleotide) in the soma than in the germline. Such event is likely to be gene- and tissue-specific.

These results also indicate that the mechanisms underlying the formation of a somatic mutation are often identical to those of a germline mutation. This is in line with recent observations made by Upadhyaya *et al.* (2004). In particular, the occurrence of substitutions at the same CpG dinucleotides in both germline and somatic spectra indicate that methylation-mediated deamination can occur in mitosis as well as meiosis.

The distribution of somatic mutations identified here did not appear to reveal any clustering, and were dispersed between exons 12b and 41. Although no mutations were identified before

exon 12b, the series number is small, and reported somatic mutations are distributed along the *NF1* gene.

There does not appear to be any correlation between the nature and location of the germline alteration and that of the somatic mutation in the same individual. As expected and as previously reported (Colman *et al.*, 1995; Serra *et al.*, 2001a, b; Wiest *et al.*, 2003; Upadhyaya *et al.*, 2004), different tumours from the same patient arise from independent somatic events. For example, in patient P141, the somatic mutation was identified in two tumours, one a missense mutation, the other a deletion. Similarly, LOH can be the second "hit" in one tumour whereas a missense mutation occurs in another, as seen in patient P140. Serra *et al.* (2001b) observed that tumours from the same patient had a tendency to harbour the same type of mutation. For example, in a patient in whom multiple neurofibromas were studied, the predominant mutation was found to be LOH resulting from mitotic recombination. Others reported that neurofibromas from a single patient harboured predominantly microlesions (Wiest *et al.*, 2003). This would suggest that the mechanism underlying or influencing the appearance of a somatic mutation in the *NF1* gene might be different between patients. For instance, factors influencing mismatch repair may lead to the appearance of point mutations, whereas other factors may drive recombination and subsequent LOH (Serra *et al.*, 2001a, b; Wiest *et al.*, 2003). It is difficult to identify a similar pattern in the present study, because the maximum number of somatic mutations identified in tumours from a single patient was three. However, in two patients, P141 and P176, 2/2 and 3/3 tumours (respectively) harboured microlesions. In the case of patient P176, all three somatic mutations were also nucleotide substitutions. In patient P151, two malignant tumours both appeared to harbour deletions encompassing all or most of the *NF1* gene. Other cases are more ambiguous, such as the two neurofibromas in patient P140, mentioned earlier, one with LOH and the other with a missense mutations.

Altogether, the somatic mutation detection rate in the present study reached 32%. While this is in accordance with the previous somatic studies using both mutation and LOH detection techniques (Serra *et al.*, 2001; Weist *et al.*, 2003; Upadhyaya *et al.*, 2004), the detection rate is still significantly lower than that observed in germline mutations screening. The use of DHPLC imparts sensitivity and practicality to the detection of microlesions. However, shortcomings from the combined mutation detection techniques may account for some of the low detection rate.

As a somatic hit was identified in more than half of malignant tumours, but in only a quarter of benign tumours, it is clear that the cellular heterogeneity of neurofibromas still represents a major obstacle to mutation detection. Evidently, a proportion of alterations could not be identified by the techniques used here, perhaps including multi-exon deletions.

Alternatively, other mechanisms may also be involved in somatic inactivation. Epigenetic inactivation by methylation, as seen in many tumour suppressor genes, has been the object of several investigations in the *NF1* gene , but does not seem to occur in NF1 tumours (Horan et *al.*, 2000; Luitjen *et al.*, 2000; Harder *et al.*, 2004; Fishbein *et al.*, 2005).

Elements regulating the *NF1* gene also remain to be investigated. Several potential transcription factor binding sites have been identified in the 5' upstream region (Lee and Friedman, 2005). A protein involved in signal transduction in various cell processes including cell-cycle progression, apoptosis and mitogenic signalling (Hermekin, 2003), 14-3-3 has been shown to negatively regulate the function of neurofibromin (Feng *et al.*, 2004). Therefore, alteration of the function of regulating factors and protein may also be involved in the loss of *NF1* gene function (This may also be the case in a proportion of patients with unidentified *NF1* germline mutations).

Loss of one allele of a tumour suppressor gene, and thus consequent reduction of the protein product quantity by 50%, is termed haploinsufficiency and is now thought to have a greater importance in tumour development (Quon and Berns, 2001). A recent review of genes thought to exhibit haploinsufficiency underscored the variety of genes (cell-cycle regulation, maintenance of genome integrity) and phenotypes that have been observed. It may be of particular interest, for example, that haploinsufficiency of certain genes (PTEN, Smad4, Lkb1) is associated with tumour onset, such as the formation of polyps, whereas LOH is required for carcinogenesis. Haploinsufficiency of one tumour suppressor gene may also have no effect on it own, but cooperate with other heterozygously mutated genes, and be pathway-specific. A mechanism of dominant-negative mutation, which results in a protein product that inhibits the function of the remaining wild-type allele, has been proposed for certain *TP53* mutations (Quon and Berns, 2001; Santarosa and Ashworth, 2004). Some evidence has been forthcoming regarding the importance of *NF1* haploinsufficiency. Perhaps the most striking is its involvement in models of NF1 tumour formation (neurofibroma, glioma), where one cellular type has been shown to harbour the complete inactivation of the gene (Schwann cell, astrocyte), but required the input of their multicellular, haploinsufficient micro-environment for tumour formation (Zhu *et al.*, 2002; Bajenaru *et al.*, 2005.). Cell types of prime importance in neurofibroma formation are thought be $Nf1^{+/-}$ mast cell and $Nf1^{+/-}$ Schwann

cells (Ingram *et al.*, 2000; McLaughlin and Jacks, 2002; Viskochil, 2003). Furthermore, the effect of *NF1* haploinsufficiency on cultured melanocytes have been investigated and the differences in cell phenotype were linked to reduced neurofibromin levels. The latter is thought to lead to a reduction of the signal-to-noise ratio in signalling pathways caused by increased noise ("noise" encapsulates the complex external and internal influences that occur in a cell; Kemkemer *et al.*, 2002). Thus haploinsufficiency of the *NF1* gene may influence different phenotypes in different cell types.

In conclusion, as previously remarked (Upadhyaya *et al.*, 2004), NF1 haploinsufficiency, through negative-dominant mutation or cooperation with non-allelic mutation, may also account for the lower somatic mutation detection rate.

6.3.4/ Conclusion

In the present study, the mutation detection rates for both germline and somatic mutations in NF1 tumours are in line with the most recent investigations. The use of DHPLC, a new mutation detection technique introduced during the time of this study, presents a rapid, sensitive and cost effective method for mutation detection, in a large gene such as *NF1*. A reliable detection system for single or multi-exon deletions has still to be developed for NF1 DNA samples. A quantitative real-time and multiplex PCR technique may provide an attractive option for detection of these deletions (Zhou *et al.*, 2003). Alternatively, the newly-designed *NF1* array-CGH may provide an insight in this type of deletion (Mantripragada *et al.*, 2005).

Despite the growing number of germline mutations identified, the percentage of novel mutations remains high. Thus a complete picture of the *NF1* germline spectrum is still required, as it may lead to future fast detection assays of recurrent mutations, of particular interest for rapid diagnosis. Furthermore, it would be useful to determine if certain regions of the gene or type of mutations are involved in particular NF1-associated traits. Interestingly, in the present study, the germline mutation of patients with gliomas appeared to be clustered in the 5' part of the gene. Similarly, no correlation between large germline deletions and MPNST occurrence was observed in this study.

Finally, the present study yielded additional data on the ever elusive *NF1* somatic mutational spectrum. Microlesions were identified in 14 tumours, and add consequently to the reported

38 somatic mutations. The detection rate however still remains low, compared to that seen in germline investigations, and this is probably attributed to the complex cellular heterogeneity of the tumours. However, other events, such as alterations in non-coding regions, haploinsufficiency, non-allelic mutations perhaps in genes involved in the pathway or alterations of factors controlling *NF1* expression, should also be fully examined.

CHAPTER 7: GENERAL DISCUSSION

7.1 Mutation detection

The search for mutations in the *NF1* gene has been hindered by the large size of the gene, the lack of mutational hotspots, and the presence of pseudogene sequences. The studies that have attempted to define the NF1 somatic mutational spectrum have also been faced with the added challenge of neurofibromas' heterogeneous cellular composition. Moreover, the diversity of *NF1* gene mutations means that mutation screening cannot be undertaken with one single mutation detection technique. Finally, and despite the bias inherent in the first published studies, the *NF1* mutations span the entire gene, implying that the screening of the gene in its entirety must be undertaken to identify any new and unknown mutation.

One of the aims of the present study has been to identify the somatic mutations in a panel of NF1 tumours, in order to gain a better understanding of the somatic mutational spectrum for which little information is as yet available. The germline mutations were also sought in the same samples in order to allow comparison between the two spectra. The screening techniques employed were chosen for their complementarity and proven efficiency. Firstly, a recently designed "microdeletion" PCR assay was used to determine the occurrence of the common 1.5Mb deletion encompassing the *NF1* gene (Lopez-Correa *et al.*, 2001), but did not yield any positive result. This result is not unexpected as a variety of other large *NF1* deletions have been described (Kayes *et al.*, 1992, 1994; Wu *et al.*, 1995;1997; Cnossen *et al.*, 1997; Leppig *et al.*, 1997; Tonsgard *et al.*, 1997; Upadhyaya *et al.*, 1998; Dorschner *et al.*, 2000; Riva *et al.*, 2000; Jenne *et al.*, 2003; Kehrer-Sawatzki *et al.*, 2003, 2004, 2005; Petek *et al.*, 2003; Venturin *et al.*, 2004a, 2004b; Gervasini *et al.*, 2005). A similar study identified the same 1.5Mb deletion in only 3% of the samples (Upadhyaya *et al.*, 2004).

Secondly, DHPLC was used for screening for micro-lesions in the coding sequence and immediately flanking intronic sequences. The advantages of this relatively new technique were its sensitivity, high throughput and semi-automation. The germline mutation detection rate obtained in this study (63%) was in line with similar studies in the *NF1* gene (66-68%, Han *et al.*, 2001; De Luca *et al.*, 2004), and remains one of the highest obtained for a single technique. Admittedly, the technique cannot identify either large DNA rearrangements or mutations located further into the intron (outside the reach or at the edge of the primers),

while mutations in the untranslated regions or alternative exons were not investigated. These may in part account for the remaining undiscovered mutations that we assume remain to be characterized. Adapted to somatic mutation detection, DHPLC screening yielded a detection rate of 18%, much lower than the rate typical for germline mutations. It is possible that, although it is more sensitive than previous techniques used to detect somatic mutations, DHPLC is still not yet sensitive enough to identify somatic mutations in Schwann cells. The latter are now accepted to be the key cells in NF1 tumours, and while Schwann cells are estimated to represent up to 80% of the neurofibroma (Peltonen *et al.*, 1988), two genetically different Schwann cell populations, $NF1^{+/-}$ and $NF1^{-/-}$, have also been identified in tumours (Rosenbaum *et al.*, 2000; Serra *et al.*, 2000). It may be that the *NF1* null cells are less represented than originally thought, as DHPLC has been estimated to detect as low an allele frequency as 5% (Xiao and Oefner, 2001), and still retrieves only 18% of the somatic mutations (21% of tumours without LOH). Of note, DHPLC has been reported to detect 21% of *TP53* somatic mutations in heterogeneous gastric carcinomas DNA (Lu *et al.*, 2003) and 27% of *PTEN* somatic mutations in gliomas (Liu *et al.*, 1998).

Alternatively, the generally low somatic mutation detection rate could be explained by other mutational mechanisms being responsible: e.g. epigenetic silencing, haploinsufficiency, mutations in gene regions not investigated here, or regulatory genes at unlinked loci (Section 6.3.3). There are, however, two observations in the present study that may implicate the cellular heterogeneity of neurofibromas as key to this issue: 4/4 somatic mutations were identified in patients harbouring a large deletion of the *NF1* gene (thereby effectively removing one copy of the gene), and 4/5 somatic mutations were identified in $NF1^{-/-}$ Schwann cells extracted from tumours. Schwann cell culture proved to be an efficient means to isolate, or at the very least to greatly enrich, $NF1^{-/-}$ Schwann cells from neurofibromas. Although it may be considered as a preliminary step to mutation detection or LOH assay, the technique has proved to be time-consuming and is therefore probably not suited for routine identification of somatic mutations.

The use of cDNA, either through DHPLC or direct sequencing, proved to be an asset in screening a large gene like *NF1*. Caution should be exercised when using RNA extracted from frozen tumours, since aberrant transcripts have been noted, in this study and by others (Ars *et al.*, 2000b, 2003; Wimmer *et al.*, 2000; Serra *et al.*, 2001a; Thomson and Wallace, 2002; Vandenbroucke *et al.*, 2001, 2002). In fresh samples ($NF1^{-/-}$ Schwann cells RNA), however,

direct sequencing of the cDNA proved an efficient technique to detect both germline and somatic mutations. The main drawback is that mutations located in the intron may not be detected.

Thirdly, an LOH assay using a panel of 13 microsatellite and RFLP markers was used to search for somatic deletions in NF1 tumours. Such assays, with different numbers of markers, have been used in the past, and have yielded widely diverging results demonstrating 3% to 57% LOH (Coleman *et al.*, 1995; Daschner *et al.*, 1997; Serra *et al.*, 1997; Kluwe *et al.*, 1999a,b; Eisenbarth *et al.*, 2000; John *et al.*, 2000; Rasmussen *et al.*, 2000; Serra *et al.*, 2001a,b; Upadhyaya *et al.*, 2004). In this study, the assay yielded a detection rate of 14%, in line with other reports (John *et al.*, 2000; Upadhyaya *et al.*, 2004). Although this was the most efficient and most used LOH assay at the time of the study, one major drawback of this methodology is the use of silver staining, which lacks sensitivity. Fluorescent detection of markers on a genomic sequencer, as used by others (Dietmaier *et al.*, 1999; Faulkner *et al.*, 2004; Cai *et al.*, 2004), would be recommended for future work, especially considering the cellular heterogeneity of neurofibromas. Since the latter represent the majority of the present panel, it is likely that the true frequency of LOH was slightly underestimated.

In conclusion, the present study made use of the most reliable and efficient techniques available that were available at the onset of the study. The germline mutation detection rate (63%) was one of the highest obtained for a single technique, while overall somatic detection rate (35%), although low by comparison, was in line with similar studies (Serra *et al.*, 2001a; Weist *et al.*, 2003; Upadhyaya *et al.*, 2004) and was considerably improved by the isolation of *NF1*$^{-/-}$ Schwann cells from the neurofibromas. Admittedly, one caveat of *NF1* mutation detection has been the lack of a technique designed to detect and characterize multi-exons deletions, thus these may be under-represented. The development of a multiplex PCR should be considered for future studies, as discussed in Section 7.5.1.

7.2 The NF1 mutational spectrum

As emphasized by the numerous studies addressing *NF1* mutations, the germline mutational spectrum is varied, both in the nature of the mutations and in its location on the *NF1* gene. With over 730 alterations now identified, the accretion rate of novel mutations is still substantial (55-74% (Ars *et al.*, 2003; Mattocks *et al.*, 2004; De Luca *et al.*, 2004), as is evident from this study (51%). Mutations resulting in a truncated protein remain high, around

70% (Shen *et al.*, 1996; 69% in the present study). Similar high percentages of truncating mutations have been reported in breast cancer gene *BRCA1* (70%, Szabo *et al.*, 2004) and adenomatous polyposis coli gene (*APC*, 95%; De Rosa *et al.*, 2003).

The two major types of mutations (nucleotide substitutions and frameshift mutations) appear to be almost equally represented. No mutational hotspot has been identified; however mutational "warm" spots in certain exons (4b, 7, 37) are starting to emerge, and approximately 25 recurrent mutations have been identified (approximately half of which are in a CpG dinucleotide). Certain mutational "warm" spots, such as nucleotides 6789-92 in exon 37 (Section 1.3.1.6), draw one's attention to the underlying nucleotide sequence, and a comprehensive approach to the sequence complexity and inherent patterns in the *NF1* gene may uncover additional regions with a propensity for mutation. These results are interesting in the context of future mutation detection studies, as they may help to prioritise the screening of the "most mutated" exons in what is a very large gene.

An interesting observation on the mutations identified was the capacity of some of these alterations to disrupt predicted splicing enhancer elements (ESEs). As recently shown by Cartegni *et al.* (2002), even nonsense mutations have been implicated in splicing, emphasizing the need to consider a mutation at both DNA and RNA levels. In the present study, in *silico* analysis was used to help determine the involvement of ESE disruption by *NF1* mutations. The results obtained from two different software programs can be contradictory, as previously noted (Zatkova *et al.*, 2004), and would require additional *in vitro* assays. Nevertheless, potential disruption of ESEs was observed following the appearance of silent, missense and nonsense mutations (Section 6.3.2.1). Of note, a silent mutation in exon 8 (C1137T) disrupted an ESE and was predicted to result in the reduction of affinity for protein SRp40 (Section 6.2.3.5). As this was the only non-polymorphic alteration in this patient, the disruption of the ESE may account for the pathogenicity of the mutation. ESE-disrupting mutations that cause pathogenic splicing abnormalities have been identified in the breast cancer genes *BRCA1* and *BRCA2* (Liu *et al.*, 2001; Fackenthal *et al.*, 2002), spinal muscular atrophy gene *SMN2* (Cartegni and Krainer, 2002) and MMR genes *MLH1* and *MSH2* (Gorlov *et al.*, 2003). ESE disruption may be more frequent than originally thought, with, for example, over 20% of *MSH2* and 16% of *MLH1* missense mutations reported to be ESE-related (Gorlov *et al.*, 2003).

As for missense and silent mutations, the pathogenicity of small in-frame deletions may be difficult to determine, and would require additional functional assay, or evaluation of the mutation in a familial (or population) context. Such studies could not be undertaken here. One in-frame deletion was however identified (4967delTCTATA); it removed two amino-acids just outside the GRD region and had been previously reported as pathogenic (Wu *et al.*, 1999).

Finally, a number of previously reported and novel polymorphisms, and putative rare variants have also been identified in more than half of the patients. Indeed several patients also harboured multiple polymorphisms. It is difficult to determine the impact of the presence of such sequence changes on the overall function of protein. The functional aspect of "normal" polymorphisms or variants has long been overlooked. A recent publication reflected on this relatively new research field, which was perhaps spurred on by the completion of the human genome project; Loktionov (2004) has stressed the importance of polymorphisms in the different stages of neoplastic growth and in functions such as cell proliferation, differentiation, angiogenesis and death. Examples include: two common SNPs (single nucleotide polymorphisms) in the 3' UTR of the *CDKN2A* gene, where both variants [(500)G and (540)T] have been associated with poorer prognosis in bladder cancer and metastatic malignant melanoma, respectively; a polymorphism at codon 72 of the *TP53* gene giving rise to two variants, Arg72 and Pro72, the latter being markedly less proficient at inducting apoptosis and associated with a poorer prognosis in cancer (Loktionov, 2004). Variants of genes involved in genome integrity have also been reported: the V2 variant (Trp65Cys) of the *MGMT* was found unstable in cell culture and resulted in reduced cell survival (Inoue *et al.*, 2000); some MLH1 variants were found to have an influence on acute lymphoblastic leukaemia when combine to other genes' variants (Mathonnet *et al.*, 2003); polymorphisms –448A and –159T in the promoter of another mismatch repair gene, MSH6, are thought to modify a Sp1 transcription factor binding site, thereby leaving the promoter open for hypermethylation, and subsequent silencing (Gazzoli and Kolodner, 2003). Gene polymorphisms are also significant with regard to therapy and pharmacogenomics; therapeutic drugs are targeted at specific biologic mechanisms, and the presence of gene variants in the underlying pathway, in a particular patient, may affect the drug's toxicity or response (Loktionov, 2004).

Generally, the *NF1* somatic micro-lesion spectrum (e.g. excluding deletion of more than a few basepairs identified by LOH), with 56 alterations so far identified (including 18 from this study), is beginning to resemble the germline spectrum; the diversity in mutation type and position on the gene is found in both, and a number of mutations (25%) are common to both spectra. Of note, CpG dinucleotides underlie 40% of somatic transitions, twice the proportion seen in germline mutations. Deletions (several exons to the whole *NF1* gene) are also prominent in the somatic spectrum, with LOH ranging from 12% to 25% in the most documented studies (Serra *et al.*, 1997; John *et al.*, 2000; Upadhyaya *et al.*, 2004). By contrast, germline large gene deletions (*NF1* locus and adjacent loci) are estimated to occur at a frequency of approximately 5-10% (Cnossen *et al.*, 1997; Lopez-Correa *et al.*, 2001; De Raedt *et al.*, 2004; Kluwe *et al.*, 2004).

In the 21 tumour samples studied here in which both germline and somatic mutations were identified, the two did not appear to be correlated, in either their type or position on the gene. Non-random "first hit-second hit" association has been reported in familial adenomatous polyposis (FAP) gene APC, where the occurrence of a germline mutation near codon 1300 is correlated with a somatic LOH (Crabtree *et al.*, 2003). It may be that modifier genes are involved in the determination of the type (LOH or micro-lesion) of the second hit, as previously postulated (Easton *et al.*, 1993; Szudek *et al.*, 2000; Serra *et al.*, 2001b; Wiest *et al.*, 2003). It was difficult to confirm a "trend" for LOH or micro-lesions in tumours from the same patient (Serra *et al.*, 2001b; Wiest *et al.*, 2003), due to the reduced number of somatic mutations identified; in three patients, this "trend" remains a possibility (Section 6.3.3).

7.3 Genotype and phenotype relationship

The wide range of phenotypic expression, even between affected family members, is characteristic of NF1. A number of studies have addressed the genotype-phenotype relationship, but no obvious correlation has been identified (Easton *et al.*, 1993; Castle *et al.*, 2003; Szudek *et al.*, 2003). Trends have been described but remain to be fully validated (Ars *et al.*, 2003). A "microdeletion syndrome" has been used to label the severe phenotype (excessive burden of neurofibromas, predisposition to MPNSTs) exhibited by a subgroup of patients harbouring large deletions of the *NF1* gene and surrounding sequences (Kayes *et al.*, 1994; Wu *et al.*, 1995; Cnosson *et al.*, 1997; Leppig *et al.*, 1997; Tonsgard *et al.*, 1997; Upadhyaya *et al.*, 1997, 1998; De Raedt *et al.*, 2003; Section 1.3.1.3). However, not all

patients with such a deletion demonstrate these features (Rasmussen *et al.*, 1998; Upadhyaya *et al.*, 1998; Mantripragada *et al.*, 2005).

In the present study, observations on the genotype-phenotype relationship were hindered by the shortage of patient information available. Two interesting results were however noted. Firstly, the "microdeletion syndrome" did not apply to any of the patients harbouring MPNSTs. A germline mutation was identified in seven of these patients, and all were micro-lesions (Section 6.3.2.1). Secondly, the mutation screen of a small panel of patients with gliomas, a feature found in 25% of NF1 patients (Listernick *et al.*, 1999), revealed two trends: a statistically significant distribution of the mutations at the 5' end of the coding region (before exon 17), and a tendency for the great majority (91%) of mutations to result in a truncated transcript (Section 6.3.2.2). These observations were in line with a previous report by Ars *et al.* (2003), but they do need to be retested in a larger panel of gliomas..

Despite these "trends", it is likely that as yet unidentified modifier genes are responsible for the observed diversity of NF1 phenotypic features (Easton *et al.*, 1993; Castle *et al.*, 2003; Szudek *et al.*, 2003). It has been proposed, for example, that MMR genes variants (with decreased activity) could be responsible for the frequent occurrence of somatic micro-lesions in a patient, thereby explaining the patient's excessive neurofibroma burden (Wiest *et al.*, 2003). This is interesting, in the light of the putative association between the *NF1* gene and MMR function, as it would appear that disruption of the latter targets the former for mutations. Mouse models (Gutmann *et al.*, 2003) and numerous case reports of MMR gene-deficient patients exhibiting NF1 features (Section 1.6.3; Ricciardone *et al.*, 1999; Wang *et al.*, 1999; Trimbath *et al.*, 2001; Vilkki *et al.*, 2001; Whiteside *et al.*, 2002; Bougeard *et al.*, 2003; Hedge *et al.*, 2003; De Vos *et al.*, 2004; Gallinger *et al.*, 2004; Menko *et al.*, 2004) support this theory.

In this study, the assessment of MMR gene involvement in NF1 tumours was limited to a one-marker LOH assay of *MLH1*, and promoter hypermethylation analysis of *MLH1* and *MSH2*. Hypermethylation of the two MMR genes was scarcely observed, suggesting that these genes are not often inactivated in this manner in NF1 tumours. Hypermethylation of *MLH1* was however observed in a rare tumour, a medullary thyroid carcinoma (MTC), in conjunction with LOH. This is consistent with the possibility of *MLH1* inactivation in that tumour, and raises two issues: is *MLH1* inactivation responsible for the occurrence of this rare

tumour in the context of NF1? Could *MLH1* inactivation be implicated in the loss of NF1 function in that tumour? Without additional information on the patient phenotype, and further investigation, it may difficult to answer these questions. Of note, *MLH1* expression appeared to be reduced in a RT-PCR assay (Section 3.2.2.4), which would correlate with the inactivating action of combined LOH/promoter hypermethylation.

LOH of the *MLH1* gene was also seen in 6 malignant tumours, but not neurofibromas, which may also indicate a role for the gene in malignant progression. One of these tumours also appeared to show reduced *MHL1* expression by RT-PCR (Section 3.2.2.4). No hypermethylation was found in the *MLH2* gene in this study, and has rarely been seen in cancer (Section 1.5.3.3.1).

The relationship between the *NF1* gene and MMR genes is complex and intriguing. No mutations have been reported in *MLH1* or MSH2 in the germlines of *de novo* NF1 patients (Wang *et al.*, 2003). This does not negate the possible occurrence of other inactivating mechanisms, or the involvement of other MMR genes. As the case report of MMR-deficient patients would suggest, the loss of MMR function, rather than any particular gene, may target the *NF1* gene (Whiteside *et al.*, 2002). Additionally, MMR genes function through the formation of heterodimers (Charames and Bapat, 2003), and the loss of 50% of one protein (e.g. haploinsufficiency) may also have consequences in a cell, as seen in mouse models (Takagi *et al.*, 2003). It is unclear whether MMR genes would have a role as modifiers, influencing the appearance of *NF1* germline and/or somatic mutations, or as mutators allowing the appearance of additional mutations in tumour suppressor genes and oncogenes in the progression to malignancy. The present study is the first report of *MLH1* LOH and promoter hypermethylation in NF1 tumours, and adds to the growing body of evidence for MMR gene involvement in NF1.

7.4 Progression to malignancy

Malignant peripheral nerve sheath tumours (MPNSTs) occur in NF1 patients with a lifetime risk of 8-13% (Evans *et al.*, 2002; Ferner and Gutmann, 2002), generally arise from plexiform neurofibromas and are some of the most aggressive tumours (King *et al*, 2000; Ferner and Gutmann, 2002). The occurrence of a somatic hit in the *NF1* gene in Schwann cells, combined with a heterozygous $NF1^{+/-}$ microenvironment, is necessary and sufficient for benign tumour formation (Zhu *et al.*, 2002). However, additional alterations in cancer-related

genes are likely to be required for progression to malignancy, but little is known of this process to date. A number of studies have addressed the issue. Cytogenetic studies (FISH and CGH) have assessed the chromosomal alterations in MPNSTs, thereby highlighting losses (potential tumour suppressor genes) and gains (potential oncogenes); the alterations were numerous and could affect virtually any chromosome (Jhanwar *et al.*, 1994; Mertens *et al.*, 1995, 2000; Lothe *et al.*, 1996; Mechtersheimer *et al.*, 1999; Schmidt *et al.*, 2000; Wallace *et al.*, 2000; Koga *et al.*, 2002; Perry *et al.*, 2002; Frank *et al.*, 2003; Bridge *et al.*, 2004; Frahm *et al.*, 2004a). Other studies focused upon a particular gene or pathway, using genetic and epigenetic mutation detection and expression assays. A number of genes and proteins have thus been found to be altered in MPNSTs, the most documented being *CDKN2A/p16^{INK4a}* (Section 1.3.4.2.1), *TP53* (Section 1.3.4.1) and *EGFR* (Section 1.3.4.3).

The present study investigated a number of candidate genes by screening for LOH and by carrying out methylation-specific PCR assays. Additionally, the presence of microsatellite instability (MSI) in NF1 tumours was also explored.

7.4.1/ Candidate genes

The present study addressed the involvement of 9 genes in NF1 tumours: *CDKN2A/p16^{INK4a}*, *RB1*, *TP53*, and *MGMT* have previously been investigated in NF1 tumours, whereas the results for *MLH1*, *MSH2*, *RARB*, *NORE1A* and *RASSF1A* are reported here for the first time. Additionally, this is the second report of the use of a MS-PCR assay to assess promoter hypermethylation of candidate genes in NF1 tumours (Gonzalez-Gomez *et al.*, 2003a). MS-PCR was found to be a rapid technique for the global assessment of the hypermethylation status of genes in NF1 tumours. However, the technique also lacked sensitivity, especially in the context of the neurofibromas' cellular heterogeneity. The present results should therefore be considered as foundations for future investigations, and techniques allowing quantification of hypermethylation are recommended. The use of a real-time PCR assay permitted additional insights into the role of the genes and their expression level, although it should be noted that the very nature of NF1 tumours makes it difficult to find an adequate control sample for the expression assays. Normal peripheral nerve sheath could be used, but obtaining biopsies from healthy individuals is always going to be problematic.

It should also be mentioned that the finding of hypermethylation in the genes raises certain issues. Firstly, the "normal" methylation status of the cell in the particular tissue should be

considered. For example the *14-3-3σ* gene, found hypermethylated in a number of cancers (discussed in 7.5.2), was found to be commonly methylated in normal and malignant lymphoid cells, an event that did not completely silence the basal expression of 14-3-3σ in these cells (Bhatia *et al.*, 2003). In the present study, malignant tumours were assessed against benign tumours, neither of which can be considered as "normal". However, as mentioned for the real-time PCR assay, "normal" tissue can be hard to obtain. Secondly, the MS-PCR assay is limited to the region bound by the primers, and may or may not reflect the overall status of the whole CpG island, or even guarantee that the hypermethylation observed has a functional consequence. In this study, the primer sets had previously been successfully used by others, in conjunction with an expression assay, and hypermethylation in the region bound by the primer had been correlated with functional inactivation. Thirdly, the question remains as to whether the hypermethylation observed is causal or consequential to MPNST formation. As proven by cytogenetic assays, MPNSTs harbour numerous aberrations; if some of these disrupt genes involved in the control of genomic methylation, hypermethylation could be frequently encountered in the tumour. For example, simultaneous inactivation of maintenance methylase DNMT1 and *de novo* methylase DNMT3b (Section 1.5.1) by homologous recombination have shown that these enzymes are responsible for gene silencing, including tumour suppressors (Rhee *et al.*, 2002; Bachman *et al.*, 2003). Alternatively, inactivation by hypermethylation of genes involved in genome maintenance (e.g. *MLH1, MGMT*) may trigger the appearance of mutations in tumour suppressor genes or oncogenes (e.g. *BAX, K-Ras*, respectively) and drive malignant progression (Esteller, 2000). Hypermethylation may also directly inactivate a gene (e.g. tumour suppressor), allowing the cell to acquire key functions, as described by Hanahan and Weinberg (2000). This may be the case for *RASSF1A* in NF1 MPNSTs, because inactivation of this gene could allow the avoidance of apoptosis.

Only recently identified, *RASSF1A* is quickly becoming recognised as a key gene in human cancers and is almost exclusively inactivated by promoter hypermethylation (Agathanggelou *et al.*, 2005). A Ras effector, RASSF1A has been implicated in the Ras-mediated pathway to apoptosis; its many functions are not completely understood, and the protein has also been implicated in microtubule stability and cell cycle regulation (Agathanggelou *et al.*, 2005). Interestingly, the *RASSF1A* promoter was found to be hypermethylated in more than half of the MPNSTs in the present study. This suggests that *RASSF1A* inactivation may not participate in NF1 MPNST formation, but may be a preferential means to acquire additional selective capacities in the malignant cells. Epigenetic inactivation of *RASSF1A* is also thought

to contribute to tumour aggressiveness, as seen in neuroblastomas (Yang *et al.*, 2004), and this may also be the case in NF1 MPNSTs. Importantly, this is the first report of *RASSF1A* involvement in NF1 tumours. A second Ras effector in the apoptotic pathway, *NORE1A,* did not appear to be inactivated in NF1 tumours.

The *CDKN2A* locus is a cornerstone in cancer as it encodes two tumour suppressors, $p16^{INK4a}$ and $p14^{ARF}$, effectively linking two major pathways, the Rb and p53 pathways respectively (Quelle *et al.*, 1995). Deletion of the *CDKN2A* locus, or 9p where it resides, is frequently seen in cancer. *CDKN2A/p16^{INK4a}* is found to be disrupted by promoter hypermethylation and mutations with a frequency that is second only to *TP53* (the most frequently inactivated gene in human cancer). *CDKN2A/p16^{INK4a}* is also found to be frequently altered in NF1 MPNSTs, but not neurofibromas (Lothe *et al.*, 1996; Berner *et al.*, 1999; Kourea *et al.*, 1999a; Nielsen *et al.*, 1999; Mertens *et al.*, 2000; Schmidt *et al.*, 2000; Birindelli *et al.*, 2001; Perry *et al.*, 2002; Perrone *et al.*, 2003; Zhou *et al.*, 2003; Bridge *et al.*, 2004; Frahm *et al.*, 2004a; Ågesen *et al.*, 2005). In accordance with these reports, the present study identified LOH at *CDKN2A*, using four microsatellite markers on 9p, in 12.5% of MPNSTs. Surprisingly, and in contrast to observations by Gonzalez-Gomez *et al.* (2003), hypermethylation of the *CDKN2A/p16^{INK4a}* promoter was not observed, although it may that the sensitivity of the detection technique may be at fault. The advantage for the tumorigenic cell of deletion over epigenetic inactivation is that the former can inactivate both two tumour suppressors at the *CDKN2A* locus, as well as tumour suppressor $p15^{INK4b}$ at the adjacent *CDKN2B* locus. Therefore, in these tumours, both the p53 and Rb pathways may be altered.

The *RB1* promoter was found to be rarely hypermethylated in 7% of NF1 tumours, both benign and malignant, confirming results obtained by Gonzalez-Gomez *et al.* (2003a). Because dermal neurofibromas do not evolve to MPNST, it would suggest that either *RB1* is disrupted early in NF1 tumour formation, or that hypermethylation of *RB1* may not completely inactivate the gene, allowing for residual activity. It may also be that *CDKN2A/p16^{INK4a}* and not *RB1* mutation is involved in NF1 tumours, or that mutation in the two genes may be mutually exclusive since they belong to the same pathway. It has also been suggested that RB1 contributes to cell-cycle regulation only under certain conditions (for example when cells differentiate or become senescent), and its actual role in different physiological settings remains to be determined (Sherr and McCormick, 2002).

The DNA repair gene *MGMT* was also found to be commonly hypermethylated, in almost half of NF1 tumours. Again, the hypermethylation status was not correlated to malignancy, suggesting an early involvement of the gene in NF1 benign tumours, but not in malignant progression. *MGMT* may be indirectly involved, as it has been correlated with the appearance of mutations in other genes (*K-ras*, *TP53*; Esteller *et al.*, 2000b; Esteller *et al.*, 2001c; Nakamura *et al.*, 2001; Park *et al.*, 2001; Whitehall *et al.*, 2001). Interestingly, LOH at *MLH1*, discussed in Section 7.3, was found in 3 MPNSTs that also harboured *MGMT* hypermethylation and microsatellite instability. It has been suggested that *MGMT* might be the first line of defence in DNA repair, and its loss may overload the pathway. Thus, while insufficient to bring about the onset of NF1 malignancy, *MGMT* loss may create a permissive background, and additional loss on *MLH1* in a subset of MPNSTs may accelerate genomic defects.

Additionally, *TP53* showed LOH in 12.5% of MPNSTs; alterations of p53 have been reported in up to 100% of MPNSTs (Section 1.3.4.1), so this result was not unexpected. Loss of p53 confers two advantages to the cell, as p53 mediates both an apoptotic pathway, and DNA damage- induced cell cycle arrest (Levine, 1997). Additionally, an interesting theory pertaining to *TP53* loss of expression and hypermethylation is worth mentioning here: Although the mechanism underlying the process of tumour suppressor gene hypermethylation is not completely understood, it has been proposed that loss of transcription factor (e.g. p53) binding from certain promoter regions (e.g. *14-3-3σ*) may leave the CpG residues "open" to hypermethylation (Hermeking, 2003).

Hypermethylation of *RARB* in the only rhabdomysarcoma (RMS) studied was interesting. RMS are soft tissue sarcomas (STS) not exclusively found in NF1, although they do occur with increased incidence in NF1 patients (Yang *et al.*, 1995). Retinoids have previously been implicated in inhibition of STS cell lines proliferation (Gabbert *et al.*, 1988; Crouch and Helman, 1991; Brodowicz *et al.*, 1999), and although hypermethylation of *RARB* has not been found in sporadic RMS (Harada *et al.*, 2002), it may be that a) the retinoic pathway is involved in NF1 RMS and b) inactivation of *RARB* by hypermethylation is specific to NF1 RMS. This is important for its implication in clinical treatment of this rare type of tumour arising in NF1 patients, because hypermethylation is a reversible mechanism, as discussed in Section 7.5.3.

Finally, MPNSTs could be distinguished from benign tumours by the presence of alterations in several genes. One example is MPNST T145, which harboured LOH at *CDKN2A* and *MLH1*, and demonstrated hypermethylation of *RASSF1A* and *MGMT*. The tumour had thus potentially acquired the capacity to evade apoptosis (*RASSF1A*, *CDKN2A/p14^{ARF}*), show limitless replicative potential (*CDKN2A/p16^{INK4a}*), metastasis (*RASSF1A*) and a mutator phenotype (*MGMT/MLH1*).

In conclusion, the present study of candidate genes has uncovered two new genes potentially involved in NF1 malignancies (*RASSF1A* in MPNSTs, and *RARB* in RMS) and identified for the first time a role for *MLH1* in NF1 MPNSTs, which adds to the previous reports of an MMR gene /NF1 relationship. Results on *RB1* and *MGMT* hypermethylation in NF1 benign and malignant tumours also echo those of Gonzalez-Gomez *et al.* (2003), suggesting an early role for these genes in NF1 tumorigenesis. Some MPNSTs were found to harbour disruptions in several genes, illustrating the multistep nature of NF1 malignant progression.

7.4.2/ Microsatellite instability

Microsatellite instability (MSI), found in a variety of human cancer, is a mark of genomic instability. This is also particularly important when repeat sequences are located in coding regions, as it then implies the disruption of these genes. Reports of MSI in NF1 tumours have been few and contradictory, especially with regard to instability in benign tumours (Ottini *et al.*, 1995; Serra *et al.*, 1997; Luijten *et al.*, 2000b). Further, as with other MSI studies in cancer, the differences in panel size and markers hinder the possibility of a direct comparison (Arzimanoglou *et al.*, 1998). The present study identified the statistically significant occurrence of MSI in MPNSTs compared to neurofibromas; approximately half of the malignant tumours showed instability. This also suggests that the presence of MSI is not an obligatory step to malignant progression, but may occur later in the tumour.

The presence of MSI also refers back to the possible involvement of MMR genes, which are responsible for the phenomenon in colorectal and other cancer (Thibodeau *et al.*, 1993; Aaltonen *et al.*, 1993; Peltomaki *et al.*, 1993; Arzimanoglou *et al.*, 1998). Certainly, the occurrence of *MLH1* LOH in tumours harbouring MSI is intriguing (Section 4.3.3.1). Additionally, one neurofibroma with both NF1-intragenic and extragenic instability may have harboured LOH at a marker closely linked to *MSH2* (Section 4.3.3.1). The *NF1* gene and

MMR relationship have been discussed in Section 7.3, and MSI may be yet another facet of its expression.

The MSI detection technique used in this study (large gel/silver staining) would certainly benefit from the use of a fluorescent marker approach, and the limited sensitivity of this methodology may have led to a slight underestimation of instability in neurofibromas. Admittedly, the appearance of additional bands is more easily visible than LOH, although the cellular heterogeneity of neurofibromas may bias the description. For example, compression may be seen as an extra band (wild-type, compression and residual allele from unaffected cell population). NF1 MSI studies would also probably benefit from the use of a standard panel and guidelines, as done for HNPCC (Boland *et al.*, 1998). The latter however had a wealth of studies with diverse markers to draw from, and similar resources are not available for NF1. In the present study, one tetra-penta-nucleotide marker (MYCL) and two dinucleotide markers (D18S58 and APC) were found to be most informative. Dietmaier *et al.* (1997) also reported other dinucleotide markers to be informative in NF1 tumours and mono- and di-nucleotide sequences present throughout the *NF1* gene could be used as markers (Inagaki et al, 2005). Diversity in the type of repeat and chromosomal location was recommended in the HNPCC guideline (Boland *et al.*, 1998). Bearing this in mind, some of the markers in the present study may be a good starting point for further investigations.

7.5 Future directions

7.5.1/ Mutation detection and functional assay

The use of DHPLC and, more recently, ACSA (Section 1.7.4) has shown that a single technique is capable of detecting most of the germline micro-lesion in the *NF1* gene. However, multi-exons and large gene deletion remains a challenge. The recently devised NF1 array-CGH (Mantripragada *et al.*, 2005) may be the answer to this problem. This genomic array spans a region of 2.24Mb on 17q11, with closer-spaced detection points on the *NF1* gene, allowing the detection of deletions even in single exon. The use of DNA as a template can be an advantage, as this is the most accessible material (especially for tumours). The array format may not however be amenable to being easily set-up in the lab. In HNPCC, where approximately 10% of patients harbour a large deletion, both semi-quantitative multiplex PCR and multiplex ligation-dependent probe amplification (MLPA)are being routinely used.. Because these techniques are PCR-based and only require fluorescence (for MLPA), and the

use of a sequencer, they may be easier to implement in a routine screening laboratory. Of note, *MLH1* and *MSH2* are usually analysed together, totalling 35 exons (Wehner *et al.*, 2005). The techniques can still be adapted to the 60 *NF1* exons, although marginally more PCRs would be required. MLPA has also been used to confirm the array-CGH results in NF1 samples (Mantripragada *et al.*, 2005).

Detection of somatic *NF1* mutations is much more problematic, and still remains to be resolved.. The present study showed that culture of the $NF1^{-/-}$ Schwann cell population prior to mutation screening greatly improved the detection rate. Similar results were also observed in previous LOH assays by Serra *et al.* (2001a). Thus, unless tested on a large panel, it will be difficult to determine whether other mechanisms (for example, haploinsufficiency, epigenetic mechanisms) can explain the lack of somatic mutation, or if the mutation detection assays are simply not sensitive enough. Admittedly, Schwann cell culture is a time-consuming process; it may be sufficient to simply enrich the Schwann cell population, for mutation detection purposes. This would still require approximately two weeks of work per sample, with the pre-culture, dissociation and three-medium phase steps (Section 2.8). Alternatively, other labs are currently using laser micro-dissection (LMD) to isolate Schwann cells. LMD has successfully been used in colorectal carcinoma tumours to dissect carcinoma cells prior to mutation screening, allowing the first report of *EGFR* somatic mutation in 12% of the tumours (Nagahara *et al.*, 2005).

Once *NF1* mutations have been identified, their functional effect remains to be determined. This has been so far greatly overlooked, and few NF1 functional assays exist, centred around the GRD (Li *et al.*, 1992; Gutmann *et al.*, 1993; Poullet *et al.*, 1994; Upadhyaya *et al.*, 1997). Despite the location bias of many earlier studies, the *NF1*-GRD does not harbour more mutations than the rest of the gene (Section 1.3.1.6). The large size of the *NF1* gene presents an obvious problem to the design of functional assays, but the problem may be circumvented by using *NF1* cDNA, effectively reducing the size from 350kb to 12.5kb (Shen *et al.*, 1996). A vector incorporating *NF1* cDNA, subjected to site-directed mutagenesis, could be used in conjunction to the phosphate-release method (using H-ras as substrate) previously used to determine the functional effect of mutations located in the *NF1*-GRD (Upadhyaya *et al.*, 1997).

The mRNA splicing machinery is extremely complex and precise, able to recognize the correct splice site prior to the cut-and-paste reaction (removal of intron and joining of exons). This is due not only to the short consensus degenerate splice site sequences, but also to the presence of exonic and intronic splicing enhancers (ESEs and ISEs) and silencers (ESS and ISS). Thus exon recognition relies on a sum of interacting signals, likely to vary for each exon (Faustino and Cooper, 2003). These observations add a new dimension to exonic and intronic alterations whose pathogenicity may have previously been unclear.

As mentioned in Section 7.2, it is becoming increasingly important to consider the effect of the mutation at the RNA level. The disruption of ESE is one example of a situation in which even a nonsense mutation may not simply be truncating, as demonstrated in *NF1* mutations (Zatkova *et al.*, 2004). Although *in silico* approaches now exist, the complexity of the mechanism is better served by *in vitro* assays. Minigene assays have successfully been used to demonstrate exon skipping and reduction of splicing enhancement (Zatkova *et al.*, 2004), and should be considered for a better understanding of the mutation and its functional consequence. For example, a nonsense mutation may generate a truncated transcript, but, if disrupting an ESE, the mutation may in fact lead to the skipping of the mutated exon, and, potentially, to a shorter protein instead.

7.5.2/ Modifier genes

The advances in technology offer two approaches to detect genes up- and down- regulated in NF1 tumours: microarray assays and real-time PCR assay. A microarray is a collection of known probes (up to tens of thousands of cDNA fragments or synthetic oligonucleotides) organised on a solid support. After hybridisation with a labelled target (for example, tumour mRNA), the signal intensity obtained from each probe should correlate with the abundance of the mRNA complementary to that probe (Brentani *et al.*, 2005). As a large number of probes can be used, a microarray generates a high output of results, and analysis relies on pattern recognition software. Microarray would thus give an overview of altered gene expression between normal and pathological samples. The technology has been widely used in cancer for candidate gene search and as a diagnostic/prognosis tool (Brentani *et al.*, 2005). A number of studies have now used microarray for expression profiling in human $NF1^{+/-}$ astrocytomas (Gutmann *et al.*, 2001, 2002), $NF1^{+/-}$ melanocytes (Boucneau *et al.*, 2005), mouse $Nf1^{-/-}$ Schwann cells (Miller *et al.*, 2003), NF1 dermal and plexiform neurofibromas, NF1 MPNSTs

and MPNSTs cell cultures, sporadic MPNSTs (Holtkamp *et al.*, 2004; Watson *et al.*, 2004) and NF1 blood samples (Tang *et al.*, 2004). As would be expected, a plethora of genes were found to be up- or down- regulated in NF1 tissues as compared to controls. Of note, genes involved in tissue remodelling, bone development and tumour suppression were down-regulated in NF1 blood samples as compared to normal blood samples (Tang *et al.*, 2004). Gene expression was most affected by genotype (rather than lesion type) in $NF1^{+/-}$ melanocytes compared to $NF1^{+/+}$ melanocytes (both in normal skin and hyper-pigmented café-au-lait spot skin; Boucneau *et al.*, 2005); *NF1* heterozygosity was found to affect cell attachment, spreading and motility in astrocytes (Gutmann *et al.*, 2001, 2002) A protein (brain lipid binding protein, BLBP) was identified in Schwann cell – axon interaction (Miller *et al.*, 2003). Thus the microarray approach generates a wealth of information that often guides additional in-depth studies.

By contrast, a quantative real-time PCR approach can be used to investigate a smaller number of genes. Real-time PCR is quickly becoming an alternative to microarray technology, as it is fast, sensitive and requires minimal handling of the samples. Real-time PCR may be more easily implemented in the lab, once the initial cost of the equipment has been borne. Larger studies can also be undertaken and Lévy *et al.* (2004) conducted on such real-time PCR study to determine the expression profiles of 489 genes in NF1 tumours. The group identified 28 genes that were up- or down- regulated in NF1 MPNSTs compared to plexiform neurofibromas, including genes involved in cell proliferation, apoptosis and in the Ras or Hedgehog pathways (Lévy *et al.*, 2004)

In both approaches, the use of an adequate control for NF1 tumours should be given particular thought. Additionally, tumour-derived $NF1^{-/-}$ Schwann cell lines and normal Schwann cell lines could be used to circumvent both issues of suitable control and cellular heterogeneity in tumours. However, the use of cell lines is debatable in that cells are taken out of their biological context and may have acquired genomic changes in culture. Alternatively, laser capture microdissection of NF1 tumours may allow one to select one tumour type, without resorting to culture, and low quantities of mRNA can be amplified prior to analysis (Feldman *et al.*, 2002; Gomes *et al.*, 2003)

To identify proteins that physically interact with neurofibromin, Feng *et al.* (2003) have immobilized a fusion protein of the NF1-CTD (C-terminal domain) on a column, then applied

a rat brain cytosolic fraction to the column. Through this method, the group described the new interaction of 14-3-3 proteins and neurofibromin. The 14-3-3 proteins bind their ligand on a consensus serine-threonine motif, one of which is present on the NF1-CTD (amino acids 2573-2580, RKVSVSES). A series of experiments (with a 14-3-3η fusion protein) have demonstrated that both phosphorylation of all PKA (cAMP-dependent protein kinase) on NF1 and 14-3-3 binding on NF1-CTD were required for neurofibromin/14-3-3 association. The interaction resulted in negative regulation of the NF1 GAP function (Feng *et al.*, 2004). The knowledge of this interaction adds functional consequences to the presence of mutations on the phosphorylation / binding site. Although it was shown that all needed to be mutated, in *vitro*, to abrogate the interaction, it remains to be determined how one mutation may modulate the interaction. Mutations that truncate the protein before the CTD would also abolish the interaction. There are seven human genes encoding the highly conserved 14-3-3 isoforms β, γ, ε, η, σ, θ and ξ, most of which are expressed in all tissues. The different isoforms are not functionally redundant, and although their mechanisms are not fully understood, the proteins are involved in many cellular processes, including apoptosis, cell cycle regulation and mitogenic signalling (Hermeking, 2003). The 14-3-3σ protein is perhaps the most documented for its involvement in cancer. Firstly, 14-3-3 σ is directly regulated by p53 following DNA damage and required for a stable G2-cell-cycle arrest (Hermeking *et al.*, 1997). Secondly, 14-3-3 σ has been found to be inactivated by promoter hypermethylation in a variety of cancers, including breast (Ferguson *et al.*, 2000) and lung cancer (Osada *et al.*, 2002). Because p53 is commonly altered in NF1 MPNSTs (Section 1.3.4.1), and hypermethylation of several genes has now been shown in NF1 tumours (Gonzalez-Gomez *et al.*, 2003a; this study), it would be of interest to assess the status of the *14-3-3* genes in NF1 tumours.

7.5.3/ Clinical prospects

Significant advances in this field have been made in developing animal models .Mouse models exhibiting a phenotype which reflects a number of human clinical features have been developed (Costa *et al.*, 2001, 2002; Zhu *et al.*, 2001; 2002; Bajenaru *et al.*, 2003; Le *et al.*, 2004). Mice in which the *NF1* gene is disrupted either in specific cell types and/or during specific time windows have been developed (Zhu *et al.*, 2001; Zhu *et al.*, 2002; Bajenaru *et al.*, 2003; Le *et al.*, 2004; Hiatt *et al.*, 2004). Mice in which multiple genes have been disrupted, have also been modelled (Cichowski *et al.*, 1999; Vogel *et al.*, 1999). Learning disability affects some 40% of all NF1 patients and is clearly a target for therapeutic

intervention (Ruggieri and Huson, 1999). It has been possible to rescue learning deficits in *Nf1*$^{+/-}$ mice by genetic and pharmacologic manipulations that decrease Ras activity (Costa *et al.*, 2002). Mice model studies have produced some promising results. Statins, which are used to lower cholesterol levels in humans have exhibited dramatic effect on the learning disability of *Nf1*$^{+/-}$ mice. As a result, statins is being used in the clinical trials (NNFF international consortium, Aspen, 2005).

In neurofibroma, Schwann cells over secrete a stem cell factor and this factor attracts mast cells due to the presence of c-Kit tyrosine kinase receptor on their surface (Hirota *et al.*, 1993; Ryan *et al.*, 1994; Badache *et al.*, 1998; Carroll and Stonecypher, 2005). A compound which directly affects the c-Kit receptor has been identified and is being tested in NF1 mouse models (Badache *et al.*, 1998; NNFF international consortium, Aspen, 2005).

To date, surgery remains the primary method of treatment for NF1 tumours. However, some deep-seated tumours can be difficult to excise and, in the case of aggressive MPNSTs, surgery may not always prevent the apparition of metastases (Wong *et al.*, 1998)

Two aspects should be considered here: the initial formation of neurofibromas, and the evolution of some tumours to MPNSTs. The first involves the germline and somatic mutations in the *NF1* gene, the loss of neurofibromin on Ras signalling and other yet-unidentified components of this pathway whereas the second involves additional factors influencing malignant evolution.

Cancer gene therapy using tumour suppressor genes is a relatively new approach, with less than a decade of experience. While this would seem a natural evolution to *in vitro* studies and a logical approach to cancer, the clinical trials have had a limited success thus far and the capability to induce cell cycle arrest and apoptosis *in vitro*, or growth arrest in mouse xenograft, did not foreshadow success in clinical trials. The most experience in the field probably comes from p53 trials: Injections (generally intratumoral) of adenovirus-encoding wild-type p53 have had limited success and this was probably due to the incomplete understanding of the pathways involved (McNeish *et al.*, 2004).

To apply gene therapy to correct *NF1* mutations, a number of obstacles would have to be overcome. Firstly, the large size of the gene may hinder the construction of an efficient vector, and using only the GRD may not provide a functional alternative. Secondly, the vector

would have to be distributed to a high percentage of cells of a different lineage. Thirdly, as Ras play a key role in many different cellular processes, exogenous *NF1* would need to be expressed at physiological concentrations (Weiss *et al.*, 1999)

At the *NF1* mutation level, the disruption of elements from the splicing machinery (Sections 7.2 and 7.5.1) may also present a therapeutic opportunity. A number of strategies have recently been devised to correct or circumvent splicing defects and include over-expression of proteins that alter splicing of the affected exon (Hofmann *et al.*, 2000; Nissim-Rafinia *et al.*, 2000), use of compounds that stabilize putative secondary structures (Varani *et al.*, 2000) and trans-splicing replacement of mutated exons with wild-type exons (Liu *et al.*, 2002). The questions of efficiency of treatment, side effect and reliable and long-lasting delivery remain to be answered (Faustino and Cooper, 2003).

Reed and Gutmann (2001) proposed that, in order to prevent NF1 tumour growth, Ras could be targeted directly by preventing secondary post-translational modification. Farnesylation, which add a farnesyl group to the Ras protein, is one such modification that allows Ras to translocate to the membrane and initiate signalling. Farnesyl transferase inhibitors (FTIs) can block farnesylation, inhibiting cell proliferation and facilitating apoptosis. FTIs have been shown to inhibit growth of MPNST cell lines derived from an NF1 patient (Yan *et al.*, 1995). Furthermore, on $Nf1^{-/-}$ mice-derived Schwann cell, FTIs were able to inhibit proliferation, but not invasion (Kim *et al.*, 1997a). This implies that FTIs may be useful in treating benign tumours. However, as the Kim study suggests, and as remarked by others (Griesser *et al.*, 1995; Corral *et al.*, 2003), neurofibromin may also exert its effect in a Ras-independent manner. FTIs have been tested in clinical trials and demonstrated evidence of anti-tumour capacities in breast cancer (Johnston *et al.*, 2003) and acute leukaemia (Karp *et al.*, 2001), but were less successful in lung cancer patients (Johnson and Heymach, 2004).

Other therapeutic approaches may also target Ras effectors, and MEK and PI3K inhibitors have been reported (Weiss *et al.*, 1999). Recently, neurofibromin has been shown to regulate mTOR, through Ras and PI3K (Johannessen *et al.*, 2005). Thus, NF1 tumours may be responsive to TOR inhibitors, like rapamycin and its derivatives.

Alternatively, other factors involved in neurofibroma formation could also be targeted for therapy. Recently, it has been suggested that, in the mouse model, *Nf1* loss predisposes

Schwann cells to up-regulate EGFR (epidermal growth factor receptor), thereby leading to neurofibroma formation (Ling *et al.*, 2005). RNA interference (RNAi) is a new technology used to silence genes by targeted mRNA degradation (Uprichard, 2005). RNAi has recently been adapted to target and knockdown human EGFR in glioma cells (Zhang *et al.*, 2004). In mouse models, human EGFR-specific RNAi has been shown to reduce EGFR expression and vascularisation in tumours, and improve survival time by 88% (Zhang *et al.*, 2004). Thus, assuming that EGFR is as crucial to neurofibroma onset as recently suggested, targeted RNAi may represent a way to down-regulate EGFR, and prevent Schwann cell growth.

A receptor from the EGF receptor family and proto-oncogene, HER-2 (c-erbB2/neu) has also recently been targeted with specific RNAi and knocked-down in ovarian carcinoma cell lines (Urban-Klein *et al.*, 2005). Use of HER-2-specific RNAi also resulted in a reduction of tumours in athymic nude mouse models, in which HER-2 expression is a rate-limiting factor for tumour growth *in vivo* (Urban-Klein *et al.*, 2005). Neuregulins bind receptors of the ErbB family, and although ErbB2 (HER-2) has no known ligand, it is often activated by heterodimerization with ErbB1, ErbB3 or ErbB4 (Rimer, 2003). As reviewed in Section 1.4.1, neuregulins are neuronally-expressed mitogens that play an essential role in normal Schwann cells proliferation, differentiation, survival and migration (Zorick and Lemke, 1996; Aldkofer and Lai, 2000; Meintanis *et al.*, 2001). A synthetic neuregulin, heregulin is also required for $NF1^{+/-}$ and $NF1^{-/-}$ Schwann cell growth in culture (Serra *et al.*, 2000). The mechanisms underlying Schwann cell growth are not completely understood, but impeding the signals promoting aberrant growth may be a therapeutic target to hinder neurofibroma formation.

The extent of involvement in NF1 tumorigenesis of genes like *MGMT* and *RB1*, found to be hypermethylated in this study and that of Gonzalez-Gomez *et al.* (2003a), remains to be determined. They however offer additional therapeutic options. For example, a variant adenovirus in which both the *E4* region (encoding the protein E4orf6/7 that binds directly to E2Fs, key downstream effectors of Rb) and mutant *E1A* genes (encoding E1A proteins that cannot bind Rb and release E2Fs) are under the control of a human *E2F1* promoter can selectively kill cells with deregulated E2Fs (Johnson *et al.*, 2002). By contrast, normal cells arrest viral replication in an Rb-dependent manner.

MPNSTs are extremely aggressive tumours, for which there is currently no other treatment approach than removal (Leroy *et al.*, 2001). The identification of genes involved in the malignant evolution may thus represent a target for therapy.

The discovery that some genes may be hypermethylated in malignant NF1 tumours (Gonzalez-Gomez *et al.*, 2003a) presents an attractive approach to treatment, as, unlike genetic mutation, this type of epigenetic alteration is reversible. As mentioned in Section 7.4.1, DNA methylases have been shown to be responsible for gene silencing by hypermethylation (Rhee *et al.*, 2002). Therapy targeting hypermethylation of genes in cancer can be approached in two ways: by using DNA methylase (DNMT) inhibitor, like the broadly used 5-aza-CdR (5-aza-2'-deoxycytidine), and deacetylase inhibitors (histone deacetylation and compaction are also crucial in the process of gene silencing by methylation, Section 1.5; Laird, 2005). The combined use of the two types of agents also appear to synergise to reactivate epigenetically silenced genes, and clinical trials are currently underway (Laird, 2005).

It is, however, of note that catalytic inhibitors of DNMT, by causing de-methylation, may also activate pro-metastatic genes, as was seen in breast cancer cells (transformed from non-metastatic to highly invasive cells; Szyf, 2005). Thus a balance must be reached between the anti-tumorigenic effect of DNMT inhibition and global hypomethylation, which may activate other cancer genes (Szyf *et al.*, 2004). Additionally, 5-aza-CdR has been found to have toxic effects and mutagenic properties *in vivo* (Laird, 2005).

Recent studies assessed RNA interference technology (RNAi) to knockdown DNMT1 in lung and breast cancer cell lines. The results were promising, with DNMT1 knockdowns showing a dramatic loss of methylation in gene promoters and subsequent re-expression of genes, including *RASSF1A* and *CDKN2A/p16*^{INK4a}. This was not sufficient to prevent cell growth in culture, although this may have been due to the transient nature of the assay (Suzuki *et al.*, 2004). Similar results were obtained in an ovarian cell line, and growth suppression was also observed (Leu *et al.*, 2003). It has been suggested that DNMTs may possess gene silencing capabilities independent of their methylation activity, including protein-protein interactions with histone deacetylase (HDAC; Szyf *et al.*, 2004). Thus the use of RNAi to prevent DNMT expression may be more promising than global de-methylating agents.

A gene like *RASSF1A*, identified in the present study, may be involved in determining MPNST aggressiveness or the cells' capacity to metastasise. Restoration of *RASSF1A* function may not interfere with the formation of the MPNST, but could be used to prevent metastasis prior to resection, and thus improve overall patient prognosis.

In the present study, RARB was found to be hypermethylated in a rhabdomyosarcoma (Section 7.4.1), an event that may have clinical significance for such rare tumours occurring in the context of NF1. Retinoids are involved in many processes, including antiproliferation and induction of apoptosis; Retinoids have been used in clinical trials to prevent the occurrence of second primary cancers in lung and head and neck cancer patients (Pastorino *et al.*, 1993). In breast cancer, ferentinide (retinoid 4-hydroxyphenylretinamide) was found to prevent tumour recurrence, although it has been suggested that the drug may be more effective in early carcinogenesis than in metastasis (Veronesi *et al.*, 1999; Widschwendter *et al.*, 2001). Because *RARB* has been found to be silenced by hypermethylation in breast cancer, and expression has been recovered by demethylating agent Aza-CdR, it has been suggested that retinoids combined with demethylating agents or deacetylase inhibitors may be a promising approach to prevent tumour growth (Widschwendter *et al.*, 2001).

It would seem that the most recently devised therapies (tumour suppressor replacement, de-methylation), although promising, are something of a double-edged sword. As with other cancers, a better understanding of *NF1* mutation and implicated pathways in both tumorigenesis and malignancy will allow for the better development of therapies.

CONCLUSION

The search for mutations in the *NF1* gene has long been hampered by the large size of the gene, the absence of mutational hotspots, and the lack of an adequately sensitive and rapid detection method. Using a single mutation detection technique, DHPLC, the present study yielded a high detection rate for *NF1* germline mutations. Additionally, genotype/phenotype relationships were noted in the absence of germline large *NF1* deletions in patients harbouring MPNSTs, and a statistically significant distribution of germline mutations in *NF1* patients with gliomas. The somatic mutation detection rate was still low, but the micro-lesions identified here nevertheless account for a third of the known *NF1* somatic spectrum. While a number of mechanisms may account for the low somatic detection rate, it was noted that enrichment of the $NF1^{-/-}$ Schwann cell population prior to mutation screening yielded a significantly higher detection rate, suggesting that the cellular heterogeneity in neurofibromas still greatly hinders mutation detection. Loss of heterozygosity studies using sensitive fluorescently-labelled markers and new promising mutation detection techniques, such as ASCA for micro-lesions and array-CGH for multi-exon deletions, now represent the future of *NF1* mutation detection.

Microsatellite instability has been reported in many cancers, but its presence in NF1 tumours has been controversial. The present study investigated a large panel of NF1-related tumours and revealed the statistically significant presence of microsatellite instability in MPNSTs as compared to benign tumours. Fluorescence-based techniques may be used for practicality and sensitivity in the future. Most importantly, a reference panel of markers should be implemented to reduce discrepancies between studies.

Furthermore, the present study identified a possible link between mismatch repair genes *MLH1* and *MSH2* and microsatellite instability in NF1 MPNSTs. These results add to the growing body of evidence documenting the relationship between the *NF1* gene and mismatch repair gene function.

Finally, little is still known of the molecular event underlying NF1 tumorigenesis and carcinogenesis, beyond the first inactivation of the *NF1* gene, and candidate genes have

therefore been investigated for their involvement in *NF1* tumours. The present study confirmed previous report for genes altered in benign (*RB1*, *MGMT*) and malignant tumours (*CDKN2A*, *TP53*), and also identified new genes involved in MPNST formation (*RASSF1A*) and in rare NF1 malignancies (*RARB*, *MLH1*). It is also the second report to describe the occurrence of tumour suppressor gene inactivation by hypermethylation in NF1 tumours, a finding of significant importance for future therapies.

A better understanding of neurofibromin's function, though the definition of a clear mutational spectrum, and of the pathways underlying the complex multi-step process of NF1 tumorigenesis, is vital to the development of effective therapies, and much remains to be investigated.

APPENDIX

Table A.1: promoter CpG islands of eight candidate genes in NF1 tumours.

The promoter region of the eight genes was downloaded from NCBI LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink) and the location of the CpG island on the promoter region was determined using the EMBOSS web server (http://www.ebi.ac.uk/emboss/cpgplot/) as described by Ding *et al.* (2004). The parameters were set as OBS/EXP (the minimum average observed to expected ratio of C plus G to CpG)=0.6, MINPC (the minimum average percentage of G plus C)=50, LENGTH (the minimum length that the CpG has to be)=200bp.

* location relative to the translation initiation codon (ATG); *ND*, not determined; *CpGs*, number of CpG dinucleotides in the island or covered by the primer set; *Fig*, corresponding figure.

Gene	CpG island location*	CpGs	Primers	Primer location*	CpGs	Fig
RASSF1A	(-) 196 to (+) 59	26	unmethylated	(-) 206 to (-) 3	6	A.1
			methylated	(-) 196 to (-) 5	7	
NORE1A	(-) 287 to (+) 31	34	unmethylated	(-) 239 to (-) 25	10	A.2
			methylated	(-) 222 to (-) 22	11	
RB1	(-) 290 to (+) 7	56	unmethylated	(-) 227 to (-) 65	9	A.3
			methylated	(-) 227 to (-) 65	9	
CDKN2A	(-) 104 to (+) 183	29	unmethylated	(-) 79 to (+) 70	8	A.4
			methylated	(-) 79 to (+) 70	8	
MLH1	(-) 778 to (-)456	33	unmethylated	(-) 723 to (-)598	8	A.5
			methylated	(-) 717 to (-) 600	8	
MSH2	(-) 259 to (+) 85	36	unmethylated	(-) 110 to (+) 33	9	A.6
			methylated	(-) 105 to (+) 27	8	
MGMT	ND	59	unmethylated	ND	9	A.7
			methylated		7	
RARB	ND	~16	unmethylated	(-) 364 to (-) 219	8	A.8
			methylated	(-) 364 to (-) 219	8	

Figure A.1: *RASSF1A* MS-PCR primers in relation to the *RASSF1A* sequence.

In Figures A.1 to A.8, unmethylated primers are in blue and methylated primers in red. ATG in red marks the gene's translation initiation codon. CpG dinucleotides are highlighted in green. When available, the delimitations of the CpG island given by the EMBOSS software are marked by red arrows.

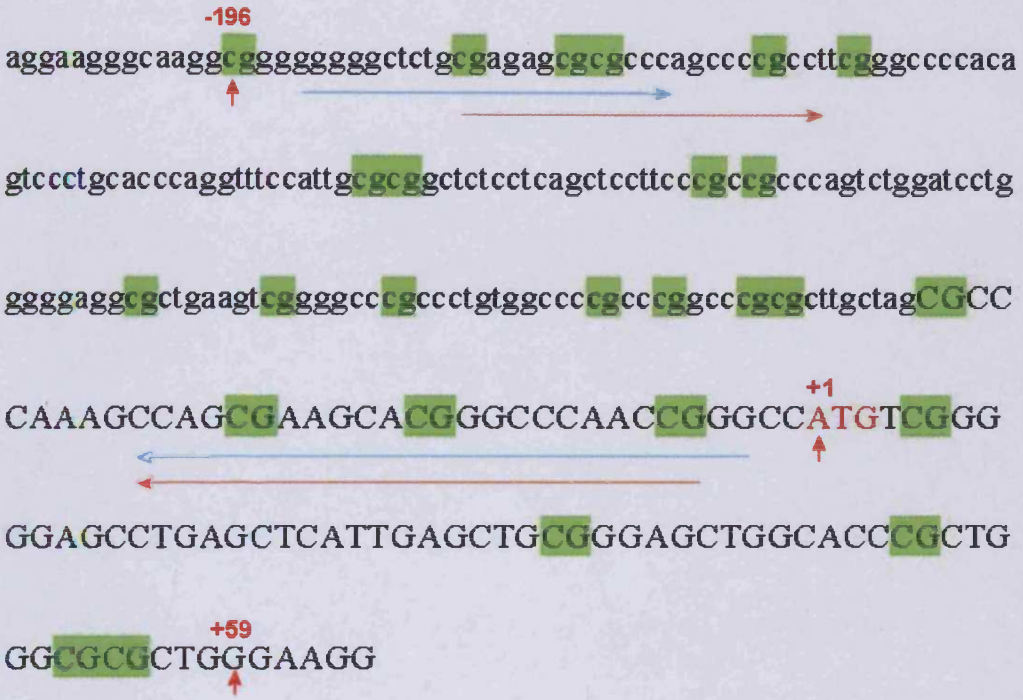

Figure A.2: *NORE1A* MS-PCR primers in relation to the *NORE1A* sequence.

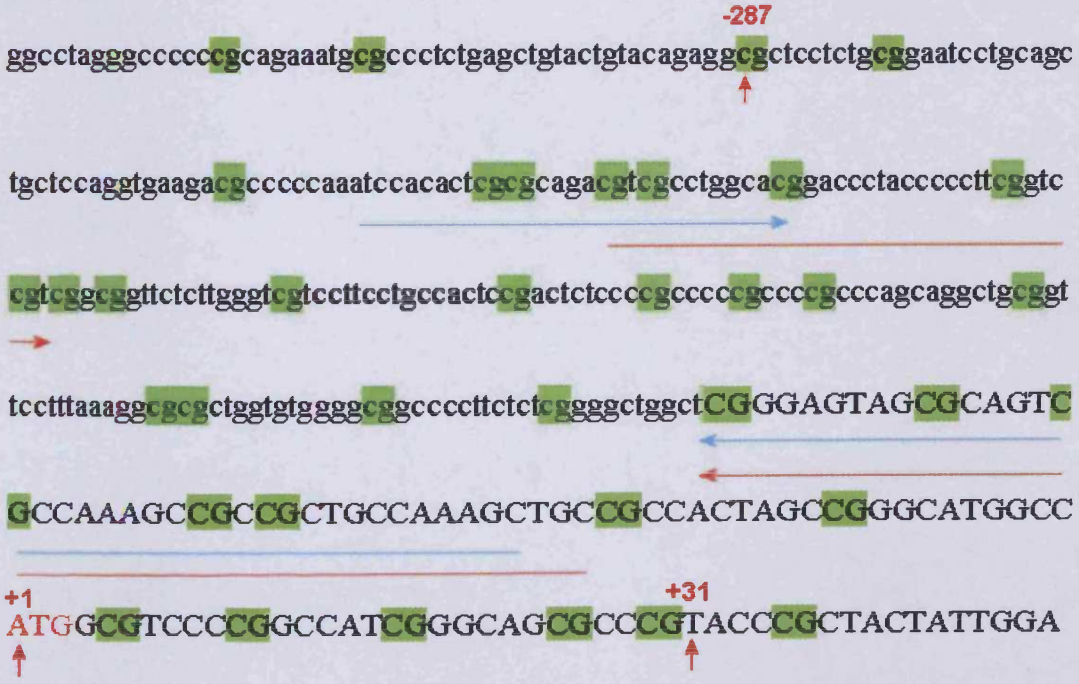

Figure A.5: *MLH1* MS-PCR primers in relation to the *MLH1* sequence.

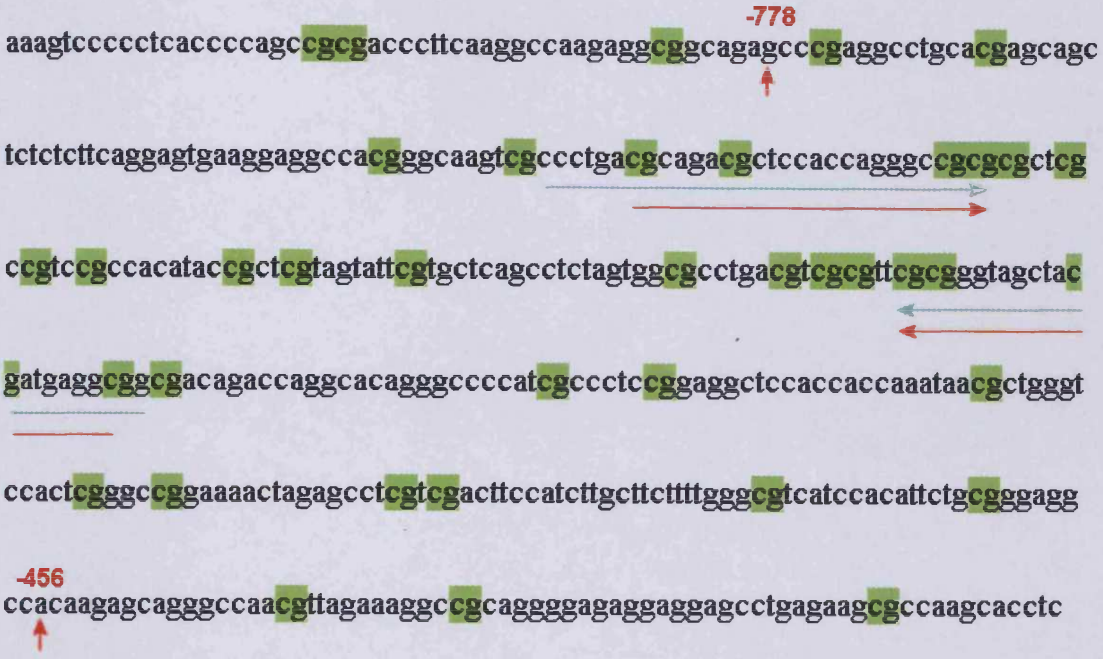

Figure A.6: *MSH2* MS-PCR primers in relation to the *MSH2* sequence.

Figure A.7: *MGMT* MS-PCR primers in relation to the *MGMT* sequence.

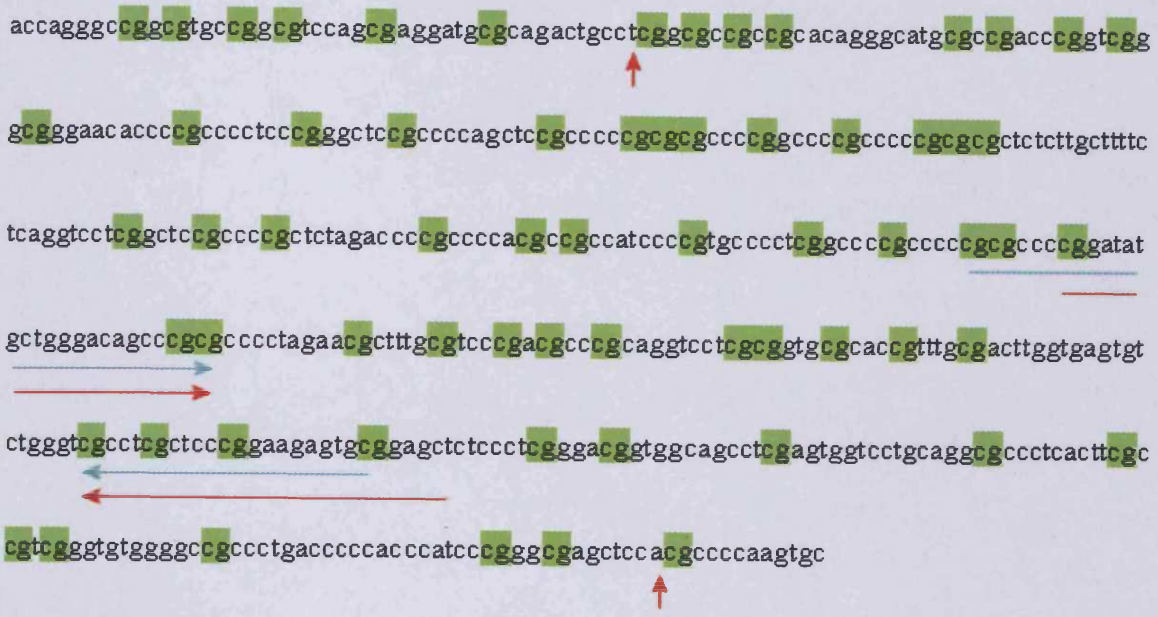

Figure A.8: *RARB* MS-PCR primers in relation to the *RARB* sequence.

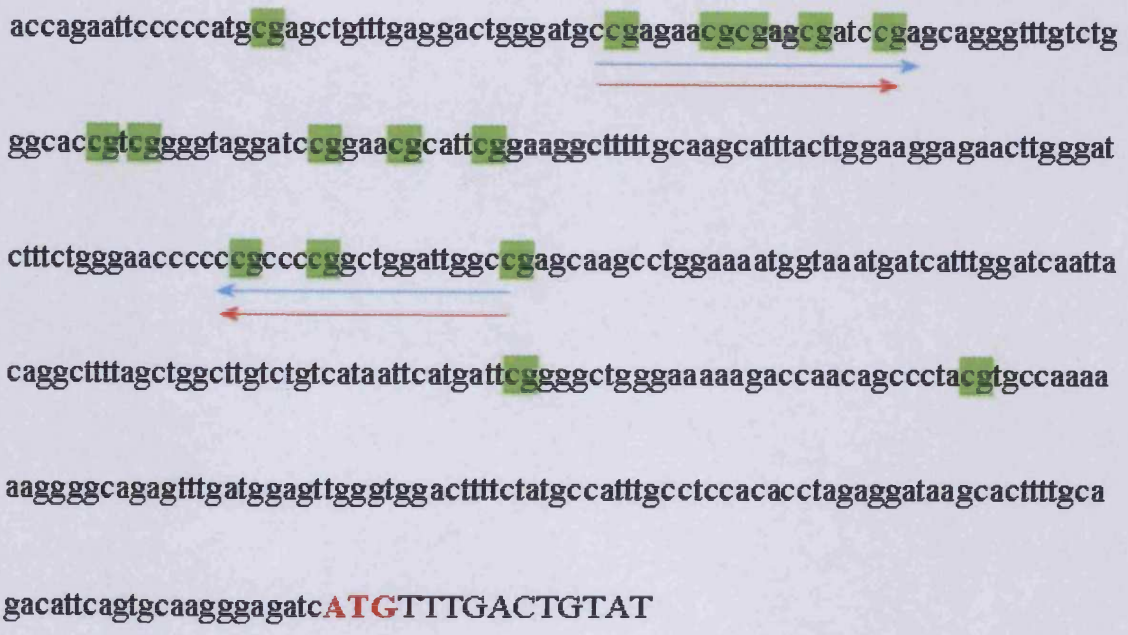

Table A.2: Germline mutations in the *NF1* gene.
The table lists all the *NF1* mutations published to date and was adapted from the HGMD database (http://www.hgmd.cf.ac.uk). *P*, number of patients reported to harbour the mutation; *ds*, splice donor site; *as*, acceptor splice site.

Exon/Intron	Genomic mutation	Aminoacid mutation	Type/effect	P	Reference
5' end	Del90kb	?	?	1	Upadhyaya *et al.*, 1990; 1992
Exon 1	1-14 to 7 del21bp	frameshift	Deletion/ truncation	1	Mattocks *et al.*, 2004
Exon 1	T2C	M68, M102 or M108	Missence/faulty initiation	1	Fahsold *et al.*, 2000
Exon 1	G62A	W9X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 1	G55T	E19X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 2	89del13	frameshift	Deletion/ truncation (38aa prot.)	1	Ars *et al.*, 2003
Exon 2	A92G	H31R	Missence/ ?	1	Mattocks *et al.*, 2004
Exon 2	127delCT	frameshift / V95X	Deletion/ truncation	1	Mattocks *et al.*, 2004
Exon 2	C147G	Y49X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 2	C168T	S56S	Silent/?	2	Fahsold *et al.*, 2000
Exon 2	197del17	frameshift	Deletion/ ?	1	Kluwe *et al.*, 2003
Intron 2	204+1 G>T	skip of exon 2?	ds/ -48aa	1	Fahsold *et al.*, 2000
Intron 2	204+1 G>A	skip of exon 2	ds/ -48aa (in-frame)	1	Ars *et al.*, 2003
Intron 2	204+2 T>G	skip of exon 2?	ds/ -48aa	2	Fahsold *et al.*, 2000 Han *et al.*, 2001
Intron 2	205-1 G>A	skip of exon 3?	as/ -28aa	1	Fahsold *et al.*, 2000
Intron 2-3	large del	loss of exon 3	Deletion/ -28	1	Hoffmeyer et al., 1994b
Exon 3	220delG	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 3	227insA	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Exon 3	C247T	Q83X	Nonsense/truncation	1	Messiaen *et al.*, 1997b
Exon 3	G271A	E91X	Nonsense/truncation	1	Mattocks *et al.*, 2004
Exon 3	G278A	C93Y	Missence/truncation	2	Messiaen *et al.*, 1997b Upadhyaya *et al.*, 1998
Intron 3	288+4insG	loss of exon 3	ds/ -28aa (in-frame)	1	Ars *et al.*, 2003
Intron 3	288+5 G>C	?	Intronic / truncation	1	Mattocks *et al.*, 2004
Exon 4a	T311A	L104X	Nonsense/truncation	1	Mattocks *et al.*, 2004
Exon 4a	347delA	frameshift	Deletion/ truncation (163aa prot.)	1	Ars *et al.*, 2003
Exon 4a	T350G	I117S	Missence/ ?	1	Ars *et al.*, 2000a
Exon 4a	413insCT	frameshift	Insertion/truncation (164aa prot.)	1	Ars *et al.*, 2003
Exon 4a	426delATTTT	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 4a	T428A	L161X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 4a	T434C	L145P	Missence/ ?	1	Mattocks *et al.*, 2004
Exon 4a	446delA	frameshift	Deletion/ truncation	1	Ars *et al.*, 2000a
Exon 4a	T470A	I157N	Missence/ normal size	1	De Luca *et al.*, 2004
Intron 4a	479+1 G>A	?	ds/?	1	John *et al.*, 2000
Exon 4b	495delTGTT	frameshift	Deletion/ ?	6	Fahsold *et al.*, 2000 Han *et al.*, 2001 De Luca *et al.*, 2004
Exon 4b	496delGT	frameshift	Deletion/ truncation	1	Toliat *et al.*, 1999
Exon 4b	496delGTTT	frameshift	Deletion/ truncation	1	De Luca *et al.*, 2003
Exon 4b	499delTGTT	frameshift / D176X	Deletion/ truncation	12	Toliat *et al.*, 1999 Fahsold *et al.*, 2000 Ars *et al.*, 2003 De Luca *et al.*, 2003 Mattocks *et al.*, 2004
Exon 4b	527delA	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 4b	527delAT	frameshift	Deletion/ truncation	1	Ars *et al.*, 2000a
Exon 4b	T528A	D176E	Missence/ ?	5	Fahsold *et al.*, 2000 Mattocks *et al.*, 2004
Exon 4b	T539A	L180X	Nonsense/truncation	1	Toliat *et al.*, 1999
Exon 4b	540insA	frameshift	Insertion/truncation	1	Daschner *et al.*, 1997
Exon 4b	546delTATC	frameshift	Deletion/ truncation	1	Sawada *et al.*, 1996
Exon 4b	550delA	frameshift	Deletion/ truncation (189aa prot.)	1	Ars *et al.*, 2003
Exon 4b	A557T	D186V	?	1	Zatkova *et al.*, 2004
Exon 4b	G560A	C187Y	Missence/ ?	1	Messiaen *et al.*, 2000

Exon/Intron	Genomic mutation	Aminoacid mutation	Type/effect	P	Reference
Exon 4b	C574T	R192X	Nonsense/truncation	6	Toliat *et al.*, 1999 Fahsold *et al.*, 2000 Messiaen *et al.*, 2000 De Luca *et al.*, 2004
Exon 4b	580delC	frameshift	Deletion/ truncation	1	Ars *et al.*, 2000a
Intron 4b	586+1 delG	skip of exon 4b?	ds deletion/ truncation	1	Fahsold *et al.*, 2000
Intron 4b	586+1 G>A	skip of exon 4b?	ds/ truncation	2	Fahsold *et al.*, 2000
Intron 4b	586+5 G>A	skip of exon 4b	ds/ truncation (163aa prot.)	2	Ars *et al.*, 2003
Intron 4b	587-3 C>A	skip of exon 4c?	as/ truncation	1	Park and Pivnick, 1998
Exon 4c	603insT	frameshift	Insertion/ ?	1	Messiaen *et al.*, 2000
Exon 4c	T647C	L216P	Missence/ ?	1	Fahsold *et al.*, 2000
Exon 4c	597/8 del23	frameshift / V207X	Deletion/ truncation	1	Mattocks *et al.*, 2004
Intron 4c	655-8del6	skip of exon 5	as/ ?	1	Serra *et al.*, 2001
Intron 4c	655-2 A>C	skip of exon 5	as/ truncation	1	Fahsold *et al.*, 2000
Intron 4c	655-1 G>A	skip of exon 5	as/ truncation	1	Horn *et al.*, 1996
Exon 5	658delT	frameshift	Deletion/ truncation	1	Abernathy *et al.*, 1997
Exon 5	686delA	frameshift	Deletion/ truncation (279aa prot.)	1	Ars *et al.*, 2003
Exon 5	T698G	G230X	Nonsense/truncation	1	Han *et al.*, 2001
Exon 5	703delTA	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 5	C715T	Q239X	Nonsense/truncation	1	Horn *et al.*, 1996
Exon 5	717insTCCCACAG	frameshift	Insertion/truncation	1	Ars *et al.*, 2000a
Exon 5	723insA	frameshift	Insertion/truncation	1	Ars *et al.*, 2000a
Intron 5	731-2 A>G	?	as/ ?	1	Kluwe *et al.*, 2003
Exon 6	large del(731-835)	frameshift	Deletion/ truncation	1	Heim *et al.*, 1995
Exon 6	754delT	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 6	A787T	K263X	Nonsense/truncation	1	Mattocks *et al.*, 2004
Exon 6	G801A	W267X	Nonsense/truncation	2	Gasparini *et al.*, 1996 Fahsold *et al.*, 2000
Exon 6	838delATAA	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 6	C844T	Q282X	Nonsense/truncation	1	Gasparini *et al.*, 1996
Exon 6	G846A	Q282Q	Silent/ truncation	2	Mattocks *et al.*, 2004
Exon 6	878delA	frameshift	Deletion/ truncation (293aa prot.)	1	Ars *et al.*, 2003
Exon 6	887delA	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Intron 6	888+1 G>A	skip of exon 6?	ds/ truncation	3	Klose *et al.*, 1999 Fahsold *et al.*, 2000
			ds/ +20 (in-frame)		Ars *et al.*, 2003
Intron 6	889-2 A>G	skip of exon 7	as/ -58aa	2	Klose *et al.*, 1999
			as/ truncation		Mattocks *et al.*, 2004
Exon 7	C910T	R304X	Nonsense/truncation	18	Hoffmeyer *et al.*, 1998 Ars *et al.*, 2000a Fahsold *et al.*, 2000 Messiaen *et al.*, 2000
		skip of exon 7	Nonsense/ -58aa		Serra *et al.*, 2000 Ars *et al.*, 2003 De Luca *et al.*, 2004 Kluwe *et al.*, 2003 Upadhyaya *et al.*, 2004
Exon 7	916delG	frameshift / L316X	Deletion/ truncation	1	Mattocks *et al.*, 2004
Exon 7	918delT	frameshift	Deletion/ truncation	3	Hoffmeyer *et al.*, 1998 Fahsold *et al.*, 2000
Exon 7	955delAG	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 7	T970C	C324R	Missence/ ?	1	Mattocks *et al.*, 2004
Exon 7	979delC>TT	979delC>UU	complex/ truncation (328aa prot.)	2	Serra *et al.*, 2001 Ars *et al.*, 2003
Exon 7	982delGT	frameshift	Deletion/ truncation	1	Abernathy *et al.*, 1997
Exon 7	985insA	frameshift	Insertion/truncation	1	Messiaen et al., 1997b
Exon 7	989insC	frameshift	Insertion/truncation (338aa prot.)	1	Ars *et al.*, 2003
Exon 7	996delT	frameshift	Deletion/ truncation	1	De Luca *et al.*, 2003
Exon 7	998dupA	frameshift	Duplication/ truncation (332aa prot.)	2	Ars *et al.*, 2003 De Luca *et al.*, 2004
Exon 7	999delC	frameshift	Deletion/ truncation (332aa prot.)	1	Ars *et al.*, 2003

Exon/Intron	Genomic mutation	Aminoacid mutation	Type/effect	P	Reference
Exon 7	G1007A	W336X	Nonsense/truncation	2	Wimmer *et al.*, 2000 De Luca *et al.*, 2004
Exon 7	A1010T	E337V	Missence/ ?	1	Mattocks *et al.*, 2004
Exon 7	A1013G	D338G	Missence/ ?	1	Upadhyaya *et al.*, 1997b
Exon 7	1019delCT	frameshift	Deletion/ truncation (350aa prot.)	5	Upadhyaya *et al.*, 1997a Fahsold *et al.*, 2000 Ars *et al.*, 2003
Exon 7	1019insT	frameshift	Insertion/truncation (352aa prot.)	1	Ars *et al.*, 2003
Exon 7	1021insTT	frameshift	Deletion/ truncation	1	Origone *et al.*, 2002
Exon 7	1021delGT	frameshift	Deletion/ ?	1	De Luca *et al.*, 2004
Exon 7	1021del11	frameshift	Deletion/ truncation (347aa prot.)	1	Ars *et al.*, 2003
Exon 7	A1060T	K354X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 7	G1062A	K354K	Silent/?	1	Fahsold *et al.*, 2000
Intron 7	1062+1 G>C	?	?	1	Upadhyaya *et al.*, 1997a
Intron 7	1062+1 G>A	skip of exon 7	ds/ -58 (in-frame)	1	Ars *et al.*, 2003
Intron 7	1062+67 T>C	?	?	1	Fahsold *et al.*, 2000
Intron 7	1063-2 A>G	?	as/?	1	Upadhyaya *et al.*, 2004
Exon 8	T1070C	L357P	Missence/ ?	1	Fahsold *et al.*, 2000
Exon 8	1096del6	in-frame del6	Deletion/ -2aa	1	Serra *et al.*, 2001
Exon 8	1111insT	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Exon 8	1119delG	frameshift	Deletion/ ?	1	De Luca *et al.*, 2004
Exon 8	1127delTGAT	frameshift	Deletion/ truncation	1	Osborn and Upadhyaya, 1999
Exon 8	1148dupG	frameshift	Duplication/ truncation	1	De Luca *et al.*, 2003
Intron 8	1185+1 G>A	skip of exon 8	ds/ -41aa	2	Hoffmeyer *et al.*, 1995 Ars *et al.*, 2000a
Intron 8	1185+1 G>T	skip of exon 8	ds/ -41aa	1	Horn *et al.*, 1996
Intron 8	1185+3insTAAA	skip of exon 8?	ds/ -41aa	1	Fahsold *et al.*, 2000
Intron 8	1186-1del15	skip of exon 9	as/ -25aa (in-frame)	1	Ars *et al.*, 2003
Exon 9	T1224G	Y408X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 9	C1246T	R416X	Nonsense/truncation	7	Osborn and Upadhyaya, 1999 Fahsold *et al.*, 2000 Eisenbarth *et al.*, 2000 Mattocks *et al.*, 2004
Exon 9	1255delA	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Intron 9	1260+1 G>A	1260ins13	ds/ truncation	2	Serra *et al.*, 2001 Eisenbarth *et al.*, 2000
Intron 9	1260+5 G>C	frameshift (1260ins13)	ds/ truncation (431aa prot.)	1	Ars *et al.*, 2003
Intron 9	1260+1604 A>G	1260ins12+9a	ds/?	1	Serra *et al.*, 2001
Intron 9	1261-2 A>C	in-frame 1261del24	as/ -8aa (2810aa prot.)	1	Ars *et al.*, 2003
Exon 10a	G1275A	W425X	Nonsense/truncation	3	Fahsold *et al.*, 2000 Messiaen *et al.*, 2000 Mattocks *et al.*, 2004
Exon 10a	C1318T	R440X	Nonsense/truncation	12	Heim *et al.*, 1995 Osborn and Upadhyaya, 1999 Fahsold *et al.*, 2000 Messiaen *et al.*, 2000 Kluwe *et al.*, 2003 Mattocks *et al.*, 2004 Frahm *et al.*, 2004
Exon 10a	1338delA	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 10a	1341delT	frameshift	Deletion/ truncation	1	De Luca *et al.*, 2003
Exon 10a	C1381T	R461X	Nonsense/truncation	4	Fahsold *et al.*, 2000 Messiaen *et al.*, 2000 Wiest *et al.*, 2003 De Luca *et al.*, 2004
Exon 10a	1448delA	frameshift	Deletion/ truncation	1	Horiguchi *et al.*, 1998
Intron 10a	1392+1 G>A	in-frame del	as/ ?		De Luca *et al.*, 2004
Intron 10a	1393-9 T>A	frameshift	as/ truncation	1	Ars *et al.*, 2000a
Exon 10b	1398insT	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Exon 10b	1399insA	frameshift	Insertion/truncation (468aa prot.)	1	Ars *et al.*, 2003
Exon 10b	A1411T	K469X	Nonsense/truncation (468aa prot.)	1	Ars *et al.*, 2003

Exon/Intron	Genomic mutation	Aminoacid mutation	Type/effect	P	Reference
Exon 10b	1414delG	frameshift	Deletion/ truncation (471aa prot.)	1	Ars *et al.*, 2003
Exon 10b	1436insA	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Exon 10b	1465insT	frameshift	Insertion/truncation	1	Ars *et al.*, 2000a
Exon 10b	1465insC	frameshift/ Y489X	Insertion/truncation		Messiaen *et al.*, 2000
Exon 10b	A1466G	Y489C	Missence/ new ds	11	Osborn and Upadhyaya, 1999 Ars *et al.*, 2000a Fahsold *et al.*, 2000 Messiaen *et al.*, 2000 Han *et al.*, 2001 Ars *et al.*, 2003 Mattocks *et al.*, 2004
Exon 10b	A1472G	Y491C	Missence/ ?	2	Fahsold *et al.*, 2000
Exon 10b	1484delCC	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 10b	A1513G	K505X	Nonsense/truncation	1	Park and Pivnick, 1998
Exon 10b	1519insT	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Intron 10b	1527+1 G>A	skip of exon 10b	ds/ -45aa (in-frame)	1	Ars *et al.*, 2000a
Intron 10b	1527+1 G>C	?	ds/ truncation	1	Mattocks *et al.*, 2004
Intron 10b	1527+4delAGTA	skip of exon 10b	ds/ -45aa (in-frame)	1	Ars *et al.*, 2003
Intron 10b	1527+5insA	skip of exon 10b	ds/ -45aa (in-frame)	1	Ars *et al.*, 2003
Exon 10c	large del(1528-1845)	frameshift	Deletion/ truncation	1	Heim *et al.*, 1995
Exon 10c	1528-14_1546del33	frameshift	as/ ?	1	Wiest *et al.*, 2003
Exon 10c	1541delAG	frameshift	Deletion/ truncation	8	Robinson *et al.*, 1996 Osborn and Upadhyaya, 1999 Ars *et al.*, 2003 Upadhyaya *et al.*, 2004
Exon 10c	1546delC	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 10c	A1556C	Q519P	Missence/ ?	1	Upadhyaya *et al.*, 2004
Exon 10c	G1570T	E524X	Nonsense/ ?	1	Messiaen *et al.*, 2000
Exon 10c	T1595C	L532P	Missence/ ?	1	Mattocks *et al.*, 2004
Exon 10c	C1607A	S536X	Nonsense/ ?	1	Messiaen *et al.*, 2000
Intron 10c	1641+1 G>T	skip of exon 10c?	ds/ -38aa	1	Fahsold *et al.*, 2000
Intron 10c	1641+1 G>A	skip of exon 10c?	ds/ ?	1	Wiest *et al.*, 2003
Intron 10c	1642-8 A>G	frameshift?	as/ truncation?	2	Side *et al.*, 1997 Fahsold *et al.*, 2000
Intron 10c	1642-5 A>T	skip of exon 11	as/ truncation	1	Serra *et al.*, 2001
Intron 10c	1642-2 A>G	skip of exon 11	as/ truncation (559aa prot.)	2	Ars *et al.*, 2003 Mattocks *et al.*, 2004
Exon 11	T1646C	L594P	Missence/ ?	1	Fahsold *et al.*, 2000
Exon 11	1673delT	frameshift	Deletion/ truncation	1	Han et al., 2001
Exon 11	1677delT	frameshift	Deletion/ truncation (566aa prot.)	1	Ars *et al.*, 2003
Exon 11	1692delT	frameshift	Deletion/ ?	1	De Luca *et al.*, 2004
Exon 11	G1721C	S574T	Missence/ ?	1	Fahsold *et al.*, 2000
Exon 11	G1721A	S574N	Missence/ ?	1	Fahsold *et al.*, 2000
Intron 11	1721+2 T>G	frameshift	ds/ truncation	1	De Luca *et al.*, 2003
Intron 11	1721+3 A>G	skip of exon 11	ds/ truncation (559aa prot.)	6	Purandare *et al.*, 1994 Fahsold *et al.*, 2000 De Luca *et al.*, 2004 Ars *et al.*, 2003
Intron 11	1722-3 C>G	frameshift	as/ +14aa	1	Ars *et al.*, 2000a
Exon 12a	C1722A	S574R	Missence/ ?	1	Mattocks *et al.*, 2004
Exon 12a	T1733G	L578R	Missence/ ?	1	Kluwe *et al.*, 2003
Exon 12a	T1742C	I581T	Missence/ ?	1	Fahsold *et al.*, 2000
Exon 12a	A1748G	K583R	Missence/ ?	2	Fahsold *et al.*, 2000
Exon 12a	1754delTAAC	frameshift	Deletion/ truncation	1	Serra *et al.*, 2000
Exon 12a	1756delACTA	frameshift	Deletion/ truncation (602aa prot.)	4	Park and Pivnick, 1998 Fahsold *et al.*, 2000 Ars *et al.*, 2003 Mattocks *et al.*, 2004
Exon 12a	1758delTA	frameshift	Deletion/ truncation (590aa prot.)	1	Ars *et al.*, 2000
Exon 12a	G1797A	W599X	Nonsense/truncation (598aa prot.)	1	Ars *et al.*, 2000a
Exon 12a	T1810G	L604V	Missence/ ?	1	Upadhyaya *et al.*, 1998

Exon/Intron	Genomic mutation	Aminoacid mutation	Type/effect	P	Reference
Exon 12a	1817insT	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Intron 12a	1845+1 G>T	skip of exon 12a	ds/ truncation	2	Abernathy *et al.*, 1997
					Messiaen *et al.*, 2000
Intron 12a	1845+1delGTAAG	frameshift	ds/ truncation	1	Fahsold *et al.*, 2000
Exon 12b	G1885A	G629R	Missence/ normal size	9	Gasparini *et al.*, 1996
					De Luca *et al.*, 2004
					Mattocks *et al.*, 2004
		1887del41	Missense/ truncation (616aa prot.)		Ars *et al.*, 2003
Exon 12b	1888delG	frameshift / 630X	Deletion/ truncation	1	Upadhyaya *et al.*, 2004
Exon 12b	1935delG	frameshift	Deletion/ truncation	2	Fahsold *et al.*, 2000
					Kluwe *et al.*, 2003
Exon 12b	C1994T	S665F	Missence/ ?	2	Fahsold *et al.*, 2000
					Mattocks *et al.*, 2004
Exon 12b	1998insCCTCT	frameshift	Insertion/truncation	1	Boddrich *et al.*, 1995
Exon 13	2124delCT	frameshift / E715X	Deletion/ truncation	1	Mattocks *et al.*, 2004
Exon 13	2027delC	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 13	2027insC	frameshift	Insertion/truncation	4	Heim *et al.*, 1995
					Side *et al.*, 1997
					Fahsold *et al.*, 2000
Exon 13	2033delC	frameshift	Insertion/truncation 687X	1	Upadhyaya *et al.*, 2004
Exon 13	2033insC	frameshift	Insertion/truncation	3	Fahsold *et al.*, 2000
					Messiaen *et al.*, 2000
Exon 13	2033insCGdelA	frameshift	Indel/ truncation	2	Origone *et al.*, 2002
					De Luca *et al.*, 2004
Exon 13	2034insC	frameshift / D699X	Insertion/truncation	1	Mattocks *et al.*, 2004
Exon 13	C2040T	C680C	Silent?	1	Mattocks *et al.*, 2004
Exon 13	C2041T	R681X	Nonsense/truncation (680aa prot.)	10	Ars *et al.*, 2000a
					Fahsold *et al.*, 2000
					Serra *et al.*, 2001
					Ars *et al.*, 2003
Exon 13	C2044T	Q628X	Nonsense/truncation	1	Horiguchi *et al.*, 1998
Exon 13	T2048C	L695P	Missence/ ?	1	Fahsold *et al.*, 2000
Exon 13	C2076G	Y629X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 13	2076insGTAC	frameshift	Insertion/ truncation (748aa prot.)	1	Ars *et al.*, 2003
Exon 13	2077delAT	frameshift	Deletion/ truncation (697aa prot.)	1	Ars *et al.*, 2003
Exon 13	2088delG	frameshift	Deletion/ truncation	1	Upadhyaya *et al.*, 2004
Exon 13	2133delCC	frameshift	Deletion/ truncation	1	Han *et al.*, 2001
Exon 13	2153delA	frameshift	Deletion/ ?	1	De Luca *et al.*, 2004
Exon 13	G2173T	E725X	Nonsense/truncation	1	De Luca *et al.*, 2003
Exon 13	2173insT	frameshift	Insertion/ truncation (729aa prot.)	1	Ars *et al.*, 2003
Exon 13	C2180G	S727X	Nonsense/truncation	1	Mattocks *et al.*, 2004
Exon 13	2190delCCTCT	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 13	C2246G	S749X	Nonsense/truncation	1	Serra *et al.*, 2001
Intron 13	2251+2 T>C	skip of exon 13	ds/ truncation (679aa prot.)	2	Serra *et al.*, 2001
					Ars *et al.*, 2003
Exon 14	C2266T	Q756X	Nonsense/ Skipping E14	1	Serra *et al.*, 2000
Exon 14	2269delAA	frameshift	Deletion/ ?	1	De Luca *et al.*, 2004
Exon 14	2271delAA	frameshift	Deletion/ truncation	1	De Luca *et al.*, 2003
Exon 14	2272delAG	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 14	T2288C	L763P	Missence/ ?	1	Fahsold *et al.*, 2000
Exon 14	2310delGCAG	frameshift	Deletion/ truncation (772aa prot.)	1	Ars *et al.*, 2003
Exon 14	2320insA	frameshift	Insertion/truncation	1	Park and Pivnick, 1998
Intron 14	2326-2 A>G	frameshift	as/ -84aa (in-frame)	1	De Luca *et al.*, 2003
Exon 15	G2330C	W777S	Missence/ normal size	2	Fahsold *et al.*, 2000
					De Luca *et al.*, 2004
Exon 15	C2339A	T780K	Missence/ normal size	5	Fahsold *et al.*, 2000
					De Luca *et al.*, 2003
					Han *et al.*, 2001
Exon 15	2341del18	in-frame (aa 781-786)	deletion/?	1	Upadhyaya *et al.*, 2004
Exon 15	A2342C	H781P	Missence/ ?	1	Fahsold *et al.*, 2000
Exon 15	A2347T	L783X	Nonsense/truncation	1	De Luca *et al.*, 2004

Exon/Intron	Genomic mutation	Aminoacid mutation	Type/effect	P	Reference
Exon 15	T2350C	W784R	Missence/ regular size	3	Kluwe *et al.*, 2001 De Luca *et al.*, 2004 Ars *et al.*, 2003
Exon 15	G2352C	W784C	Missence/ ?	1	Han *et al.*, 2001
Exon 15	C2356T	Q786X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Intron 15	2409+2insT	skip of exon 15	ds/ -28aa (in-frame)	1	Ars *et al.*, 2003
Intron 15	2410-16 A>G	frameshift	as/ truncation (807aa prot.)	8	Ars *et al.*, 2000a Serra *et al.*, 2001 Ars *et al.*, 2003
Intron 15	2410-15 A>G	frameshift 2409ins14	as/ truncation (822aa prot.)	1	Ars *et al.*, 2003
Intron 15	2410-12 T>G	frameshift 2409ins11	as/ truncation (823aa prot.)	1	Ars *et al.*, 2003
Exon 16	2427insGTCTT/2430delG	frameshift	Indel/ truncation	1	Fahsold *et al.*, 2000
Exon 16	A2428T	K810X	Missence/ truncation	1	Origone *et al.*, 2002
Exon 16	2438delTTinsA	frameshift	Indel/ ?	1	De Luca *et al.*, 2004
Exon 16	C2446T	R816X	Nonsense/truncation	10	Maynard *et al.*, 1997 Bahuau *et al.*, 1998 Fahsold *et al.*, 2000 John *et al.*, 2000 De Luca *et al.*, 2004
Exon 16	2497delT	frameshift	Deletion/ ?	1	Kluwe *et al.*, 2003
Exon 16	C2514G	I838M	Missence/ ?	1	Mattocks *et al.*, 2004
Exon 16	C2530T	L844F	Missence/ ?	1	Mattocks *et al.*, 2004
Exon 16	T2531G	L844R	Missence/ ?	1	Maynard *et al.*, 1997
Exon 16	T2531C	L844P	Missence/ ?	2	Mattocks *et al.*, 2004
Exon 16	2536insTG	frameshift	Insertion/truncation	1	Osborn and Upadhyaya, 1999
Exon 16	T2540C	L847P	Missence/ regular size	3	Fahsold *et al.*, 2000 Messiaen *et al.*, 2000 De Luca *et al.*, 2003
Exon 16	G2543A	G848E	Missence/ regular size	1	De Luca *et al.*, 2003
Exon 16	2545delGAGinsA	frameshift	Indel/ truncation	1	De Luca *et al.*, 2003
Exon 16	2590insTATA	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Exon 16	C2617T	R873C	Missence/ ?	1	Mattocks *et al.*, 2004
Exon 16	2665delA	frameshift	Deletion/ truncation	1	Maynard *et al.*, 1997
Exon 16	2666delC	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 16	2674delA	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 16	T2693C	L898P	Missence/ ?	1	Maynard *et al.*, 1997
Exon 16	A2722T	K908X	Nonsense/truncation	1	Maynard *et al.*, 1997
Exon 16	T2759C	L920P	Missence/ ?	1	Kluwe *et al.*, 2003
Exon 16	2760delG	frameshift	Deletion/ truncation	1	Maynard *et al.*, 1997
Exon 16	G2764A	G922S 2761del90	Missence/ -30aa (in-frame)	1	Ars *et al.*, 2000a
Exon 16	2779dupCCTGCTC	frameshift	Duplication/truncation	1	Maynard *et al.*, 1997
Exon 16	T2790G	Y930X	Nonsense/truncation	1	Maynard *et al.*, 1997
Exon 16	2815delA	frameshift	Deletion/ ?	1	Serra *et al.*, 2001
Exon 16	C2819T	T940I	Missence/ ?	1	Wu *et al.*, 1999
Exon 16	2830delT	frameshift	Deletion/ ?	1	De Luca *et al.*, 2003
Exon 16	C2842T	Q948X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 16	2844delA	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 16	2845insT	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Exon 16	2850insTT	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Intron 16	2850+1 G>A	frameshift 2176del233	ds/ truncation (876aa prot.)	3	Maynard *et al.*, 1997 Ars *et al.*, 2003
Intron 16	2850+3del6	2850+3del233	intronic/?	1	Messiaen *et al.*, 2000
Intron 16	2850+7 G>A	?	intronic/?	1	Han *et al.*, 2001
Intron 16	2851-6del4	skip of exon 17	intronic/ del140bp	1	Messiaen *et al.*, 2000
Intron 16	2851-2 A>G	?	as/ truncation	1	Mattocks *et al.*, 2004
Intron 16	2851-1delGGTTT	skip of exon 17	as/ truncation (972aa prot.)	1	Ars *et al.*, 2003
Exon 17	C2875T	Q959X	Nonsense/ ?	1	Messiaen *et al.*, 2000
Exon 17	C2887T	Q963X	Nonsense/ ?	1	Messiaen *et al.*, 2000
Exon 17	T2903G	M968R	Missence/ regular size	1	De Luca *et al.*, 2003
Exon 17	2928del13	2928del13	Deletion/?	1	Serra *et al.*, 2000

Exon/Intron	Genomic mutation	Aminoacid mutation	Type/effect	P	Reference
Exon 17	2942ins7	frameshift	Insertion/truncation (982aa prot.)	2	Serra *et al.*, 2001
					Ars *et al.*, 2003
Exon 17	2970delAA	frameshift	Deletion/ truncation (1018aa prot.)	1	Ars *et al.*, 2003
Exon 17	2970delAAT	991delM	Deletion/ -1aa (in-frame)	4	Shen *et al.*, 1993
					Messiaen *et al.*, 2000
					Ars *et al.*, 2003
					Mattocks *et al.*, 2004
Intron 17	2990+2insT	skip of exon 17	ds/ truncation (972aa prot.)	1	Ars *et al.*, 2003
Intron 17	2991-4delATA	?	as/ truncation	1	Mattocks *et al.*, 2004
Intron 17	2991-2 A>G	in-frame del	as/ -41aa	3	Fahsold *et al.*, 2000
					John *et al.*, 2000
					Han *et al.*, 2001
					Upadhyaya *et al.*, 2004
Intron 17	2991-1 G>A	skip of exon 18	as/ -41aa	2	Perrin *et al.*, 1996
					Fahsold *et al.*, 2000
Intron 17	2991-1 G>C	skip of exon 18	as/ -41aa	1	Fahsold *et al.*, 2000
Exon 18	T2994A	Y998X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 18	T3044C	L1015P	Missence/ ?	1	Kluwe *et al.*, 2003
Exon 18	C3049T	Q1017X	Nonsense/truncation	2	Heim *et al.*, 1995
					Weist *et al.*, 2003
Exon 18	3050delAATT	frameshift	Deletion/ truncation	1	Heim *et al.*, 1995
Exon 18	3060delA	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 18	T3104G	M1035R	Missence/ ?	1	Wu *et al.*, 1996
Intron 18	3113+1 G>A	skip of exon 18	ds/ -41aa (in-frame)	2	Purandare *et al.*, 1995a
					Ars *et al.*, 2000a
Intron 18	3113+1 G>T	skip of exon 18	ds/ -41aa (in-frame)	1	Ars *et al.*, 2003
Intron 18	3113+3insA	?	Intronic / truncation	1	Mattocks *et al.*, 2004
Intron 18	3113+5 G>C	skip of exon 18	ds/ -41aa (in-frame)	1	Ars *et al.*, 2003
Exon 19a	A3148G + 3149delT	frameshift	complex/ truncation	1	Abernathy *et al.*, 1997
Exon 19a	3151delGG	frameshift	Deletion/ truncation	1	Park and Pivnick, 1998
Exon 19a	C3163T	Q1055X	Nonsense/truncation	1	Mattocks *et al.*, 2004
Exon 19a	3178delG	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 19a	3192delT	frameshift	Deletion/ truncation	1	Abernathy *et al.*, 1997
Exon 19a	3193insA	frameshift	Insertion/truncation	1	Klose *et al.*, 1999
Exon 19a	3193delC	frameshift	Deletion/ ?	1	Messiaen *et al.*, 2000
Intron 19a	3197+1 G>A	skip of exon 19a	ds/ -28aa (in-frame)	1	Ars *et al.*, 2000a
Exon 19b	3198insT	frameshift	Insertion/truncation	1	Han *et al.*, 2001
Exon 19b	3214del11bp	frameshift	Insertion/truncation (1080aa prot.)	1	Ars *et al.*, 2000a
Exon 19b	A3217G	M1073V	Missence/ ?	1	Mattocks *et al.*, 2004
Exon 19b	3239insT	frameshift	Insertion/truncation (1087aa prot.)	1	Ars *et al.*, 2003
Exon 19b	G3277A	V1093M/ frameshift	Missence/ Skip part E19b	2	Messiaen *et al.*, 2000
					Ars *et al.*, 2003
Exon 19b	3303del19	frameshift	Deletion/ splicing defect	1	Weist *et al.*, 2003
Intron 19b	3315-3 C>G	frameshift	as/ skip E20	1	Messiaen *et al.*, 1997b
Exon 20	G3367T	E1123X	Nonsense/?	1	Messiaen *et al.*, 2000
Exon 20	3394insAG	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Exon 20	3394delCGTGGCA	frameshift	Deletion/ truncation (1138aa prot.)	1	Ars *et al.*, 2003
Exon 20	3403delTC	frameshift	Deletion/ truncation (1193aa prot.)	1	Ars *et al.*, 2003
Exon 20	C3419G	S1140X	Nonsense/truncation (1139aa prot.)	2	Ars *et al.*, 2000a
					Serra *et al.*, 2001
Exon 20	C3427T	H1140Y 3425del71	Missence/ truncation (1169aa prot.)	1	Ars *et al.*, 2003
Exon 20	T3440C	L1147P	Missence/ ?	1	Han *et al.*, 2001
Exon 20	3456delACTC	frameshift	Deletion/ truncation (1155aa prot.)	10	Upadhyaya *et al.*, 1997b
					Osborn and Upadhyaya, 1999
					Ars *et al.*, 2000a
					Fahsold *et al.*, 2000
					Ars *et al.*, 2003
					Frahm *et al.*, 2004
Exon 20	3457delCTCA	frameshift	Deletion/ ?	1	Kluwe *et al.*, 2003
Exon 20	3460delAA	frameshift	Deletion/ truncation	1	Upadhyaya *et al.*, 1997b

Exon/Intron	Genomic mutation	Aminoacid mutation	Type/effect	P	Reference
Exon 20	A3467G	N1156S	Missence/ ?	1	Fahsold *et al.*, 2000
Intron 20	3496+2 T>C	skip of exon 20	ds/ truncation	1	Klose *et al.*, 1998a
Exon 21	G3497A	G1166D	Missence/ ?	1	Purandare *et al.*, 1994
Exon 21	3509delA	frameshift	Deletion/ truncation (1182aa prot.)	1	Ars *et al.*, 2003
Exon 21	C3520T	Q1174X	Nonsense/?	1	Messiaen *et al.*, 2000
Exon 21	3525delAA	frameshift	Deletion/ truncation (1192aa prot.)	8	Fahsold *et al.*, 2000 Serra *et al.*, 2000 Ars *et al.*, 2003
Exon 21	3526delAG	frameshift	Deletion/ ?	1	Kluwe *et al.*, 2003
Exon 21	3528delA	frameshift / L1183X	Deletion/ truncation	1	Mattocks *et al.*, 2004
Exon 21	3538delATGGAAinsG	frameshift	Indel/ truncation (1191aa prot.)	1	Ars *et al.*, 2003
Exon 22	3546delT	frameshift / L1183X	Deletion/ truncation	1	Mattocks *et al.*, 2004
Exon 21	T3578G	F1193C	Missence/ ?	1	Han *et al.*, 2001
Exon 21	T3587G	L1196R	Missence/ ?	1	Mattocks *et al.*, 2004
Exon 21	3599insG	frameshift	Insertion/truncation	1	Han *et al.*, 2001
Exon 21	C3610T	R1204W	Missence/ regular size	1	Ars *et al.*, 2000a
Exon 21	G3628T	E1210X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 21	3643delATG	frameshift	deletion M	1	Fahsold *et al.*, 2000
Exon 21	3686delA	frameshift	Deletion/ ?	1	Kluwe *et al.*, 2003
Exon 21	C3703T	Q1235X	Nonsense/truncation	1	De Luca *et al.*, 2004
Exon 21	G3707A	W1236X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 21	C3721T	R1241X	Nonsense/?	4	Eisenbarth *et al.*, 2000 Han *et al.*, 2001 Mattocks *et al.*, 2004
Exon 22	3731delT	frameshift	Deletion/ truncation	2	Upadhyaya *et al.*, 1997 Upadhyaya *et al.*, 2004
Exon 22	3737delT	frameshift	Deletion/ truncation	1	Upadhyaya *et al.*, 1997
Exon 22	3737delTGTT	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 22	3739delTTTG	frameshift	Deletion/ truncation (1263aa prot.)	1	Ars *et al.*, 2003
Exon 22	G3749C	R1250P	Missence/ ?	1	Fahsold *et al.*, 2000
Exon 22	3758delTCTAC	frameshift / F1261X	Deletion/ truncation	1	Mattocks *et al.*, 2004
Exon 22	3759delCTACC	frameshift	Deletion/ truncation (1260aa prot.)	1	Ars *et al.*, 2000a
Exon 22	G3773A	W1258X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 22	3818delCT	frameshift	Deletion/ truncation	1	Ars *et al.*, 2000a
Exon 22	3822delCT	frameshift	Deletion/ truncation (1281aa prot.)	2	Fahsold *et al.*, 2000 Ars *et al.*, 2003
Exon 22	C3826T	R1276X	Nonsense/truncation	8	Heim *et al.*, 1995 Side *et al.*, 1997 Upadhyaya *et al.*, 1997b Osborn and Upadhyaya, 1999 Fahsold *et al.*, 2000 Messiaen *et al.*, 2000
Exon 22	C3826G	R1276G	Missence/ ?	2	Mattocks *et al.*, 2004
Exon 22	G3827A	R1276Q	Missence/ ?	4	Fahsold *et al.*, 2000 Mattocks *et al.*, 2004
Exon 22	G3827C	R1276P	Missence/ ?	1	Klose *et al.*, 1998
Exon 22	C3831T	G1277G	Silent/?	1	Fahsold *et al.*, 2000
Exon 22	3847delA	frameshift	Deletion/ ?	1	De Luca *et al.*, 2004
Exon 22	3861delC	frameshift	Deletion/ ?	1	De Luca *et al.*, 2004
Intron 22	3870+1 G>T	3844del26	ds/ truncation	1	Serra *et al.*, 2000
Intron 22	3870+1 G>C	3844del26	ds/ truncation (1303aa prot.)	1	Ars *et al.*, 2003
Intron 22	3871-2 A>G	?	intronic/?	1	Upadhyaya *et al.*, 1997b
Exon 23.1	3909delT	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 23.1	3911delT	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 23.1	C3916T	R1306X	Nonsense/truncation	10	Park and Pivnick, 1998 Fahsold *et al.*, 2000 Han *et al.*, 2001 Weist *et al.*, 2003 De Luca *et al.*, 2003 Mattocks *et al.*, 2004

Exon/Intron	Genomic mutation	Aminoacid mutation	Type/effect	P	Reference
Exon 23.1	3921insT	frameshift	Deletion/ truncation	1	Origone *et al.*, 2002 De Luca *et al.*, 2004
Exon 23.1	G3942A	W1314X	Nonsense/truncation	1	Upadhyaya *et al.*, 1997b
Intron 23.1	3974+1 G>C	?	intronic/?	1	Han *et al.*, 2001
Intron 23.1	3975-2 A>G	skip of exon 23.2	as/ truncation (1330aa prot.)	2	Fahsold *et al.*, 2000 Ars *et al.*, 2003
Exon 23.2	3982insA	frameshift / E1333X	Insertion/truncation	1	Mattocks *et al.*, 2004
Exon 23.2	C4006T	Q1336X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 23.2	4016delT	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 23.2	C4021T	Q1341X	Nonsense/ ?	1	Eisenbarth *et al.*, 2000
Exon 23.2	4024delTA	frameshif	Deletion/ truncation	1	John *et al.*, 2000
Exon 23.2	4045insT	frameshift / S1373X	Insertion/truncation	1	Mattocks *et al.*, 2004
Exon 23.2	4071delC	frameshift	Deletion/ truncation	1	Upadhyaya *et al.*, 1997
Exon 23.2	4083insT	frameshift	Insertion/truncation	1	Upadhyaya *et al.*, 2004
Exon 23.2	C4084T	R1362X	Nonsense/ ?	7	Upadhyaya *et al.*, 1997 Messiaen *et al.*, 2000 Han *et al.*, 2001 Kluwe *et al.*, 2003 Eisenbarth *et al.*, 2000 De Luca *et al.*, 2004
Exon 23.2	4088del23bp	frameshift	Deletion/ truncation	1	Heim *et al.*, 1995
Exon 23.2	4095insTG	frameshift	Insertion/truncation	2	Mattocks *et al.*, 2004 Baralle *et al.*, 2003
Exon 23.2	4106insTA	frameshift	Insertion/truncation	1	Origone *et al.*, 2002
Intron 23.2	4110+1 G>C	skip of exon 23.2	ds/ truncation	1	Fahsold *et al.*, 2000
Intron 23.2	4110+1 G>A	?	intronic/?	1	Upadhyaya *et al.*, 1997b
Intron 23.2	4111-8delGTT	?	intronic/ truncation	1	Mattocks *et al.*, 2004
Intron 23.2	4111-2 A>G	?	intronic/?	1	Upadhyaya *et al.*, 1997b
Exon 24	4152delA	frameshift	Deletion/ truncation	1	Abernathy *et al.*, 1997
Exon 24	4155delA	frameshift	Deletion/ truncation	1	Upadhyaya *et al.*, 1997b
Exon 24	A4173T	R1392S	Missence/ ?	1	Upadhyaya *et al.*, 1997b
Exon 24	4190delT	frameshift	Deletion/ truncation	1	Anglani *et al.*, 1993
Exon 24	T4193A	V1398D	Missence/ ?	1	Upadhyaya *et al.*, 1998
Exon 24	4203delTGinsA	frameshift	Indel/ ?	1	De Luca *et al.*, 2004
Exon 24	G4243T	E1415X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 24	4247ins74bp from intron 25	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Exon 24	A4255C	K1419Q	Missence/ ?	1	Upadhyaya *et al.*, 1997b
Exon 24	A4255G	K1419E	Missence/ ?	1	Mattocks *et al.*, 2004
Exon 24	A4256G	K1419R	Missence/ ?	1	Purandare *et al.*, 1997a
Exon 24	C4265A	S1422X	Nonsense/truncation	1	Mattocks *et al.*, 2004
Exon 24	A4267G	K1423E	Missence/ regular size	7	Li *et al.*, 1992 Upadhyaya *et al.*, 1997b Ars *et al.*, 2003 Upadhyaya *et al.*, 2004
Exon 24	A4268G	K1423R	Missence/ ?	1	Han *et al.*, 2001
Exon 24	G4269T	K1423N	Missence/ regular size	1	De Luca *et al.*, 2003
Exon 25	T4274C	L1425P	Missence/ regular size	5	Peters *et al.*, 1999 Ars *et al.*, 2000a Fahsold *et al.*, 2000 Ars *et al.*, 2003
Exon 25	4311delAGAA	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 25	4312delGAA	in-frame 1438delQ	Deletion/ -1aa (in-frame)	3	Ars *et al.*, 2003 Mattocks *et al.*, 2004 Baralle *et al.*, 2003
Exon 25	4323insGC	frameshift	Insertion/truncation	1	Han *et al.*, 2001
Intron 25	4367+1 G>A	skip of exon 25	ds/ truncation (1426aa prot.)	1	Ars *et al.*, 2003
Intron 25	4368-46 G>C	?	intronic/?	1	Fahsold *et al.*, 2000
Intron 25	4368-1 G>T	skip of exon 26	as/ -49aa	1	Fahsold *et al.*, 2000
Exon 26	4374insT	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Exon 26	4374delCC	frameshift	Deletion/ truncation 1459X	1	Upadhyaya *et al.*, 2004

Exon/Intron	Genomic mutation	Aminoacid mutation	Type/effect	P	Reference
Exon 26	A4402G	S1468G	Missence/ ?	2	Upadhyaya *et al.*, 1997b Mattocks *et al.*, 2004
Exon 26	4426delT	frameshift	Deletion/ truncation (1477aa prot.)	1	Ars *et al.*, 2003
Exon 26	4431delC	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 26	G4473A	W1491X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 26	4481delAG	frameshift	Deletion/ truncation	1	De Luca *et al.*, 2003
Exon 26	4486delA	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 26	G4493A	G1498E	Missence/ regular size	1	Ars *et al.*, 2003
Exon 26	4497insG	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Intron 26	4514+1 G>A	skip of exon 26?	ds/ -49aa	1	Fahsold *et al.*, 2000
Intron 26	4514+1 G>C	skip of exon 26	ds/ ?	1	Serra *et al.*, 2001
Intron 26	4515-2 A>G	skip of exon 27a?	as/ -49aa	1	Fahsold *et al.*, 2000
Intron 26	4515-2 A>T	frameshift	as/ +14aa /+17aa	1	Messiaen *et al.*, 2000
Intron 26	4515-1 G>A	?	as/ truncation	1	Mattocks *et al.*, 2004
Exon 27a	C4537T	R1513X	Nonsense/truncation (1512aa prot.)	18	Side *et al.*, 1997 Ars *et al.*, 2000a Fahsold *et al.*, 2000 Messiaen *et al.*, 2000 Kluwe *et al.*, 2003 Ars *et al.*, 2003 De Luca *et al.*, 2003 Mattocks *et al.*, 2004 Upadhyaya *et al.*, 2004 Frahm *et al.*, 2004
Exon 27a	4568insC	skip of exon 27a	Insertion/ -49aa (in-frame)	1	Ars *et al.*, 2003
Exon 27a	4572delC	frameshift	Deletion/ truncation	1	Upadhyaya *et al.*, 1995
Exon 27a	G4614A	W1538X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 27a	4614delGT	frameshift	Deletion/ truncation (1552aa prot.)	1	Ars *et al.*, 2003
Exon 27a	4630delA	frameshift	Deletion/ truncation	1	Upadhyaya *et al.*, 1995
Exon 27a	4649insG	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Intron 27a	Large del - hemizygosity	frameshift	Deletion/ truncation	2	Lazaro *et al.*, 1993
Exon 27b	4696delTT	frameshift	Deletion/ ?	1	Kluwe *et al.*, 2003
Exon 27b	4703delC	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 27b	T4706G	L1569X	Nonsense/truncation	1	Upadhyaya *et al.*, 1997b
Exon 27b	C4719G	Y1573X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 27b	A4750G	I1584V	Missence/ ?	1	Fahsold *et al.*, 2000
Exon 27b	4750insT	frameshift	Insertion/ ?	1	Weist *et al.*, 2003
Exon 27b	C4768T	R1590W	Missence/ ?	1	Upadhyaya *et al.*, 1997b
Intron 27b	4772+1del9bp	frameshift	ds/ -23aa (in-frame)	1	Ars *et al.*, 2000a
Intron 27b	4773-5 C>T	?	intronic/?	1	Upadhyaya *et al.*, 2004
Intron 27b	4773-2 A>T	frameshift	as/ -433aa / -293aa	1	Messiaen *et al.*, 2000
Exon 28	4798insC	frameshift	Insertion/truncation	1	Han *et al.*, 2001
Exon 28	4829del15	frameshift	Deletion/ truncation (1616aa prot.)	1	Ars *et al.*, 2003
Exon 28	T4839G	Y1613X	Nonsense/truncation	1	Peters *et al.*, 1999a
Exon 28	4868delAC	frameshift	Deletion/ truncation	1	Xu *et al.*, 1992b
Exon 28	4873insA	frameshift	Insertion/truncation	1	Colman *et al.*, 1997
Exon 28	4905ins11bp	frameshift	Insertion/ ?	1	Kluwe *et al.*, 2003
Exon 28	4907dup14bp	frameshift	Duplication/ truncation	1	Eisenbarth *et al.*, 2000
Exon 28	4913delTCTCT	frameshift / C1661X	Deletion/ truncation	1	Mattocks *et al.*, 2004
Exon 28	4914delCTCT	frameshift	Deletion/ truncation	1	Side *et al.*, 1997
Exon 28	4936insT	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Exon 28	4950insA	frameshift / Y1650X	Insertion/truncation	1	Mattocks *et al.*, 2004
Exon 28	4967delTCTATA	in-frame del	Deletion/ -2aa	1	Wu *et al.*, 1999
Exon 28	4973del6	delIY1658-9	Deletion/ -2aa (in-frame)	1	Mattocks *et al.*, 2004
Exon 28	5010delG	frameshift	Deletion/ truncation	1	Colman *et al.*, 1993
Exon 28	5024delT	frameshift	Deletion/ truncation	1	Side *et al.*, 1997
Exon 28	5033delG	frameshift	Deletion/ ?	1	Messiaen *et al.*, 2000
Exon 28	5050delAGGCTTG	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 28	5055insT	frameshift	Insertion/truncation	1	Peters *et al.*, 1999
Exon 28	5077del13bp	frameshift	Deletion/ truncation	1	Shen and Upadhyaya, 1993
Exon 28	5094delAG	frameshift	Deletion/ truncation (1699aa prot.)	1	Ars *et al.*, 2003

Exon/Intron	Genomic mutation	Aminoacid mutation	Type/effect	P	Reference
Exon 28	5095dup42bp	frameshift	Duplication/ +44aa	1	Tassabehji *et al.*, 1993
Exon 28	A5106G	Q1702Q	Silent/?	1	Peters *et al.*, 1999
Exon 28	5108delAG	frameshift	Deletion/ truncation	1	Zhong *et al.*, 1993
Exon 28	5123delCCACC	frameshift	Deletion/ truncation	1	Stark *et al.*, 1991
Exon 28	5152delG	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 28	5168delTC	frameshift	Deletion/ truncation	1	Peters *et al.*, 1999
Exon 28	G5172A	K1724K	Silent/ truncation	3	Peters *et al.*, 1999 Mattocks *et al.*, 2004
Exon 28	5194insA	frameshift	Insertion/truncation (1734aa prot.)	1	Ars *et al.*, 2003
Exon 28	5205delAGTAA	frameshift	Deletion/ truncation	1	Peters *et al.*, 1999
Exon 28	Del11kb	frameshift	Deletion/ truncation	1	Martinez *et al.*, 1996
Exon 28	Del40kb	frameshift	Deletion/ truncation	1	Viskochil *et al.*, 1990
Intron 28	5205+1 G>A	frameshift	ds/ -18aa (in-frame)	2	Ars *et al.*, 2000a Weist *et al.*, 2003
Intron 28	5205+5 G>A	5152del54	ds/ -18aa (in-frame)	1	Ars *et al.*, 2003
Intron 28	5206-2 A>G	skip of exon 29	as/ truncation (1739aa prot.)	1	Ars *et al.*, 2003
Exon 29	C5224T	Q1742X	Nonsense/truncation	2	Han *et al.*, 2001 De Luca *et al.*, 2003
Exon 29	5227delGTAinsT	frameshift	Indel/ ?	1	Kluwe *et al.*, 2003
Exon 29	C5242T	R1748X	Nonsense/truncation (1747aa prot.)	7	Valero *et al.*, 1994 Peters *et al.*, 1999 Fahsold *et al.*, 2000 Kluwe *et al.*, 2003 Ars *et al.*, 2003
Exon 29	5248delAAA	frameshift	delK	1	Fahsold *et al.*, 2000
Exon 29	C5260T	Q1754X	Nonsense/truncation	1	Valero *et al.*, 1994
Exon 29	C5264G	S1755X	Nonsense/ ?	2	Messiaen *et al.*, 2000 De Luca *et al.*, 2004
Exon 29	5272delC	L1758X	frameshift/truncation	1	John *et al.*, 2000
Exon 29	T5286G	Y1762X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 29	5289insAA	frameshift	Insertion/truncation	1	Colman *et al.*, 1997
Exon 29	G5290T	A1764S	Missence/ ?	1	Han *et al.*, 2001
Exon 29	C5294A	S1765X	Nonsense/ -90aa (E29)	1	Messiaen *et al.*, 2000
Exon 29	5303delA	frameshift	Deletion/ truncation (1771aa prot.)	1	Ars *et al.*, 2003
Exon 29	C5329T	Q1777X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 29	T5339G	L1780X	Nonsense/truncation	3	Fahsold *et al.*, 2000 De Luca *et al.*, 2004
Exon 29	5351insC	frameshift	Insertion/truncation (1796aa prot.)	1	Ars *et al.*, 2003
Exon 29	C5353T	Q1785X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 29	5368delTG	frameshift	Deletion/ truncation	1	Upadhyaya *et al.*, 1997b
Exon 29	C5380T	Q1794X	Nonsense/truncation	1	Heim *et al.*, 1994
Exon 29	5399delT	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 29	C5427T	R1808R	Silent / truncated	1	Mattocks *et al.*, 2004
Exon 29	5449insC	frameshift	Insertion/truncation	2	Upadhyaya *et al.*, 1992
Exon 29	5453delT	frameshift	Deletion/ truncation	1	Park and Pivnick, 1998
Exon 29	C5458T	Q1820X	Nonsense/truncation	1	Peters *et al.*, 1999a
Exon 29	5460delAC	frameshift	Deletion/ truncation (1838aa prot.)	1	Ars *et al.*, 2003
Exon 29	5466insT	frameshift	Insertion/truncation	2	Upadhyaya *et al.*, 1992
Exon 29	5484delT	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 29	5486insC	frameshift	Insertion/truncation (1839aa prot.)	1	Ars *et al.*, 2003
Exon 29	G5546A	R1849Q/ delE29	Missence/ truncation (1739aa prot.)	7	Ars *et al.*, 2000a Fahsold *et al.*, 2000 Messiaen *et al.*, 2000 Ars *et al.*, 2003
Intron 29	5546+1 G>A	skip of exon 29	ds/ truncation (1739aa prot.)	2	Fahsold *et al.*, 2000 Ars *et al.*, 2003
Intron 29	5546+1 G>C	frameshift	ds/ truncation	1	Faravelli *et al.*, 1999
Intron 29	5546+2 T>G	skip of exon 29?	ds frameshift/ ?	1	Fahsold *et al.*, 2000
Exon 30	5556_5578dup	frameshift	Tandem duplication	1	De Luca *et al.*, 2004
Exon 30	5567delT	frameshift	Deletion/ ?	1	Messiaen *et al.*, 2000
Exon 30	5584delAC	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000

Exon/Intron	Genomic mutation	Aminoacid mutation	Type/effect	P	Reference
Exon 30	5592delTTTAA	frameshift	Deletion/ truncation	1	Harder *et al.*, 1999
Exon 30	5617del16bp	frameshift	Deletion/ truncation	1	Ars *et al.*, 2000a
Exon 30	5618del16bp	frameshift	Deletion/ truncation (1897aa prot.)	1	Ars *et al.*, 2000a
Exon 30	5656delT	frameshift	Deletion/ ?	1	De Luca *et al.*, 2004
Exon 30	5673delTAAG	frameshift	Deletion/ truncation	1	Abernathy *et al.*, 1997
Exon 30	5679delACTG	frameshift	Deletion/ truncation	1	Hatta *et al.*, 1994
Exon 30	G5719T	E1907X	?	1	Zatkova *et al.*, 2004
Exon 30	5738insAT	frameshift	Insertion/truncation (1920aa prot.)	1	Ars *et al.*, 2003
Intron 30	5749+2 T>G	skip of exon 30	ds/ truncation	1	Purandare *et al.*, 1994
Intron 30	5749+332 A>G	frameshift	Intronic/ truncation (1927aa prot.)	6	Perrin *et al.*, 1996
					Side *et al.*, 1997
					Ars *et al.*, 2000a
		5749ins177	Intronic/ IF +59aa		Serra *et al.*, 2001
					Ars *et al.*, 2003
Intron 30	5749+4046 A>G	frameshift	Intronic/ + 57aa	1	Osborn and Upadhyaya, 1999
Exon 31	5767delTTTG	frameshift	Deletion/ ?	1	Weist *et al.*, 2003
Exon 31	5774delT	frameshift	Deletion/ ?	1	Serra *et al.*, 2001
Exon 31	5788delC	frameshift	Deletion/ ?	1	Kluwe *et al.*, 2003
Exon 31	T5791C	W1931R	Missence/ ?	1	Hudson *et al.*, 1997
Exon 31	5794delCTG	in-frame 1932delL	Deletion/ -1aa	1	De Luca *et al.*, 2003
Exon 31	T5795C	L1932P	Missence/ ?	1	Cawthon *et al.*, 1990b
Exon 31	5798delC	frameshift	Deletion/ ?	1	Messiaen *et al.*, 2000
Exon 31	5816insG	frameshift	Insertion/truncation	1	Zhong *et al.*, 1993
Exon 31	C5839T	R1947X	Nonsense/truncation (1946aa prot.)	21	Cawthon *et al.*, 1990b
					Estivill *et al.*, 1991
					Ainsworth *et al.*, 1993
					Horiuchi *et al.*, 1994
					Valero *et al.*, 1994
					Dublin *et al.*, 1995
					Lazaro *et al.*, 1995b
					Abernathy *et al.*, 1997
					Hudson *et al.*, 1997
					Klose *et al.*, 1999
					Fahsold *et al.*, 2000
					Messiaen *et al.*, 2000
					Ars *et al.*, 2003
					De Luca *et al.*, 2004
Exon 31	5843delAA	frameshift	Deletion/ truncation	1	Valero *et al.*, 1994
Exon 31	5843insA	frameshift	Insertion/truncation	1	Hatta *et al.*, 1995
Exon 31	5847delAG	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 31	5852insTT	frameshift	Insertion/truncation	1	Ainsworth *et al.*, 1993
Exon 31	Ins200bp, del28bp	in-frame	Gross rearrangement/ ?	1	Skuse, 1998
Exon 31	5887delA	frameshift	Deletion/ truncation (1989aa prot.)	1	Ars *et al.*, 2000a
Exon 31	A5893T	K1965X	Nonsense/truncation	1	De Luca *et al.*, 2003
Exon 31	C5896T	Q1966X	Nonsense/?	1	Messiaen *et al.*, 2000
Exon 31	5898delGA	frameshift / D1987X	Deletion/ truncation	1	Mattocks *et al.*, 2004
Exon 31	G5938A	G1980R	Missence/ regular size	1	De Luca *et al.*, 2003
Intron 31	5943+1 G>A	skip of exon 31?	ds/ truncation	1	Fahsold *et al.*, 2000
Intron 31	5943+1 G>T	?	ds/ truncation	1	Mattocks *et al.*, 2004
Intron 31	5944-5 A>G	skip of exon 32?	as/ -47aa	1	Fahsold *et al.*, 2000
Intron 31	5944-1 G>C	?	as / truncation	1	Mattocks *et al.*, 2004
Intron 31	Del12kb	frameshift	Deletion/ truncation	1	Lazaro *et al.*, 1994a
Exon 32	5949delA	frameshift	Deletion/ truncation	1	Hatta *et al.*, 1994
Exon 32	G5971A	D1991N	Missence/ ?	1	Han *et al.*, 2001
Exon 32	6020dupCTGAGGTG	frameshift	Duplication/ ?	1	Kluwe *et al.*, 2003
Intron 32	6084+1 G>A	in-frame del	ds/ ?	1	De Luca *et al.*, 2004
Intron 32	Ins320bp	frameshift	Insertion/truncation	1	Wallace *et al.*, 1990
Intron 32	Del517bp	frameshift	Deletion/ truncation	1	Xu *et al.*, 1992b
Exon 33	6117delG	frameshift	Deletion?	1	Upadhyaya *et al.*, 2004
Exon 33	6181del8	frameshift / D2074X	Deletion/ truncation	1	Mattocks *et al.*, 2004
Exon 33	6220delG	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000

Exon/Intron	Genomic mutation	Aminoacid mutation	Type/effect	P	Reference
Exon 33	6292del31	frameshift	Deletion/ ?	1	Serra *et al.*, 2001
Exon 33	T6339A	C2113X	Nonsense/truncation	1	Heim *et al.*, 1995
Intron 33	6364+2 T>G	skip of exon 33	ds/ truncation	1	Osborn and Upadhyaya, 1999
Intron 33	6364+4 A>G	skip of exon 33	ds/ truncation	1	Hutter *et al.*, 1994
Exon 34	A6387C	R2129S	Missence/ ?	1	Upadhyaya et al., 2004
Exon 34	6395del10bp	frameshift	Deletion/ truncation (2174aa prot.)	1	Ars *et al.*, 2000a
Exon 34	C6427A	L2143M	Missence/ ?	1	Upadhyaya *et al.*, 1992
Exon 34	6468delC	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 34	6470delT	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 34	C6483G	Y2161X/ skip of E34	Nonsense/truncation (2160aa prot.)	1	Ars *et al.*, 2003
Exon 34	6519insG	frameshift	Insertion/truncation	1	Purandare *et al.*, 1994
Exon 34	T6566A	L2189X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 34	6577delGAGGTA	frameshift	Deletion/ -215 (E34)	1	Messiaen *et al.*, 2000
Exon 34	6579insGCAT	frameshift	Insertion/ truncation (2221aa prot.)	1	Ars *et al.*, 2003
Intron 34	6579+2 T>G	skip of exon 34	ds/ truncation	1	Fahsold *et al.*, 2000
Intron 34	6579+45 T>A	?	Intronic/?	1	Fahsold *et al.*, 2000
Intron 34	6579+87 G>A	?	Intronic/?	1	Fahsold *et al.*, 2000
Exon 35	6593insT	frameshift	Insertion/truncation (2219aa prot.)	1	Ars *et al.*, 2000a
Exon 35	C6598G	P220A	Missence/ ?	1	Kluwe *et al.*, 2003
Exon 35	6604delT	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 35	G6624A	W2208X	Nonsense/truncation	1	Heim *et al.*, 1995
Exon 35	G6628T	E2210X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 35	A6640T	R2214X	Nonsense/truncation	1	Osborn and Upadhyaya, 1999
Intron 35	6641+1 G>T	skip of exon 35	ds/ truncation	1	Park and Pivnick, 1998
Intron 35	6641+1 G>A	frameshift	ds/ ?	1	De Luca *et al.*, 2004
Intron 35	6641+1delG	?	ds/ truncation	1	Mattocks *et al.*, 2004
Intron 35	6641+2delT	skip of exon 35?	ds/ truncation	1	Fahsold *et al.*, 2000
Intron 35	6641+2 T>A	skip of exon 35	ds/ truncation (2198aa prot.)	1	Ars *et al.*, 2003
Intron 35	6642-1 G>T	skip of exon 36?	as/ truncation	1	Fahsold *et al.*, 2000
Exon 36	6649delT	frameshift	Deletion/ truncation (2242aa prot.)	1	Ars *et al.*, 2003
Exon 36	6704insC	frameshift	Insertion/truncation	1	Han *et al.*, 2001
Exon 36	A6706T	K2236X	Nonsense/?	1	Kluwe *et al.*, 2003
Exon 36	6709insC	frameshift	Insertion/truncation	1	Ars *et al.*, 2000a
Exon 36	C6709T	R2237X	Nonsense/ truncation	6	Messiaen *et al.*, 2000 De Luca *et al.*, 2004 Mattocks *et al.*, 2004
Exon 36	6711insC	frameshift	Insertion/truncation	1	Abernathy *et al.*, 1997
Exon 36	C6724T	Q2242X/ skip of E36	Nonsense/truncation (2258aa prot.)	1	Ars *et al.*, 2003
Intron 36	6756+1 G>A	skip of exon 36?	ds/ truncation	1	Fahsold *et al.*, 2000
Intron 36	6756+2 T>A	skip of exon 36	ds/ truncation (2220aa prot.)	2	De Luca *et al.*, 2003 Ars *et al.*, 2003
Intron 36	6756+3 A>G	skip of exon 36	ds/ truncation (2220aa prot.)	2	Side *et al.*, 1997 Ars *et al.*, 2003
Intron 36	6756+6delTCG	skip of exon 36	ds/ truncation	1	Side *et al.*, 1997
Exon 37	6757delG	frameshift	Deletion/ ?	1	Kluwe *et al.*, 2003
Exon 37	6772delT	frameshift	Deletion/ truncation	2	Origone *et al.*, 2002 De Luca *et al.*, 2004
Exon 37	6789delTTAC	frameshift	Deletion/ truncation	13	Robinson *et al.*, 1995 Boddrich *et al.*, 1997 Hoffmeyer *et al.*, 1998 Fahsold *et al.*, 2000 Messiaen *et al.*, 2000 De Luca *et al.*, 2004 Upadhyaya *et al.*, 2004
Exon 37	6790insTT	frameshift	Insertion/truncation	1	Boddrich *et al.*, 1997
Exon 37	6791insA	frameshift	Insertion/truncation	8	Upadhyaya *et al.*, 1996b, 2004 Abernathy *et al.*, 1997 Colman *et al.*, 1997 Osborn and Upadhyaya, 1999 Fahsold *et al.*, 2000 De Luca *et al.*, 2004

Exon/Intron	Genomic mutation	Aminoacid mutation	Type/effect	P	Reference
Exon 37	C6792A	Y2264X	Nonsense/truncation	20	Robinson *et al.*, 1995
					Messiaen *et al.*, 1997a
					Hoffmeyer *et al.*, 1998
					Ars *et al.*, 2000a
					Fahsold *et al.*, 2000
		IF skip of exon 37	Nonsense/ -34aa		Serra *et al.*, 2001
					Messiaen *et al.*, 2000
					Kluwe *et al.*, 2003
					Ars *et al.*, 2003
					De Luca *et al.*, 2004
					Mattocks *et al.*, 2004
					Frahm *et al.*, 2004
Exon 37	C6792G	Y2264X	Nonsense/truncation	4	Messiaen *et al.*, 1997
					Fahsold *et al.*, 2000
		IF skip of exon 37	Nonsense/ -34aa		Messiaen *et al.*, 2000
					Mattocks et al., 2004
Exon 37	6792insA	frameshift	Insertion/ truncation	3	Fahsold *et al.*, 2000
		IF skip of exon 37	Intronic/ -34aa (in-frame)		Ars *et al.*, 2003
Exon 37	6797delGT	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 37	6819delAGAAG	frameshift	Deletion/ truncation	1	Abernathy *et al.*, 1997
Exon 37	T6839G	L2280X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 37	6839delTAC	frameshift	Deletion/ truncation	1	Upadhyaya *et al.*, 1996b
Exon 37	G6858C	K2286N	Missence/ -102bp (E37) IF	1	Messiaen *et al.*, 2000
Intron 37	6858+1 A>G	?	ds/ ?	1	Han *et al.*, 2001
Intron 37	6858+2 T>G	skip of exon 37	ds/ -34aa	1	Ars *et al.*, 2000a
Intron 37	Del2892bp	frameshift	Intronic/ -47aa	1	Osborn and Upadhyaya, 1999
Exon 38	6922ins10bp	frameshift	Insertion/truncation	1	Legius *et al.*, 1994b
Exon 39	G7006A	E2336K	Missence/ ?	1	Han *et al.*, 2001
Exon 39	7032insG	frameshift	Insertion/truncation	1	Han *et al.*, 2001
Exon 39	7080insA	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Exon 39	7095delT	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 39	7096insA	frameshift / N2400X	Insertion/truncation	1	Mattocks *et al.*, 2004
Exon 39	7096delAACTTT	in-frame del	Deletion/ -2aa	8	Abernathy *et al.*, 1997
					Ars *et al.*, 2000a
					Messiaen *et al.*, 2000
					Ars *et al.*, 2003
					Mattocks *et al.*, 2004
Intron 39	7126-12 T>A	del132nt/ins10nt	Intronic/ complex splicing	1	Messiaen *et al.*, 2000
Exon 40	7132insG	frameshift	Insertion/truncation	1	Han *et al.*, 2001
Exon 40	7149insC	frameshift	Insertion/truncation		Origone *et al.*, 2002
Exon 40	7188delA	frameshift	Deletion/ truncation	1	Han *et al.*, 2001
Exon 40	7168delGA	frameshift	Deletion/ ?	1	John *et al.*, 2000
Exon 40	7190delCT	frameshift	Deletion/ truncation	1	Origone *et al.*, 2002
Exon 40	A7201T	K2401X	Nonsense/ ?	1	Messiaen *et al.*, 2000
Exon 40	7204delCA	frameshift	Deletion/ truncation (2404aa prot.)	1	Ars *et al.*, 2003
Exon 40	7206delCA	frameshift / C2405X	Deletion/ truncation	1	Mattocks *et al.*, 2004
Exon 40	7208delGA	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 40	C7237T	Q2413X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 40	7257del2bp	frameshift	Deletion/ ?	1	Kluwe *et al.*, 2003
Exon 40	G7258C	A2420P	Missence/ ?	1	Fahsold *et al.*, 2000
Intron 40	7258+1 G>T	in-frame del	ds/?	1	De Luca *et al.*, 2001
Intron 40	7259-17 C>T	?	Intronic/ ?	1	Fahsold *et al.*, 2000
Intron 40	7259-14 C>T	?	Intronic/ ?	1	Fahsold *et al.*, 2000
Exon 41	7260delT	frameshift	Deletion/ truncation	1	Heim *et al.*, 1995
Exon 41	7267insA	frameshift	Insertion/ truncation (2425aa prot.)	2	Ars *et al.*, 2003
					Upadhyaya *et al.*, 2004
Exon 41	7268delCA	frameshift	Deletion/ truncation	2	Fahsold *et al.*, 2000
					Upadhyaya *et al.*, 2004
Exon 41	7285delC	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000

Exon/Intron	Genomic mutation	Aminoacid mutation	Type/effect	P	Reference
Exon 41	C7285T	R2429X	Nonsense/truncation	5	Fahsold *et al.*, 2000 Messiaen *et al.*, 2000 Mattocks *et al.*, 2004 Upadhyaya *et al.*, 2004
Exon 41	C7296A	frameshift	Deletion/ ?	1	Kluwe *et al.*, 2003
Exon 41	7308insA	frameshift	Deletion/ truncation (2436aa prot.)	1	Ars *et al.*, 2003
Exon 41	7627delAC	frameshift	Insertion/truncation	1	John *et al.*, 2000
Exon 41	7331delA	frameshift	Deletion/ truncation	1	Origone *et al.*, 2002
Exon 41	7337delC	frameshift	Deletion/ truncation (2466aa prot.)	1	Ars *et al.*, 2000a
Exon 41	7367delCC	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 41	7372delC	frameshift	Deletion/ truncation (2466aa prot.)	2	Serra *et al.*, 2001 Ars *et al.*, 2003
Intron 41	7395-1 G>A	skip of exon 42?	as/ truncation	1	Fahsold *et al.*, 2000
Exon 42	CT7424AG	S2475X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 42	7427insTC	frameshift / S2502X	Insertion/truncation	1	Mattocks *et al.*, 2004
Exon 42	C7457T	T2486I	Missence/ ?	1	Fahsold *et al.*, 2000
Exon 42	7458delC	frameshift	Deletion/ truncation	1	Osborn and Upadhyaya, 1999
Exon 42	7485insGG	frameshift	Insertion/truncation	1	Purandare *et al.*, 1994
Exon 42	C7486T	R2496X	Nonsense/ ?	3	Purandare *et al.*, 1994 Messiaen *et al.*, 2000 Mattocks *et al.*, 2004
Exon 43	7633insC	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Intron 43	7676-2 A>G	skip of exon 43	as/ truncation	1	Osborn and Upadhyaya, 1999
Exon 44	7682delAG	frameshift	Deletion/ ?	1	De Luca *et al.*, 2004
Exon 44	C7699T	Q2567X	Nonsense/truncation	1	Fahsold *et al.*, 2000
Exon 44	A7701G	Q2567Q	Silent/?	1	Fahsold *et al.*, 2000
Exon 44	C7702T	Q2568X	Nonsense/truncation (2567aa prot.)	3	Fahsold *et al.*, 2000 Ars *et al.*, 2003
Exon 44	7719insA	frameshift	Insertion/ truncation (2537aa prot.)	1	Ars *et al.*, 2003
Exon 44	7720delA	frameshift	Deletion/ truncation (2601aa prot.)	1	Ars *et al.*, 2003
Exon 44	7745del10bp	frameshift	Deletion/ truncation	1	Shen *et al.*, 1993
Exon 44	C7783T	Q2595X	Nonsense/truncation	1	Origone *et al.*, 2002
Intron 44	7806+1 G>T	skip of exon 44	ds/ truncation (2567aa prot.)	1	Ars *et al.*, 2003
Intron 44	7807-2 A>T	skip of exon 45	as/?	1	Serra *et al.*, 2001
Exon 45	C7846T	R2616X	Nonsense/truncation	2	Fahsold *et al.*, 2000 De Luca *et al.*, 2004
Exon 45	7884delGT	frameshift	Deletion/ ?	1	Messiaen *et al.*, 2000
Exon 45	7900delC	frameshift	Deletion/ ?	1	De Luca *et al.*, 2004
Intron 45	7907+1 G>A	skip of exon 45	ds/ truncation	1	Fahsold *et al.*, 2000
Intron 45	7907+5 G>A	frameshift	ds/ ?	1	De Luca *et al.*, 2004
Intron 45	7907+790 C>G	7907ins70	ds/ truncation (2637aa prot.)	1	Ars *et al.*, 2003
Intron 45	7908-2 A>G	skip of exon 46?	as/ truncation	1	Fahsold *et al.*, 2000
Exon 46	7926insT	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Exon 46	8016delA	frameshift	Deletion/ ?	1	Messiaen *et al.*, 2000
Exon 46	8024delC	frameshift	Deletion/ truncation	1	Fahsold *et al.*, 2000
Exon 46	8040delT	frameshift	Deletion/ truncation (2716aa prot.)	1	Ars *et al.*, 2003
Exon 46	8042insA	frameshift	Insertion/ truncation (2680aa prot.)	1	Ars *et al.*, 2000a
Exon 46	C8047T	E2683X	Nonsense/ truncation (2682aa prot.)	1	Ars *et al.*, 2003
Exon 47	8081delC	frameshift	Deletion/ truncation	1	Osborn and Upadhyaya, 1999
Exon 47	8092insTT	frameshift	Insertion/truncation	1	Fahsold *et al.*, 2000
Exon 47	C8093G	S2698X	Nonsense/ ?	1	Kluwe *et al.*, 2003
Exon 48	8134delAA	frameshift	Deletion/ truncation (2713aa prot.)	1	Ars *et al.*, 2000a
3' end	Del11kb	?	?	1	Viskochil *et al.*, 1990

Table 4.3: Polymorphisms in the *NF1* gene
Het, heterozygosity

Exon/Intron	Genomic mutation	Aminoacid mutation	Type	Het	Reference
Intron 1	1-22 G>C		Intronic	3%	Mattocks *et al.*, 2004
Intron 2	61-4delT		Intronic	0.8%	Mattocks *et al.*, 2004
Exon 2	C168T		Silent	8%	Mattocks *et al.*, 2004
Intron 3	288+41 G>A		Intronic	21%	De Luca *et al.*, 2003
Exon 4b	T528A	D176E	Missence	0.8%	Mattocks *et al.*, 2004
				?	Fahsold *et al.*, 2000
				1%	De Luca *et al.*, 2003
				?	Ars *et al.*, 2003
Intron 5	730-6 A>C		Intronic	2%	De Luca *et al.*, 2003
Exon 5	A702G			53%	Mattocks *et al.*, 2004
				?	Fahsold *et al.*, 2000
Exon 6	G846A	Q282Q	Silent	2%	De Luca *et al.*, 2003
Intron 7	889-31delATTAT		Intronic	0.8%	Mattocks *et al.*, 2004
Intron 7	1063-24delT		Intronic	2%	De Luca *et al.*, 2003
Intron 7	1063-28 G>C		Intronic	8%	De Luca *et al.*, 2003
Intron 10a	1393-32 C>T		Intronic	57%	Mattocks *et al.*, 2004
Intron 10b	1528-29delT		Intronic	44%	Mattocks *et al.*, 2004
				?	Fahsold *et al.*, 2000
Intron 10c	1641+39 C>T			?	Fahsold *et al.*, 2000
Intron 11	1642-25 T>C		Intronic	5%	Mattocks *et al.*, 2004
Exon 12a	T1810C	L604L	Silent	1.4%	Mattocks *et al.*, 2004
Exon 13	G2034A			?	Fahsold *et al.*, 2000
Intron 13	2252-31 A>T		Intronic	?	Fahsold *et al.*, 2000
Intron 16	C2617T	R873C	Missence	0.8%	Mattocks *et al.*, 2004
Intron 16	G2531A	G844G	Silent	0.8%	Mattocks *et al.*, 2004
Intron 17	2851-16 T>C		Intronic	4.5%	Mattocks *et al.*, 2004
Intron 19a	3198-111 C>T		Intronic	5%	Han *et al.*, 2001
Intron 19b	3315-142 T>C		Intronic	3%	Han *et al.*, 2001
Intron 19b	3315-130 G>C		Intronic	?	Fahsold *et al.*, 2000
Exon 21		N1229S	Missence	?	Ars *et al.*, 2003
Intron 20	3496+33 C>A		Intronic	33%	De Luca *et al.*, 2004
Intron 20	3497-88 C>A		Intronic	3%	Han *et al.*, 2001
Intron 22	C3867T	F1289F	Silent	0.8%	Mattocks *et al.*, 2004
Intron 23.1	3974+27 insC/delC		Intronic	3%	Han *et al.*, 2001
Intron 25	4368-46G>C		Intronic	?	Fahsold *et al.*, 2000
				8%	De Luca *et al.*, 2004
Exon 28	G4866C	V1622V	Silent	3%	De Luca *et al.*, 2004
Exon 28	G5172A	K1724K	Silent	?	Fahsold *et al.*, 2000
				3%	De Luca *et al.*, 2004
Intron 28	5205+23 T>C		Intronic	?	Fahsold *et al.*, 2000
				7%	Han *et al.*, 2001
Exon 29		R1809C	Missence	?	Ars *et al.*, 2003
Exon 29		R1825W	Missence	?	Ars *et al.*, 2003
Intron 29	5546+19 T>A		Intronic	41%	De Luca *et al.*, 2003
				7%	Han *et al.*, 2001
Intron 29	5546+25 G>A		Intronic	3%	Han *et al.*, 2001
Intron 29	5546-19 T>A		Intronic	56%	Mattocks *et al.*, 2004
Intron 31	5943+11 A>T		Intronic	2%	De Luca *et al.*, 2004

Exon/Intron	Genomic mutation	Aminoacid mutation	Type	Het	Reference
Intron 32	6048+8 C>G		Intronic	0.8%	Mattocks *et al.*, 2004
Intron 33	C6173A	A2058D	Missence	1%	Mattocks *et al.*, 2004
Intron 34	6579+45 T>A		Intronic	?	Fahsold *et al.*, 2000
				5%	De Luca *et al.*, 2004
Intron 37	6859-17 G>A		Intronic	1%	Han *et al.*, 2001
Intron 38	7000-13 T>C		Intronic	3%	Han *et al.*, 2001
Intron 39	7126+37 G>C		Intronic	?	Rodenhiser and Hovland, 11995
				5%	De Luca *et al.*, 2004
				24%	Han *et al.*, 2001
				12%	Mattocks *et al.*, 2004
Intron 41	7395-29 G>A		Intronic	?	Fahsold *et al.*, 2000
				10%	De Luca *et al.*, 2004
				49%	Han *et al.*, 2001
Intron 42	7395-29 A>G			44.5%	Mattocks *et al.*, 2004
Intron 46	8050+20 A>G		Intronic	?	Fahsold *et al.*, 2000
				2.2%	Mattocks *et al.*, 2004

Table A.4: Somatic mutations in the *NF1* gene in NF1 tumours

P, number of patients. * indicates that the same mutation was reported in the germline of unrelated NF1 patient(s).

Exon/Intron	Genomic mutation	Aminoacid mutation	Type/effect	P	Reference
Exon 4b	546delTATC	frameshift	Deletion/ truncation	1	Sawada *et al.*, 1996
Intron 4c	655-8del6	skip of exon 5	as/ ?	1	Serra *et al.*, 2001
Exon 7	C910T	R304X	Nonsense/truncation	1*	Upadhyaya *et al.*, 2004
Intron 9	1260+1 G>A	1260ins13	ds/ truncation	2	Serra *et al.*, 2001 Eisenbarth *et al.*, 2000
Intron 9	1260+1604 A>G	1260ins12+9a	ds/?	1	Serra *et al.*, 2001
Exon 10a	C1381T	R461X	Nonsense/truncation	1*	Wiest *et al.*, 2003
Exon 10c	1528-14_1546del33	frameshift	as/ ?	1	Wiest *et al.*, 2003
Exon 10c	1541delAG	frameshift	Deletion/ truncation	1*	Upadhyaya *et al.*, 2004
Exon 10c	A1556C	Q519P	Missence/ ?	1	Upadhyaya *et al.*, 2004
Intron 10c	1641+1 G>A	skip of exon 10c?	ds/ ?	1	Wiest *et al.*, 2003
Exon 12b	1888delG	frameshift / 630X	Deletion/ truncation	1	Upadhyaya *et al.*, 2004
Exon 13	2033delC	frameshift	Insertion/truncation 687X	1	Upadhyaya *et al.*, 2004
Exon 13	2088delG	frameshift	Deletion/ truncation	1	Upadhyaya *et al.*, 2004
Exon 13	C2246G	S749X	Nonsense/truncation	1	Serra *et al.*, 2001
Exon 14	C2266T	Q756X	Nonsense/ Skipping E14	1	Serra *et al.*, 2000
Exon 15	2341del18	in-frame (aa 781-786)	deletion/?	1	Upadhyaya *et al.*, 2004
Exon 16	C2446T	R816X	Nonsense/truncation	1*	John *et al.*, 2000
Exon 16	2815delA	frameshift	Deletion/ ?	1	Serra *et al.*, 2001
Exon 17	2928del13	2928del13	Deletion/?	1	Serra *et al.*, 2000
Exon 18	C3049T	Q1017X	Nonsense/truncation	1*	Weist *et al.*, 2003
Exon 19b	3303del19	frameshift	Deletion/ splicing defect	1	Weist *et al.*, 2003
Exon 21	C3721T	R1241X	Nonsense/?	1*	Eisenbarth *et al.*, 2000
Exon 23.1	C3916T	R1306X	Nonsense/truncation	1*	Weist *et al.*, 2003
Exon 23.2	C4021T	Q1341X	Nonsense/ ?	1	Eisenbarth *et al.*, 2000
Exon 23.2	C4084T	R1362X	Nonsense/ ?	1*	Eisenbarth *et al.*, 2000
Intron 26	4514+1 G>C	skip of exon 26	ds/ ?	1	Serra *et al.*, 2001
Exon 27b	4750insT	frameshift	Insertion/ ?	1	Weist *et al.*, 2003
Intron 27b	4773-5 C>T	?	intronic/?	1	Upadhyaya *et al.*, 2004
Intron 27b	4773-2 A>T	frameshift	as/ -433aa / -293aa	1	Messiaen *et al.*, 2000
Exon 28	4907dup14bp	frameshift	Duplication/ truncation	1	Eisenbarth *et al.*, 2000
Intron 28	5205+1 G>A	frameshift	ds/ -18aa (in-frame)	1*	Weist *et al.*, 2003
Exon 31	5767delTTTG	frameshift	Deletion/ ?	1	Weist *et al.*, 2003
Exon 31	5774delT	frameshift	Deletion/ ?	1	Serra *et al.*, 2001
Exon 33	6292del31	frameshift	Deletion/ ?	1	Serra *et al.*, 2001
Exon 34	A6387C	R2129S	Missence/ ?	1	Upadhyaya et al., 2004
Exon 40	7168delGA	frameshift	Deletion/ ?	1	John *et al.*, 2000
Exon 41	C7285T	R2429X	Nonsense/truncation	1*	Upadhyaya *et al.*, 2004
Intron 44	7807-2 A>T	skip of exon 45	as/?	1	Serra *et al.*, 2001

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