THE GENETIC BASIS OF MEDULLOBLASTOMA

presented by

Paula Elizabeth Finn

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University Department of Neurosurgery, Institute of Neurology, Queen Square, LONDON, WC1N 3BG

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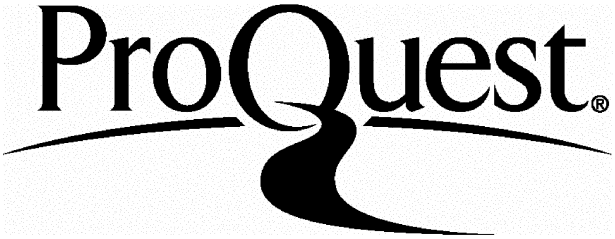


ABSTRACT

Medulloblastoma accounts for 25% of all paediatric brain tumours. Most of these tumours respond poorly to therapy and whilst advances in chemotherapy and radiotherapy have improved long term survival, many patients suffer late relapses that are usually fatal. Long term survival is only about 30%. Loss of chromosome 17p is the most common cytogenetic abnormality, observed in 25-50% of cases. Loss of heterozygosity (LOH) analysis has defined the minimum region of deletion distal to p53 suggesting that another gene on 17p contributes to the pathogenesis of medulloblastoma. The aim of the present study was to define regions of allelic loss on chromosome 17p, to identify other genetic changes by comparative genomic hybridisation (CGH) and to correlate the findings with clinical parameters.

LOH analysis was used to identify allele loss at which putative tumour suppressor genes may be located on chromosome 17p in a series of medulloblastoma. LOH was determined using polymorphic microsatellite markers to amplify the DNA from specific regions of chromosome 17p between 17p13.1 and 17p13.3. The frequency of LOH was higher than seen in previous studies and was found in all tumours with the most consistent region of loss at 17p13.1, distal to p53. CGH was used to screen the entire genome and a number of non-random regions of genomic imbalance were observed including gain at 2q, 4q, 5q, 6q, 9p and 13q and loss at 1p, 9q, 10, 12q, 16, 17, 19 and 22. The most consistent regions of loss detected by CGH, at 9q, 10q, 12q, 16q and 22q were then studied using LOH analysis.

It was found that those children ≤ age 3 years had a significantly better prognosis than those > than 3 years of age. Loss of 16q in the CGH studies and LOH 16q in the microsatellite analysis was significantly associated with longer survival. In conclusion, this study has demonstrated that medulloblastomas are an extremely heterogeneous tumour type. It has provided new evidence of the involvement of several chromosomes in their development, which shows that their pathogenesis is far more complicated than has previously been thought.

CONTENTS

FIGURES AND TABLES

SUMMARY OF ABBREVIATIONS OF TERMS USED

ABR	Active BCR related
APC	Adenomatous Polyposis Coli
AT/RTs	atypical teratoid/rhabdoid tumours
BCCs	Basal Cell Carcinomas
BrDU	Bromodeoxyuridine
CAS	Capillary Assay Solution
CGH	Comparative genomic hybridisation
CNAs	Copy Number Aberrations
CNS	Central Nervous System
CSART	Craniospinal Radiation Therapy
CSF	Cerebrospinal Fluid
DMBT1	Deleted in Malignant Brain Tumours 1
DMs	Double Minutes
DMSO	Dimethyl Sulphoxide
EDTA	Ethylene Diamine Tetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
FAP	Familial adenomatous polyposis
FISH	Fluorescence In Situ Hybridisation
FCS	Foetal Calf Serum
GEU	Gel Electrophoresis Unit
GFAP	Glial Fibrillary Acidic Protein
GS	Gorlin Syndrome
HBSS	Hanks Balanced Salt Solution
HIC-1	Hypermethylated in Cancer-1
i(17q)	Isochromosome 17q
LI	Labelling Index
LOH	Loss of heterozygosity
NBCC	Nevoid Basal Cell Carcinoma
NP-40	Nonidet P-40
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PNETs	Primitive Neuroectodermal Tumours
Rb	Retinoblastoma
RFLP	Restriction Fragment Length Polymorphism
SHH	Sonic Hedgehog
SMO	Drosophila's smoothened
SSC	Sodium Chloride/Sodium citrate
SSCP	Single Strand Conformation Polymorphism
SV40	Simian virus 40
TAE	Tris Acetate
TE	Tris Edta
TrkC	Tyrosine Kinase C
WHO	World Health Organisation

Chapter 1

INTRODUCTION

With the exception of leukaemia and lymphoma, brain tumours are the most frequent type of childhood cancer (Bigner et al., 1997). Most children who suffer from a brain tumour will die from the disease despite the advances made in radiation therapy and chemotherapy over the past few years (Belza et al., 1991).

Advances in molecular biology during the past 15 years have contributed to the understanding of the pathogenesis of many types of neoplasms. Improved insights into the causes of brain tumour development may eventually lead to more specific therapies. Currently, however, the interest of most of those involved in cancer research is focussed on the search for new and better prognostic markers in brain tumours since long term survivors are often confronted by serious late sequelae caused by therapy (Michiels et al., 2000). Such indicators may help to decide which patients with brain tumours will gain benefit from radiotherapy and/or chemotherapy as well as surgery whilst suffering from a minimum of late effects.

Age and sex distribution of primitive neuroectodermal tumours

Primitive neuroectodermal tumours (PNETs) are the most frequently occurring malignant childhood brain tumours. The most common form of this type of neoplasm is medulloblastoma which constitutes 80% of PNETs. Medulloblastoma accounts for nearly 30% of childhood CNS neoplasms (Russell and Rubenstein, 1989) and the peak incidence is at 7 years of age (Giangaspero et al., 1997). In the United States alone, approximately 350 new cases of medulloblastoma are identified each year (Friedman et al., 1991). Males

are traditionally affected more frequently than female children. In a Swedish Cancer Registry study, the overall male to female ratio for medulloblastoma was 1.8 : 1 in comparison to a ratio of 1.01 : 1 for primary brain tumours as a whole (Lannering et al., 1990). Seventy per cent of medulloblastoma occur in individuals younger than 16 years of age (Arseni et al., 1981; Roberts et al., 1991) and overall, about half of medulloblastoma occur in the first 10 years of life (Russell and Rubinstein, 1989). In adults, 80% of neoplasms of this type arise in the 21-40 years age group (Baker et al., 1991; Hubbard et al., 1989). Critically, young age (< 3 years old) is associated with a worse prognosis (Kopelson et al., 1983).

Location and differentiation of medulloblastoma

PNETs may occur anywhere in the brain but have a propensity for the posterior fossa and are divided into two types; supratentorial and infratentorial. Medulloblastoma is a subset of PNETs, it is an infratentorial tumour and three quarters of them occur in a midline or vermal location within the cerebellum. The tumours can grow in almost any direction with a propensity to invade the fourth ventricle as well as the overlying subarachnoid spaces (Tomlinson et al., 1992).

Currently, the WHO classifies embryonal tumours as those composed mainly of undifferentiated cells (Kleihues et al., 1993). PNETs fall into this category and are small cell pluripotent neoplasms thought to arise from a neural stem cell which has the capacity to differentiate along neuronal, glial, ependymal and/or mesenchymal pathways (Molenaar et al., 1989).

Relationship of medulloblastoma to PNETs

An issue which has aroused great debate is the actual relationship of medulloblastoma to the general PNET classification, i.e. whether the medulloblastoma is a unique tumour with a unique cell of origin, such as the external granule cell, as hypothesised above, or whether it has arisen from a non-site-specific disseminated undifferentiated neuroepithelial cell which also gives rise to other members of the PNET group such as neuroblastoma, pineoblastoma, retinoblastoma and ependymoblastoma. The debate remains unresolved but medulloblastoma still exists as a clinical entity. However, a Working Group of the World Health Organisation (WHO) recommended that the diagnosis of PNET be used as an operative term for cerebellar medulloblastoma as well as for neoplasms that are indistinguishable morphologically from the medulloblastoma but which occur at other sites in the CNS (Kleihues and Scheithauer, 1993).

At the time of diagnosis, medulloblastoma may present without evidence of differentiation or with differentiation along one or more lineages. This differentiation is most often defined by immunohistochemical studies with antibodies to neurofilament proteins, such as nestin (Tohyama et al. 1992), glial fibrillary acidic protein, vimentin, desmin (Gould et al. 1990) and retinal S antigen (Maraziotis et al., 1992). One hypothesis suggests that medulloblastoma originates from the external granular layer of the developing cerebellum (Tomlinson et al., 1992; Kosmik et al., 1995), and genes which are expressed in these cells are also expressed in medulloblastoma biopsies (Biegel, 1999).

Histopathology and medulloblastoma variants

Several histological variants of medulloblastoma are recognised. Classical medulloblastoma is the most common variant, constituting approximately 65% of this

tumour type (Provias and Becker, 1996). It is characterised by a highly cellular small cell appearance and has a high nuclear to cytoplasm ratio. However, round cells with less condensed chromatin are often intermingled and occasionally form the main population. Cells are often clustered as Homer-Wright rosettes and although mitoses are usually numerous, they are infrequent in approximately 25% of cases (Burger et al., 1987; Burger and Scheithauer, 1994). Apoptosis is frequent whilst geographic areas of necrosis are less common. As in all tumours, angiogenesis is present and plays an important role in tumour development and progression. However, morphological evidence of angiogenesis is less striking than in malignant glial tumours such as glioblastoma multiforme (Provias and Becker, 1996). The tumour cells of the classical medulloblastoma form patternless sheets of undifferentiated cellular elements. Vascular proliferation, calcification and massive haemorrhages are observed in a minority of cases.

A subset of medulloblastoma tumours show divergent cellular morphology and architecture which has given rise to histologic subtypes including the desmoplastic form as well as the medullomyoblastoma and the pigmented, or melanocytic, medulloblastoma (Kalino et al., 1987) which has focal areas of rhabdomyosarcomatous, glial, neuronal and melanocytic differentiation (Russell and Rubenstein, 1989).

After the classical type, the desmoplastic medulloblastoma is the most common variant, representing approximately 10% of childhood tumours and a third of medulloblastoma (Provias and Becker, 1996). It is often associated with a more lateral position in the hemisphere and arises partially as a result of tumour invasion into the overlying meninges. The name 'desmoplastic' refers to a prominent fibrous stromal element which is rich in collagen and reticulin and which separates the small tumour cells into rows. At surgery it may appear as a lobulated and well-defined lesion. In different areas of desmoplastic medulloblastoma there are often nodular, reticulin-free areas, known as 'pale islands',

which contain small neuronal cells with reduced proliferative activity. These islands in the desmoplastic medulloblastoma represent areas of greater neuronal differentiation (Trojanowski et al., 1992).

The term cerebellar neuroblastoma has been used to describe desmoplastic medulloblastoma showing extreme lobularity (Burger and Scheithauer, 1994; Pearl and Takei, 1981). In these cases, the nodules contain small round cells which are similar to the neurocytes of central neurocytoma, whilst the nodules themselves have unusual, elongated profiles. The nodular cells often form linear streams and are embedded in a fibrillar neuropil-like matrix. Neoplasms such as these sometimes develop into more-differentiated ganglion cell tumours (de Chadarevian et al., 1987).

In the medullomyoblastoma variant, striated and, occasionally, smooth muscle cells are present. In addition, neuronal and glial cell differentiation may coexist in the same lesion (Schiffer et al., 1992). When the medullomyoblastoma contains differentiated cells along ectodermal, mesodermal and endodermal lines it is reclassified as a teratoma (Berger et al., 1995).

So called melanocytic medulloblastomas are quite unusual (Maire et al., 1992). In this variant, melanin-containing cells may be found between the typical small undifferentiated medulloblastoma cells (Dolman, 1988).

Another possible variant of medulloblastoma, termed large cell medulloblastoma, was reported in 1992 when Giangaspero and colleagues described 4 patients with cerebellar tumours consisting of unusually large round cells with prominent nucleoli and large areas of necrosis, and whose clinical course evolved and terminated in death relatively rapidly (Giangaspero et al., 1992). However, a comparison of the clinical and pathological features

of these cases suggested that they actually belong to the diagnostic entity known as atypical teratoid/rhabdoid tumour of the cerebellum (Packer et al., 1999).

Aetiology

The aetiology of medulloblastoma is unknown. Despite some preliminary observations to the contrary, epidemiological studies have not shown an undisputed link between parental occupation or exposures in the development of this tumour type (Packer et al., 1999). Similarly, there is conflicting evidence as to whether exposure to N-nitroso compounds leads to the development of medulloblastoma (Bunin et al., 1993). In experimental animals, cerebellar neoplasms with a phenotype similar to that of medulloblastoma have been induced in hamsters by intracerebral injection with JC virus (Matsuda et al., 1987; Zu Rhein and Varakis, 1979) and in rats by retrovirus-mediated transfection of foetal rat brain cells with the large T antigen of simian virus 40 (SV40) (Eibl et al., 1994). Other models of medulloblastoma-like PNETs have been produced using transgenic mice that express SV40 large T antigen under the influence of different promoters (Fung and Trojanowski, 1995). It is a topic of debate as to whether SV40 is found in medulloblastomas. For example, in one study, it is argued that although SV40 DNA has been identified in choroid plexus tumours and ependymomas, the available evidence from the transgenic mice work described above is not sufficient to support a role for SV40 or related oncogenic DNA viruses in the aetiology of human medulloblastoma (Lednicky et al., 1995). However, in another investigation, SV40 large tumour antigen (Tag) was found in 33% (2/6) of medulloblastoma samples studied (Zhen et al., 1999) and the authors argued that SV40 is involved in medulloblastoma tumourigenesis. Conversely, an even more recent study found SV40-like sequences in only 2/116 medulloblastoma samples and it is asserted that this low frequency of virus sequences argues against a major role for SV40 in the pathogenesis of medulloblastoma (Weggen et al., 2000). It is clear that more work is needed in order to

establish a clear-cut relationship between viral exposure and the development of medulloblastoma in humans.

Summary of current treatment strategies

(i) Surgery

Surgery is a critical component of treatment for patients with medulloblastoma. However, although surgical mortality is low, the consequences of radical surgery may be profound effects on neurocognitive functioning and quality of life (Kao et al., 1994). For example, there has been increasing concern about a surgically related complication known as the cerebellar mutism syndrome (Packer, 1999). Patients become mute 1 or 2 days after tumour operation and the disorder lasts for 1 to 4 months although the pathogenesis is still unknown (Janssen et al., 1998). A growing number of paediatric patients with mutism following posterior fossa surgery have been recognised. Problems such as this must be considered alongside the issue of the arguable success of radiotherapy and chemotherapy as postoperative adjuncts (Rorke et al., 1997).

There still remains some debate as to whether a patient who has had a 'total' resection will do better than one who has had a subtotal resection; as yet it is unknown as to whether the extent of resection favourably affects survival. For example, one study found that there is no significant advantage in terms of event-free survival between patients who undergo a total or near-total removal and those who have a less aggressive resection (Evans et al., 1990). Other investigators report that partial excision and biopsy are not justified as they are associated with poorer outcomes than more aggressively resected tumours (Packer et al., 1994). A more recent study evaluating prognostic factors, including extent of surgical intervention, in medulloblastoma looked at 173 patients between 1988 and 1998 (Jenkin et

al., 2000). It was found that whilst 78% of patients underwent at least 90% tumour resection, total resection was not a sensitive prognostic factor. Furthermore, the authors assert that in order to improve the number of patients able to complete subsequent radiation therapy, consideration should be given to limiting resection when the attainment of total resection is likely to render a morbid outcome (Jenkin et al., 2000). Another found that when assessing the effect of extent of surgery on progression free survival (PFS), the type of statistical analysis used was critical (Michiels et al., 2000). When using univariate analysis to examine the data from a series of children with medulloblastoma extent of resection was found to have a significant influence on PFS, but on multivariate analysis this factor had no significance.

In summary however, the consensus of opinion is that the aggressive tumour removal approach is the one that should be pursued, and that radical surgery is generally safe. However, it should be considered that it is difficult to determine what constitutes a total resection, and such a procedure is basically impossible in those patients whose tumour has already disseminated at the time of diagnosis. In addition, there is no benefit to be gained by attempting to remove tumours from within the brain stem or by re-operating on patients diagnosed with small amounts of residual disease prior to radio or chemotherapy as in these cases, these adjuvant treatments are sufficient without the added trauma of surgery (Rorke et al., 1997).

(ii) Radiotherapy

Developments in the treatment of medulloblastoma with radiotherapy have progressed at the same rate as those in surgery, chemotherapy and diagnosis. Although failure of radiotherapy at the primary site continues to be the predominant barrier to a cure for patients with medulloblastoma, improvements in radiotherapy equipment and techniques as well as

the use of altered fractionation regimens, optimised radiation dosage and other adjuvant therapies have at least combined to produce a more favourable outcome. However, survival is not the only important consideration. Administration of radiotherapy may produce considerable long term toxicity and treatment must be adjusted accordingly. Therefore, in recent years, adaptations of treatment have concentrated as much on improvements in quality of life as in survival.

Toxicity caused by craniospinal radiation therapy (CSART) can be high, particularly in young children (Johnson et al., 1994; Packer et al., 1989). Attempts have been made to reduce toxicity by lowering dosage after some studies demonstrated favourable outcomes in select children treated with doses considerably lower than the usual 3600cGy (Brand et al., 1987; Halberg et al., 1991; Hughes et al., 1988). A Paediatric Oncology Group/Children's Cancer Group study was undertaken which compared reduced CSART doses (2340 cGy) against standard radiation for patients with low stage disease. The trial closed prematurely because there was an increased number of failures among patients who received reduced doses (Rorke et al., 1997). There has also been an unsuccessful attempt to completely eliminate the whole-brain component of the CSART. The results of the trial were unfavourable as only 3 of the 16 children survived (Bouffet et al., 1992). It has been argued that the differences between reduced radiation and standard radiation have become less significant over time but that the more favourable outcome is still observed in those patients who receive standard CSART doses (Deutsch et al., 1996). However, one 1996 study found an event free survival rate of greater than 80% at a median of 3 years in children treated with reduced dose CSART (2400cGy) and chemotherapy (Packer et al., 1996). Moreover, a more recent investigation of 65 children with non-disseminated medulloblastoma found more favourable survival rates in those who had undergone reduced-dose CSART (2340cGy) with adjuvant chemotherapy when compared with rates

obtained in studies of patients who had received standard dose radiotherapy alone or standard dose radiotherapy plus chemotherapy (Packer et al., 1999).

Thus, it is still arguable as to whether it is more beneficial for patients with medulloblastoma treated with adjuvant radiotherapy alone to have the standard or reduced dose. Overall though, CSART is necessary in the treatment of medulloblastoma as well as a posterior fossa boost. Without this boost, the disease may progress leading to poorer survival rates which outweigh the effects of toxicity. Such treatment results in a current survival rate of 40-90% at 5 years depending on such factors as age, stage, and extent of surgical resection (Deutsch et al., 1996; Evans et al., 1990; Packer et al., 1994; Packer et al., 1999).

A new approach to improve the efficacy of radiotherapy which is currently under study for children with late stage medulloblastoma is hyperfractionated radiotherapy. The aim is to gain greater control over tumour progression in those patients with advanced disease by increasing radiation dose without a consequent increase in toxicity. Although single institutional series' of patients treated with hyperfractionated radiotherapy effectively have been published (Allen et al., 1991; Weil et al., 1994), larger studies conducted over greater periods of time are needed before this form of radiotherapy can be reasonably compared with conventional therapy.

Infants (< 3 years of age) with medulloblastoma are arguably the most difficult patients to treat with radiotherapy as there is greater likelihood of dissemination of disease at diagnosis. Moreover, due to the plasticity of the brain in such young patients, these children are more susceptible to the side effects of the treatment (Allen and Epstein, 1982). The brain is still immature in this age group with a very high mitotic rate of spongioblasts and incomplete myelination (Massimino et al., 2000). This is important in that it poses a

problem with regard to radiation therapy; the essential radiation insult is a demyelinating lesion which is more severe in such young children and results in well recognised cognitive and endocrine sequelae (Massimino et al., 2000). A recent investigated outcome status in long term survivors in a consecutive series of 111 children and young adults treated for medulloblastoma (Helseth et al., 1999). It was found that 61% of patients had deficits with respect to learning ability, locomotion and sociability and a younger age was correlated with more serious problems. In all but 3 cases, the major cause of permanent deficits was radiotherapy. It is thus arguable as to whether younger children (particularly those < 2 years of age) should be treated with radiotherapy. Since improvements in this treatment have contributed to improvements in the prognosis of medulloblastoma patients generally (Rorke et al., 1997), and radiation to the posterior fossa has been found to have a positive significant influence on PFS in patients with these tumours (Michiels et al., 2000), the issue with such young patients is whether radiotherapy should be administered to improve their prognosis in terms of survival when the long term effects on quality of life are so damaging.

(iii) Chemotherapy

Chemotherapy has become a crucial part of the treatment of most, if not all, children with medulloblastoma over the last 25 years since its use has shown significant indications of efficacy. Some vital information came from studies in which children with newly diagnosed disease were treated with a variety of chemotherapeutic agents (Packer et al., 1991). Beginning in 1983, the children were assigned to risk groups; those with 'standard risk' medulloblastoma were treated with radiotherapy alone whilst those in the 'poor risk' group received radiation therapy as well as adjuvant chemotherapy in the form of CCNU, vincristine and cisplatin. In comparison with children treated between 1975 and 1983 who had only received radiotherapy, it was found that there was no difference in disease-free

survival rates over time for children with standard risk factors, but that there was a significant difference in the 5-year survival rate for poor-risk patients treated prior to 1983 (35%) compared to those treated later (87%). The authors argued that their results strongly suggest that chemotherapy has a role for some, and possibly all, children with medulloblastoma.

A variety of different chemotherapeutic agents, including cisplatin, carboplatinum, cyclophosphamide and etoposide are those used in treatment at the time of disease recurrence (Allen et al., 1987; Ashley et al., 1996; Bertolone et al., 1989; Moghrabi et al., 1995). Despite these high response rates long term survival (over 1 year) has rarely been demonstrated in patients who relapse after primary treatment with any combination of the chemotherapeutic agents listed above and radiotherapy (Allen et al., 1987; Ashley et al., 1996; Bertolone et al., 1989; Packer, 1990).

More recently, higher doses of chemotherapy have been administered to children with recurrent tumours. A variety of agents have been used in these treatment regimens, and the most favourable results have been reported after the use of thiotepa, etoposide and carboplatinum (Finlay et al., 1994; Finlay et al., 1996). In one study, 8 of 23 patients treated for recurrent medulloblastoma survived without disease progression for a median of 36 months from treatment (Finlay et al., 1994). In another investigation, busulfan and thiotepa were shown to successfully control tumour growth in 11 of 13 children with progressive medulloblastoma where previous high-dose conventional chemotherapy had failed (Dupuis-Girod et al., 1996). However, all patients received local radiotherapy after treatment with the chemotherapeutic agents and thus long-term control with chemotherapy alone cannot be assessed.

Chemotherapy is most beneficial to children with high risk medulloblastoma when it is combined with radiotherapy after surgery. Two large studies performed simultaneously by the Children's Cancer Group and the International Society of Pediatric Oncology in the late 1970s and early 1980s showed that addition of CCNU and vincristine therapy used during (vincristine) and after (vincristine and CCNU) radiotherapy increased survival as compared to treatment with radiotherapy alone (Evans et al., 1990; Finlay et al., 1994). In the Childrens Cancer Group trial, children with the most extensive tumours (in terms of both large tumours at the primary site and dissemination at the time of diagnosis) who had received chemotherapy showed an event-free survival rate of 48% compared to 0% for those who received radiotherapy alone (Evans et al., 1990). In 2 subsequent Childrens Cancer Group studies, initially described in the radiotherapy section (above) large groups of children (68 and 65 respectively) *without* disseminated medulloblastoma were treated with reduced dose CSART (Packer et al., 1996; Packer et al., 1999). In the first of these studies, the CSART (2400 cGy) was combined with local radiotherapy (5400cGy) and vincristine during radiotherapy and CCNU with vincristine and cisplatin following radiotherapy. Event-free survival at a median of three years was greater than 80% (Packer et al., 1996). In the second study, CSART (2340 cGy) was given with local radiation therapy (5580 cGy) and vincristine during radiotherapy and lomustine with vincristine and cisplatin after radiation. The progression free survival rate after 3 years was 86% +/- 4% (Packer et al., 1999).

Thus, these studies suggest that adjuvant chemotherapy is beneficial for children with medulloblastoma. Children with non-disseminated posterior fossa tumours have five-year survival rates of at least 80% after treatment with combination chemotherapy. Comparatively, treatment with radiotherapy alone has resulted in five-year disease-free survival rates ranging from 55-65% only. For children with disseminated medulloblastoma, treatment with radiotherapy and adjuvant chemotherapy has not resulted

in such high survival rates as for those patients with non-disseminated disease (Packer et al., 1990; Packer et al., 1994; Zeltzer et al., 1995).

Overall, whilst advances in chemotherapy and radiotherapy have improved long-term survival for medulloblastoma (Packer et al, 1999), many patients suffer late relapses which are usually fatal. Currently, 5-year survival rates are approximately 50% -70% (Kleihues, 1997) but long-term survival is only about 30% (Friedman et al., 1991).

Prognostic factors

(i) Clinical and histopathological (therapy, age, histology, differentiation, proliferation)

The prognostic significance of age and extent of surgical resection are complicated and controversial issues (Giangaspero et al., 1997). Overall, extensive residual tumour following surgical intervention is regarded as less favourable. It is generally agreed that radical surgery is generally safe and therefore the aggressive tumour removal approach is the one that should be undertaken .

One recent study found that age < 14 years was an unfavourable prognostic factor (Jenkin et al., 2000). However, it is generally it is children of a much younger age (< 3 years old) who are associated with a worse prognosis since such patients may have biologically more aggressive lesions (Allen and Epstein, 1982).

Chang et al. (1996) have addressed prognostic factors identifiable at the time of diagnosis in medulloblastoma. These investigators identified 2 characteristic stages for newly diagnosed disease, a 'T' for tumour size stage and an 'M' for metastasis stage. Children

with low 'T' and 'M' characteristics, i.e. with small tumours that did not disseminate, had the best prognosis. This system of staging at diagnosis is now the most popular and is known as the Chang staging scheme. Cases are divided, judging by the staging criteria described, into those considered as 'good risk' and those deemed 'poor risk', and in children this scheme plays a prominent role in accurate predictions of recurrence-free survival (David et al., 1997)

The relevance of histological features as prognostic factors is equivocal. Although one study has reported that conventional cytological and histological characteristics such as nuclear atypia, high mitotic index, necrosis, vascular proliferation and lack of cytoplasmic processes are associated with a poorer prognosis (Maire et al., 1992), others have not found this connection (Giordana et al., 1995; Hubbard et al., 1989). Similarly, the relationship between the desmoplastic variant of the tumour and prognosis is controversial. It has been related to a worse prognosis in children, to a better prognosis in adults and to no difference in survival in either age group (Friedman et al., 1991; Walker and Rosenblum, 1992; Katsetos and Burger, 1994).

The prognostic significance of cellular differentiation, whether neuronal or glial, remains unclear. Glial fibrillary acidic protein (GFAP) expression appears to denote a poor prognosis (Goldberg et al., 1991; Janss et al., 1996), whilst expression of retinal s-antigen and rhodopsin have been shown to signify a more favourable outlook (Czerwionka et al., 1989; Maraziotis et al., 1992).

With regard to tumour cell proliferation and apoptosis, the prognostic value is limited. The labelling index (LI) with bromodeoxyuridine (BrDU) is an indicator of survival in that patients whose tumour has a LI of more than 20% appear to have a worse prognosis (Ito et al., 1992). A comparison of proliferating cell nuclear antigen (PCNA) and Ki67 LI

between children and adults showed that the proliferation potential of childhood medulloblastoma may be lower than those in adults (Giordana et al., 1997) thus implicating a worse prognosis for those adult patients with these tumours.

(ii) Molecular genetic or cytogenetic

Efforts are now being made to identify molecular genetic or cytogenetic features that may be of prognostic value. The use of molecular genetic markers to guide the course of treatment and predict the outcome of intervention for cancers of many different types has been steadily increasing. For instance, previous studies have shown that in neuroblastoma, another small round blue cell tumour of children, the copy number of the MYCN oncogene is a better predictor of treatment outcome than any of the clinical parameters evaluated to date (Seeger et al., 1985).

Early cytogenetic studies demonstrated that the most frequent cytogenetic abnormality found in medulloblastoma is isochromosome 17q [i(17q)] (Bigner et al., 1988; Griffin et al., 1988; Biegel et al., 1989). This results in loss on the short arm of chromosome 17 as well as duplication of the long arm (17q). It is estimated that a region of chromosome 17p13 is deleted in medulloblastoma in between 30 and 50% of cases. Although the presence of i(17q), and/or 17p deletion is not specific for medulloblastoma, it is seen in this tumour at a higher frequency than in any other tumour type (Biegel, 1999). Additionally, the finding of an i(17q) as a single structural abnormality in karyotypes suggests that it is a primary cytogenetic event and not a cytogenetic change associated with clonal evolution (Biegel et al., 1989). For this reason, there has been great interest in assessing whether deletion of 17p is associated with clinical outcome in medulloblastoma. However, a number of studies have reported conflicting results.

Loss of 17p was correlated, without statistical significance, with poor response to treatment in a small number (4) of medulloblastoma patients in one independent study (Cogen al., 1990). However, a study conducted in 1995 reported that deletion of 17p is associated with a shortened survival period that is statistically significant in medulloblastoma (Batra et al., 1995), when loss of heterozygosity (LOH) for 17p was found in 29% of a group of 28 patients. A slightly larger patient group (31 cases) was used for a correlative study a year later (Cogen and McDonald, 1996). The patients were divided into 'good risk' and 'poor risk' groups based on established clinical parameters (Kopelson et al., 1983). Patients were considered 'good risk' if their age at diagnosis was greater than 3 years, if their tumour had undergone total or near-total resection and if there was an absence of metastatic deposits in the brain or spinal cord. All of the patients who did not meet one or more of these criteria were categorised as 'poor risk'. 'Good risk' patients underwent post-operative radiation to the posterior fossa and the craniospinal axis. Patients in the 'poor risk' category below the age of 3 years at the time of diagnosis initially received a mult-drug chemotherapy regimen post-operatively, followed by the same radiation protocol as those in the 'good risk' group. The 'poor risk' patients above the age of 3 years at diagnosis received the radiation therapy as their first form of post-operative treatment with subsequent multi-drug chemotherapy. The patients in each group were then further subdivided into those with and without demonstrable 17p deletions in their tumour specimens.

It was found that the patients in the 'poor risk' group did badly whether or not there were evident 17p deletions. The results of the analysis for the 'good risk' patients was more interesting. The 'good risk' patients without 17p deletions did well after treatment; all of the patients were alive and free of tumour at the time the study was completed over 7 years later. In comparison, all except one of the 'good risk' patients with 17p deletions recurred

by the completion of the study and all of these patients have since died. The results were determined to be statistically significant.

Thus, the results of this study showed that 'good risk' patients with 17p deletions had essentially the same negative prognosis as 'poor risk' patients with or without deletions. Although the results of this study are revealing, they require replication on further series' of patients to ensure validity.

This is particularly pertinent when one considers that in other recent studies findings are varied. For example, in one multivariate analysis of 56 patients with medulloblastomas, patients with and without deletions had similar overall recurrence-free survival rates of 66% (Biegel and Wentz, 1997). Furthermore, Emadian et al. (1996) did not find a statistically significant difference in outcome in patients with or without tumour-associated 17p deletions. These authors also reported that clinical risk factors such as extent of surgical resection were better prognostic indicators than LOH, with significantly prolonged survival in patients free of craniospinal metastasis following gross total tumour resection (Emadian et al., 1996). A 1997 study addressed the question of whether or not 17p deletions are associated with a poor prognosis by identifying deletions from this region by cytogenetic and/or molecular biology methods in tumour biopsy samples from 56 patients with medulloblastoma (Biegel et al., 1997). Associations between clinical characteristics or survival outcomes and 17p status were examined by multivariate analysis and 41% of cases were found to have a deletion of 17p. No significant association was found between 17p deletion and shorter survival time or higher metastatic stage. Moreover, multivariate analysis did not find independent prognostic significance for 17p deletions after accounting for the effects of significant clinical variables such as patient age and extent of tumour resection (Biegel et al., 1997). Conversely however, another 1997 study reports that deletion of 17p can be used as a prognostic biological marker (Steichen-Gersdorf et al.,

1997). In a study of 18 medulloblastomas it was found that children with an allelic loss had a poorer prognosis than those patients without LOH ($p<0.05$). Similarly, in a study of 28 medulloblastoma samples, LOH of 17p was observed in 8 of 28 samples and was found to be indicative of a significantly worse prognosis (Batra et al., 1995).

In a recent study by Scheurlen et al. (1998), 30 primary medulloblastoma samples were examined for LOH on 17p13 as well as amplification of the MYCC oncogene. Loss of 17p13 was found in 14 of 30 (47%) tumours and was found to be linked to a poor outcome. However, MYCC amplification was also shown to predict a poor response to therapy and every case that showed amplification of MYCC also had loss of 17p.

The *HER2/neu/ERBB2* oncogene, a member of the EGFR family, and its expression may also be related to poor outcome. This gene, which is part of the RTK 1 family, maps to 17q11.2-12 and thus is duplicated in tumours with an i(17q) (Plowman et al., 1990). The c-erbB-2 oncogene product has been found to be expressed in the majority of medulloblastomas (Gilbertson et al., 1995). In a retrospective immunohistochemical study of 55 tumours conducted by these authors, patients whose neoplasms contained >50% of cells which expressed the c-erbB-2 protein had a significantly worse prognosis than patients with <50% positive tumour cells. Although the mechanism by which over-expression of c-erbB-2 contributes to malignancy is not yet understood, the confirmation of these results in a prospective clinical trial which controls for disease stage and treatment may provide a means of identifying patients with a high risk of recurrence.

A role for the neurotrophin receptor TrkC (tyrosine kinase) has been postulated for medulloblastoma (Packer et al., 1999). Previously, studies of the neurotrophin receptor genes in neuroblastoma showed an association between expression of TrkA and TrkC in tumour speciments with a favourable prognosis, whilst TrkB expression was linked to a

poor outcome (Nakagawara et al., 1993). In an effort to identify biologic prognostic factors in PNETs, including medulloblastoma, one recent study determined expression levels of TrkC mRNA in tumour samples from 87 patients and compared these levels with clinical and other laboratory variables using univariate and multivariate analysis (Grotzer et al., 2000). It was found that in comparison with established clinical prognostic factors that TrkC expression, by univariate analysis was the most powerful predictor of favourable outcome exceeding all clinical prognostic factors. The authors concluded that assessment of TrkC mRNA levels may aid in treatment planning for patients with PNETs and should be incorporated prospectively into PNET clinical trials. It is thus not clear as to which genetic markers will prove to be the most useful for predicting prognosis for patients with medulloblastoma. However, it is without doubt that the definition of a molecular variable capable of identifying medulloblastoma cases that are likely to progress rapidly would provide a powerful tool for the management of these patients.

Oncogenes and tumour suppressor genes

There is strong evidence to support the conclusion that cancer is a genetic disease of somatic cells. For example, almost all carcinogens induce mutations. Genetically determined traits associated with a deficiency in the enzymes necessary to repair lesions in DNA are associated with an increased risk of cancer (Ames, 1983). Factors which compromise the genome also increase the probability of neoplasia, and therefore several inherited diseases are associated with an increased incidence of cancer such as retinoblastoma (Knudsen, 1971). Genetic lesions may occur in the germline of an individual and be represented in every cell in the body, or they may occur in a single somatic cell and be identified in a tumour following clonal proliferation.

Recent studies have identified at least two groups of genes that play an important role in tumourigenesis; oncogenes and tumour suppressor genes (Friend et al., 1988). Oncogenes are dominantly acting growth enhancing genes whose normal cellular homologue, when transformed by amplification, mutation or rearrangement, leads an increase in their function and uncontrolled cellular proliferation. Tumour suppressor genes are recessively acting growth control genes whose inactivation by mutation, deletion or rearrangement leads to a decrease or loss of functions and neoplastic transformation.

(i) Oncogenes

The oncogenes associated with various tumour types arise from preexisting genes that are present in the normal human genome. These genes, proto-oncogenes, have essential roles in normal cellular physiology and are often involved in the regulation of normal cellular growth or proliferation. These genes become involved in neoplastic transformation as a result of somatic alterations that occur in specific target tissues and convert these genes into oncogenic alleles (Varmus, 1984).

Examples of oncogenes formed by somatic alterations of normal genetic loci are the *abl* oncogene (activated in chronic myelogenous leukaemia), *myc* oncogene (Burkitt's lymphoma), *N-myc* oncogene (neuroblastoma) and *neu/erbB2* oncogene (mammary carcinoma). Other oncogenes, in particular those of the *ras* family, are associated with a wide variety of tumours (Weaver and Hedrick, 1989). The pivotal role of these genes in tumour development has been highlighted by the capability of each to generate many of the phenotypes characteristic of cancer cells. For instance, when these genes are isolated by molecular cloning and reintroduced into normal cells, the cells assume many of the growth properties of tumour cells (Varmus, 1984).

In a small number of human cancers, alternative molecular mechanisms exist in which viral agents have major causal roles. For example, particular papillomaviruses and the Epstein-Barr virus induce disease in Burkitt's lymphoma and cervical carcinoma respectively (Nonoyama et al., 1973; McCance, 1985). Viral oncogenes are carried by the viruses into target cells where they become firmly established in the host genome. The descendants of these cells continue to carry the viral genes, and the latter play major roles in maintaining the malignant phenotype of the cells.

(ii) Tumour suppressor genes

In 1971, a theory that explained the development of the two different forms of retinoblastoma, hereditary and sporadic, was proposed (Knudsen, 1971). Knudsen observed that the two types of retinoblastoma could be distinguished by their distinct clinical patterns (Knudsen, 1971). The hereditary type is autosomal dominant with high penetrance and presents in very young children with lesions often occurring in both eyes. Up to a 1000 fold increase in secondary malignancies was also observed in these children. In comparison, the sporadic disease was more often seen in older children, usually only affected one eye and was not associated with an increased incidence in secondary malignancies.

Knudsen hypothesised that this pattern of retinoblastoma presentation could result from as few as two genetic events if each event lead to the inactivation of one copy of a gene which controls cellular proliferation. Knudsen termed this gene a 'tumour suppressor', loss of both copies of which would result in neoplasia. According to this theory, the initial inactivation of a gene, or 'hit', would take place in the germline in those individuals with hereditary retinoblastoma and would thus be carried in all of the cells of this individual. The second inactivating genetic event would be to the somatic cells, in this case the

retinoblasts, resulting in the formation of a particular tumour. Those individuals with the sporadic type of retinoblastoma would also develop tumours after the two 'hits', both of which would be found only in the somatic cells.

The Rb gene and other tumour suppressor loci

The initial evidence in support of Knudsen's hypothesis came from cytogenetic studies. Individuals suffering from both the hereditary and sporadic forms of retinoblastoma were examined and translocations and deletions of chromosome 13 were detected in the blood cells of patients with the hereditary type and in the tumour cells from patients with both forms of the disease (Sparkes et al., 1980). A number of different cases were compared and a minimal affected region, 13q14, was identified. Knudsen's hypothesis was then proved using restriction fragment length polymorphism (RFLP) technology which allows for tumour deletion mapping and genetic linkage studies (Cavenee et al., 1983). Using RFLP, as well as other techniques, more detailed mapping and eventually identification and cloning of the gene for retinoblastoma (Rb) was achieved (Friend et al., 1985).

The Rb gene has been found to be deleted in a number of different tumour types, including osteogenic sarcoma (Friend et al., 1985) and small-cell lung carcinoma (Harbour et al., 1988). These studies as have revealed that a single tumour suppressor gene may be aberrant in a number of different tumour types.

Other tumour suppressor loci have been identified subsequent to the cloning of the Rb gene. For example, one of the genes for Wilms' tumour, a common paediatric renal cancer, has been cloned; Wt1, which is located on chromosome 11 (Call et al., 1990). Several of the genes that participate in the neoplastic pathway leading to colorectal cancer have also been identified, including the gene for familial adenomatous polyposis (FAP)

which is located on chromosome 5 (Kinzler et al., 1991). The genes for neurofibromatosis have also been cloned; the gene for type 1 is located on chromosome 17q (Marchuk et al., 1991),whilst that for type 2 is found on chromosome 22 (Rouleau et al., 1993), as well as one of the genes for familial breast cancer, BRCA1, on chromosome 17 (Miki et al, 1994) and one gene that seems to act as multiple tumour suppressor for a variety of neoplasms, including some astrocytomas. This gene is MTS1, located on chromosome 9 (Kamb et al., 1994). However, the most frequently occurring genetic event across the broad spectrum of human cancer development involves mutation and/or deletion of yet another tumour suppressor gene, p53, located on chromosome arm 17p13 (Soussi et al., 1990).

The p53 gene

Functional inactivation of the p53 gene has been found in about half of all human tumours (Hollstein et al., 1996). This gene was originally detected as the 53 kD binding product of the large T antigen of the SV40 tumour virus and was initially classified as an oncogene as the result of NIH 3T3 cell transformation assay (Cogen and McDonald, 1996). It was discovered that the p53 gene used for this assay had a mutation and that instead of being oncogenic, the wild-type p53 was actually growth suppressive and prevented abnormal cell proliferation (Lamb and Crawford, 1986). The p53 gene modulates cell cycle events in response to DNA damage or stress conditions such as hypoxia by inhibition of cell cycle progression or the induction of apoptosis. Loss of function of wild-type p53 can thus lead to genomic instability and the perpetuation of gene abnormalities as well as uncontrolled cell proliferation (Yin et al., 1992).

p53 functions as a growth inhibitor in response to intracellular stress signals. Under certain conditions, p53 protein induces cell cyle arrest in the G_1 S-phase which can allow DNA to be repaired before replication (Mercer et al., 1990). p53 induces cell cycle arrest

primarily by transactivating genes that control cell proliferation and cell cycle progression, including IMP dehydrogenase (Sherley, 1991) and *cdk 2* - inhibitory protein (CIP1/WAF1) (El-Deiry et al., 1993). If CIP1/WAF1 is inactivated or repressed, cellular response to p53 over-expression may change from growth arrest to apoptosis (Allan and Fried, 1999), whilst high levels of CIP1/WAF1 can delay but not block the apoptotic response of the cells (Allan and Fried, 1999) These capacities of p53 are lost in most mutant alleles of the gene (Raycroft et al., 1991) with the consequent loss of the p53 mediated pauses which are required to repair DNA damage, a prerequisite for preserving the integrity of the genome (Yin et al., 1992).

The p53 gene product may autoregulate its own activity through regulation of the expression of the *mdm2* gene (Barak et al., 1993). Transcriptional activation of p53 can be inhibited if it is bound by *mdm2* (Momand et al., 1992), but conversely, this function can be increased by conjugation to the small ubiquitin-like protein SUMO-1, which attaches to p53 at the C-terminal (Gostissa et al., 1999). Amplification of *mdm2* gene has been shown in sarcomas and astrocytomas (Oliner, 1992; Ladanyi et al., 1993), and may occur as part of an amplicon including the cdk4 gene and, less often, the *gli* gene (Khatib et al., 1993). mdm2 gene amplification provides another mechanism for p53 inactivation in tumours that do not have p53 gene mutation.

As well as the induction of growth arrest or inhibition of cell growth, the p53 protein is also an important regulator of cell death via the apoptotic pathway in certain cell types (Agarwal et al., 1998). p53 probably plays a role in apoptosis by regulating genes which are involved in this pathway such as bcl-2, bax, cd95/fas and insulin-like growth factor binding protein-3 (IGFBP-3) (Miyashita et al., 1994; Buckbinder et al., 1995; Miyashita and Reed, 1995) and indeed it has been demonstrated that p53 can induce expression of both the bax gene (Miyashita and Reed, 1995) and the cd95/fas gene (Owen-Schaub et al.,

1995) which promote apoptosis. There is evidence that the allelic status of the p53 gene plays a role in apoptotic response also. Thymocytes which contain homozygous wild-type p53 rapidly undergo apoptosis after treatment with ionising radiation, while cells heterozygous for a p53 deletion show partial apoptosis and homozygous null cells are resistant to the induction of apoptosis (Clarke et al., 1993).

It was the work of Baker et al (1989) on the aetiology of colorectal cancer which initially revealed that p53 had an important role in human neoplasia. RFLP analysis demonstrated that loss of chromosome arm 17p was a common event in colorectal tumourigenesis. The area of loss encompassed 17p13.1; the locus of the p53 gene. Data gathered from several tumour specimens showed one copy of the p53 gene to be deleted whilst the remaining allele was mutated. These alterations inactivated both copies of the gene, identifying its role as a tumour suppressor (Baker et al., 1989).

Subsequent studies have demonstrated mutations of the p53 gene in many divergent tumour types including cancer of the breast, lung, oesophagus, bladder and liver (Batra et al., 1995) as well as some of those of the brain (Metzger et al., 1991) such as astrocytomas (Chung et al., 1991). Indeed, in so-called secondary astrocytomas, it has been shown that these tumours acquire mutations of p53 during malignant progression (Sidransky et al., 1992).

Most of the mutations of p53 seen in human cancers are localised in four regions of the gene (residues 117-142, 171-181, 234-258 and 270-286) which are highly conserved (Soussi et al., 1990). There are at least 3 mutation 'hot spots' which affect residues 175, 248 and 273. The frequency and distribution of these hot spots differs among cancers from different tissue types, and it is not known why these differences exist. They could

arise from different mutagens or other aspects of the environment in the tissues, or from the different selective pressures for promoting cell growth.

The p53 gene has been implicated in inherited as well as spontaneous malignancies. One such inherited form of cancer in which p53 mutations are seen is Li-Fraumeni syndrome. This is a rare autosomal dominant syndrome characterised by diverse neoplasms at many different sites in the body. Affected families have a high incidence of cancer and of 6 families examined, all have germline p53 mutations clustering between codons 245 and 258 (Levine et al., 1991) with at least 2 inheriting a mutation for codon 248 which leads to the substitution of glutamic acid for the wild-type arginine at this position (Santibanez-Koref et al., 1991). These inherited mutations are found in the heterozygous state in noncancerous cells of Li-Fraumeni family members and do not interfere with the developmental process and normal functions of the individual bearing them. Rather, they could have a predisposing effect on carcinogenesis (Levine et al., 1991). In fact, transgenic mice carrying a mutant murine p53 allele (along with 2 wild-type copies) have offspring with a much higher risk of developing cancer (Lavigueur et al., 1989). If a mutant allele provides a growth advantage in the heterozygous state, then the number of cells with one mutation at this locus will increase, as will the probability of a reduction to homozygosity or of a second mutation arising in the cell harbouring the first mutation (Levine et al., 1991). This could explain why p53 mutations are seen so commonly in human cancers. Moreover, the restricted distribution of Li-Fraumeni family mutations at the p53 locus compared with those found in the wide range of sporadic cancers lends support to the idea that different p53 alleles have different properties (Levine et al., 1991).

Microsatellite instability

It is possible that p53 and other tumour suppressor genes may be affected by a phenomenon known as microsatellite instability (MIN). MIN as a widespread genomic instability has been suggested as a genetic mechanism leading to inactivation of tumour suppressor genes (Honchel et al., 1995). Microsatellites, or somatic alterations in simple repeat sequences, have been found in a variety of human cancers, especially in colorectal tumours and other carcinomas in people with hereditary nonpolyposis colon cancer (Aaltonen et al., 1993; Thibodeau et al., 1993). MIN can be seen as the shift or the addition of an allele in a tumour sample at 2 or more independent loci when compared with the matched normal somatic tissue sample after electrophoresis through polyacrylamide gels (Aaltonen et al., 1993). The understanding of the origin of MIN phenotypes has been aided by the discovery of mutations in two candidate DNA mismatch repair genes (hMSH2 and hMLH1) in tumours from both hereditary nonpolyposis colon cancer patients and sporadic colorectal tumours (Fishel et al., 1993; Papadopoulos et al., 1994). Deficiencies in mismatch repair have also been found in colorectal and endometrial cancer cell lines with MIN (Umar et al., 1994). These findings support the hypothesis that mutations affecting one or more DNA repair pathways probably play a role in the development or progression of some human cancers. With regard to brain tumours, several studies looking at the frequency of MIN have been conducted. One comprehensive study examined 144 such tumours (Zhu et al., 1996) and found instability ranging only from between 0% and 3.2% in astrocytic tumours, meningiomas, schwannomas, pituitary adenomas and low-grade oligodendrogliomas suggesting that MIN is not an important pathogenetic mechanism in these tumours. However, 4/8 anaplastic oligodendrogliomas exhibited instability. Another study found that MIN is infrequent in sporadic adult gliomas (Lundin et al., 1998), whilst a third investigation looking at paediatric medulloblastomas specifically, demonstrated that MIN is uncommon in this tumour type (Lescop et al., 1999).

The multi-step process of carcinogenesis

Single oncogenes acquired by target cells through mutation or virus are usually not sufficient to complete neoplastic transformation (Land et al., 1983). Although there are forms of cancer such as retinoblastoma, where the tumour is the result of a single gene defect, often tumour cells acquire multiple genetic changes which interact to produce the full repertoire of neoplastic characteristics. The necessity for a wide variety of independently activated genes may explain the multi-step process of carcinogenesis,. As a cell clone progresses from normal to malignant, each stage of malignancy may be marked by the acquisition of one or more genetic abnormalities. For instance, it is well established that a *ras* oncogene is carried by pre-malignant papillomas on mouse skin (Balmain and Pragnell, 1983) and development of malignant papillomas is dependant on the subsequent acquisition of a second collaborating genetic change.

Familial medulloblastomas and genetic factors

Familial cases of medulloblastoma are rare. Prior to 1990, only 14 had been identified (Hung et al., 1990); 4 of which involved monozygotic twins whilst the remaining 10 tumours were found in siblings. A more recent study has, however, reported another case of medulloblastoma in monozygotic male twins in which concordant desmoplastic medulloblastoma was diagnosed at the age of 20 months (Scheurlen et al., 1996). The authors asserted that the clinical presentation supports the assumption of an inherited genetic predisposition to develop medulloblastoma in at least some cases.

Certain hereditary syndromes may predispose individuals to the development of medulloblastomas in addition to other clinical features. Nevoid basal cell carcinoma (NBCC) or Gorlin syndrome (GS) is an autosomal dominant disorder that predisposes an

individual to develop multiple basal cell carcinomas (BCCs) by puberty, multiple odontogenic keratocysts of the jaws, palmar and plantar dyskeratoses, as well as skeletal anomalies, especially malformed ribs (Gorlin, 1987). Over 30 different types of malformations, harmartomas and benign and malignant neoplasms have been associated with the syndrome (Gorlin, 1987). In a population-based study of GS undertaken in 1991, the prevalence of the syndrome was found to be approximately 1: 56,000 (Evans et al., 1991). There is a well known association between GS and medulloblastoma with around 40 GS patients who developed this tumour reported in the literature (Evans et al., 1991; Lacombe et al., 1990). In general, between 3 and 5% of patients afflicted by GS develop medulloblastoma (Evans et al., 1991; Vorechovsky et al., 1997)). Even though the GS-associated medulloblastomas are histologically indistinguishable from sporadic cases there are some clinical differences. The GS associated cases occur earlier with a mean age at diagnosis of around 2 years of age (Evans et al., 1991) as compared with an average age of 7 years for sporadic medulloblastomas (Giangaspero et al., 1997). Additionally, there is a tendency towards longer survival in GS-patients with medulloblastoma as opposed to patients with sporadic tumours. At least 10 cases of GS patients surviving from between 10 and 30 years after diagnosis of medulloblastoma have been reported (Evans et al., 1991; Lacombe et al., 1990). However, despite these differences it is still possible that there is underestimation of Gorlin syndrome patients in childhood medulloblastoma and the true incidence may be even higher (Cowan et al., 1997). This assertion is borne out by the fact that in children aged under 2 years with medulloblastoma the incidence of Gorlin syndrome is as high as 10% and it is advisable to examine the family pedigree of these early-onset cases in detail (Cowan et al., 1997).

In recent years, the genetic locus for GS has been mapped to 9q22.3 (Farndon et al., 1992; Gailani et al., 1992) and is the human homologue of the Drosphila *patched* gene, *PTC* (Hahn et al., 1996., Johnson et al., 1996). Somatic mutations in *PTC* have been

shown in approximately 10% of patients with sporadic medulloblastomas (Raffel et al., 1997). One study showed that mutation/deletion of the *PTC* locus is restricted to the desmoplastic variant of this neoplasm (Cowan et al., 1997). Furthermore, the Gorlin patients in this study presented with only the desmoplastic type which is further confirmation of this correlation between abnormalities of *PTC* and desmoplasia. Loss of 9q alleles and mutations of the remaining copy of *PTC* suggest that *PTC* may function as a tumour suppressor gene (Biegel 1997), and many features of Gorlin syndrome suggest that the primary function of the gene is the control of cell growth (Farndon et al., 1992).

In Drosophila, the patched protein has an important regulatory role in the 'hedgehog' signaling pathway (Perrimon, 1995) that is critical to embryo segmentation and other developmental steps. The regulatory element, hedgehog, stimulates the expression of the *wingless* and *cubitus interruptus* genes. Hedgehog also up-regulates *patched*, which in turn down-regulates *hedgehog, cubitus interruptus, wingless* and also *patched* itself (Raffel et al., 1997). *Cubitus interruptus* plays an essential role between *patched* and *hedgehog* (Dominguez et al., 1996). *Hedgehog* up-regulates *cubitus interruptus* which in turn increases the expression of other proteins in the pathway. *Patched*, however, down-regulates *cubitus interruptus* (Raffel et al., 1997). Each of these proteins has human homologues. The *hedgehog* protein has 3: *sonic hedgehog (SHH), indian hedgehog (IHH)* and *desert hedgehog (DHH). SHH* has a tissue distribution similar to *PTC* (Hahn et al., 1996) and it is suggested that *PTC* functions as a receptor for this protein (Stone et al., 1996). The human homologue of *cubitus interruptus* is a transcription factor called *gli*, the gene for which is occasionally amplified in glioblastoma multiforme (Kinzler and Vogelstein, 1990). The human homologue of *wingless* is *wnt-1*, a protein whose overexpression is associated with mammary tumours in mice (Tsukamoto et al., 1988).

In addition to *SHH*, *PTC* interacts with *SMO*, the mammalian homologue of *Drosophila's smoothened (smo)* (Zurawel et al., 2000). When *SHH* and *SMO* are cotransfected into cells, the 2 proteins can be coimmunoprecipitated and binding of *SHH* to the *SMO/PTC* complex has been demonstrated (Stone et al., 1996). Work on Drosophila has indicated that *SMO* is the effector of *SHH* binding to *PTC* (Chen and Struhl, 1998). One medulloblastoma has been shown to contain a mutation in *SHH* (Oro et al., 1997) and a mutation of*SMO* has also been identified in a medulloblastoma (Reifenberger et al., 1998). However, in an analysis of *PTC/SMO/SHH* pathway genes (*SHH* and*SMO* as well as other genes that were identified as candidate tumour suppressors based on their roles in controlling hh/ptc signaling in Drosophila: EN-1, EN-2, SMAD family members 1-7 and protein kinase A subunits RIα, RIβ, RIIβ, Cα and Cβ) Zurawel et al. (2000), screened 27 medulloblastomas and found no evidence for mutations in any genes other than PTC. Clearly, further studies are required to elucidate the role of these genes in the development of medulloblastomas, although some of the above studies demonstrate that the identification of genes involved in normal development will provide good candidates for embryonal tumours such as medulloblastomas.

Patients with Turcot's syndrome have multiple colorectal neoplasms and malignant epithelial tumours so when medulloblastomas are found in patients with familial polyposis it is indicative of this syndrome (Jarvis et al., 1988). Germ-line mutations in the adenomatous polyposis coli gene (*APC*) have been demonstrated in patients with Turcot's and medulloblastoma, but not in patients with isolated medulloblastoma (Hamilton et al., 1995., Mori et al., 1994). *APC* is part of a pathway that regulates *b-catenin*, a key component in cell-cell adhesive junctions and transduction of wingless-Wnt signalling. Mutations in *b-catenin* were identified in 3 of 67 (4%) of sporadic medulloblastomas (Zurawel et al., 1998). A subset of those tumours had been studied previously for mutations in *PTC* (Raffel et al., 1997) and none of the tumours with *PTC* mutations

contained a *β-catenin* mutation. Whether these mutations can be linked to the same site in developmental pathways, the disruption of which leads to medulloblastoma, is unclear at this time.

Medulloblastoma has been identified in a 3 year old child who came from a family affected by Li-Fraumeni syndrome (Levine et al., 1991). The role of p53 in sporadic medulloblastomas shall be discussed in greater depth later.

Genetic changes in spontaneous medulloblastomas

Loss of chromosome 17p and presence of i(17q)

Translocations and chromosomal gains and losses in medulloblastomas affecting almost every chromosome have been reported, however, the most common, consistent structural chromosomal abnormality found is an i(17q), which has been equated with a deletion of the short, or p, arm of chromosome 17 and a duplication of the long, or q, arm (Bigner et al., 1988; Biegel et al., 1992,1995; Fujii et al., 1994; Schütz et al., 1996). This has been observed in 25-50% of medulloblastomas (Thomas and Raffel, 1991; Cogen et al., 1992). In a large interphase cytogenetic study, Biegel et al. (1995) detected deletions of chromosome arm 17p in 44% and an isochromosome 17q in 30% of cases by using appropriate DNA probes. Another interphase cytogenetic study (Giordana et al., 1998) used a chromosome 17 centromeric probe to detect i(17q) in paraffin sections in a series of 20 medulloblastomas as well as in some other brain tumour types (ependymomas and cerebellar astrocytomas) for comparative purposes. The hybridisation signal strongly suggestive of an i(17q), i.e. a paired spot, was found in every medulloblastoma in the series but not in the other primary CNS tumours tested. There have been several tumours in which an i(17q) was the only structural abnormality seen (Biegel et al, 1989) and the non-

random occurrence of this aberration suggests that the gain of sequences on 17q with a corresponding loss of material on 17p plays a role in the pathogenesis of these tumours. These studies suggest the existence of a tumour suppressor gene, or genes, within the deleted region on 17p or the presence of a gene on 17q the increased copy number of which may result in a higher dosage of normal or abnormal product. The chromosome 17p locus does not appear to be imprinted as equal numbers of paternally and maternally-derived deletions of chromosome 17 have been identified in medulloblastomas with an i(17q) (Scheurlen et al. 1995; Biegel 1997).

Approaches to the molecular study of solid tumours have used LOH for polymorphic loci to identify allelic loss and thus pinpoint areas that may contain tumour suppressor loci. Therefore, on the basis of cytogenetic findings, several investigators have studied LOH of chromosome 17p loci in medulloblastomas. Thomas and Raffel (1991) localised allelic loss to 17p11.2 - pter in 6 out of 23 tumours studied (26%), whilst James et al (1990) found loss on 17p in 5 tumours out of 11 (45%) and Steichen-Gersdorf et al. (1997) observed LOH on 17p in 9 out of 18 of patients (50%). LOH of D17S34, the most telomeric locus on 17p13.3, has been found in a small number of tumours in one study (Biegel et al., 1992) and of the 14/32 (44%) of tumours that showed LOH in another study, all 14 showed loss of D17S34 (McDonald et al., 1994). Further evidence that a tumour suppressor locus or loci resides within the more distal region of 17p has been provided in other studies. For example, in a study comparing medulloblastoma with supratentorial PNETs, Burnett et al. (1997) found loss of 17p in 37% of the former, all of which lost the most distal marker at 17p13.3. Steichen-Gersdorf et al. (1997) observed allelic loss at 17p13.3-13.2 in 9 out of 18 tumours. Furthermore, all tumours were informative for D17S796 and 9 out of 9 samples were deleted at this locus which is located at 17p13.3. The authors conclude that genes within this locus are strong candidates for putative medulloblastoma genes.

Microsatellite analysis and interphase fluorescence in situ hybridisation (FISH) experiments have shown that the breakpoints in chromosome 17 are contained between the centromere and the proximal region of 17 (17p11.2), a region commonly deleted in patients with the Smith-Magenis syndrome (Scheurlen et al., 1998). Smith-Magenis syndrome is characterised by multiple congenital abnormalities and mental retardation but patients do not appear to have an increased risk of developing medulloblastomas (Biegel 1997). Chromosome 17 tends to undergo breakage in this region where repetitive sequences are present. These sequences may predispose the chromosome 17 homologues to undergo unequal crossing over events, leading to duplications/deletions and formation of isochromosomes. Other breakpoints were identified in a more recent study (Aldosari, 2000). Routine karyotyping, FISH and LOH analysis were conducted on 4 medulloblastoma cell lines and 1 xenograft in order to demonstrate the spectrum of chromosome 17 abnormalities which occur in these tumours. One cell line showed an i(17q), another showed 2 normal copies of chromosome 17 by all methods, whilst a third showed loss of the terminal region of 17p by LOH but what appeared to be an i (17q) on the karyotype. FISH and chromosome 17 painting however, showed that the abnormal chromosome 17 was actually formed through an unbalanced translocation involving 2 copies of the chromosome with breakpoints at 17p12 and 17q11-1. The fourth cell line had a terminal deletion at 17p11.2 and 2 cells had i(17q) , as found by interphase FISH, suggesting that the terminal deletion arose from breakage of an i(17q). The xenograft showed LOH for regions distal to 17p12, whereas karyotyping, FISH using probes on 17p and chromosome 17 painting showed 2 intact copies of the chromosome which pattern, the authors assert, can be explained by homologous recombination. Their data support the concept that the critical deletion of 17p can occur through a variety of mechanisms in medulloblastoma. The losses may occur through typical i(17q) as well as other

mechanisms such as terminal deletions, possibly through breakage of i(17q), unbalanced translocations and homologous recombination (Aldosari et al., 2000).

Deletion of most or all of 17p in medulloblastoma instead of mitotic recombination (leading to LOH) or interstitial deletions within 17p13 suggests that loss of more than one gene on 17p may be important in tumourigenesis (Biegel, 1999). Additionally, the identification of tumours with extra copies of 17q, in the absence of a 17p deletion, suggests that the duplication of sequences on 17q may confer a selective growth advantage to tumour cells (Biegel, 1999). From the above evidence then, it appears that there may be several tumour suppressor genes which play a role in the development of medulloblastoma are present on 17p. This realisation initially led to the assumption that one critical gene may be p53.

p53 and medulloblastoma

p53 was considered a candidate gene for medulloblastoma primarily because of its location, 17p13.1, but also because a high proportion of p53 mutations have been reported in adult malignant gliomas (Chung et al., 1991). The frequency of p53 gene mutation and *mdm2* gene amplification appears to differ significantly among different solid childhood tumours, with a high frequency of p53 mutation (Felix et al., 1992; Ueda et al., 1993) or *mdm2* gene amplification (Oliner et al., 1992; Ladanyi et al., 1993) detected in rhabdomyosarcoma and osteogenic sarcoma and a low frequency in Wilm's tumour, neuroblastoma and hepatoblastoma (Waber et al., 1993) suggesting that other tumour suppressor genes may be involved in the aetiology of some paediatric neoplasms.

One study investigated p53 mutation and *mdm2* gene amplification in paediatric medulloblastoma specifically (Adesina et al., 1994). This group analysed PCR-amplified products of exons 4-9 (95% of reported p53 mutations occur within this region) of the p53

gene in 9 medulloblastomas for potential mutations using single strand conformation polymorphism analysis (SSCP) and DNA sequencing. A mutation was found in only 1 of 9 samples and was due to an A-T to T-A transversion involving the second base of codon 285 resulting in the substitution of valine for glutamic acid. Mutations within this region have been reported to alter the conformation of the p53 protein product resulting in a change in its transcriptional activity and half-life (Raycroft et al., 1991). The tumours analysed in the Adesina et al. (1994) study had been shown previously to have LOH on 17p in 3/9 tumours (James et al. 1990) and only one of these had a demonstrable p53 mutation. The other 6 tumours retained both alleles for markers on 17p13. Other reports show p53 mutations in medulloblastoma/PNET to range from 0 of 12 tumours and 1 of 3 cell lines (Saylors et al., 1991), to 2 of 11 (Oghaki et al., 1991), 2/20 (Cogen et al., 1992), 2 of 22 (Badiali et al., 1993) and 0 of 7 (Biegel et al., 1992) tumours. Thus the overall incidence of p53 mutation in medulloblastomas is approximately 8%, indicating the presence of another tumour suppressor gene locus on 17p which is critical in the development and/or progression of medulloblastomas.

p53 mutations in various tumours, including malignant glial tumours and colorectal carcinoma, show a predominance of G-C to T-A transitions. Additionally, p53 mutations in liver and lung are often dominated by G-C to T-A transversions, a finding which suggests selective targeting of mutational hotspots by specific carcinogens such as benzypyrene through the formation of DNA adducts with guanine (Pusieux et al., 1991). As most of the tumours with a high incidence of p53 mutations are adult tumours, it could be that subsequent to prolonged exposure to environmental carcinogens, p53 mutational hotspots are selectively targeted. However, in contrast, medulloblastoma is predominantly a paediatric tumour with a very low incidence in adults. The low frequency of p53 mutations in these tumours is consistent with the hypothesis that prolonged exposure to

certain environmental mutagenic agents may not play a large part in the pathogenesis of medulloblastomas (Giangaspero et al., 1997).

Another mechanism of p53 inactivation is through the amplification of the *mdm2* gene, the protein product of which binds and inactivates wild type p53 (Oliner et al., 1992). Amplification of this gene has been reported in some sarcomas (Oliner et al., 1992; Ladanyi et al., 1993). One study was not able to show *mdm2* gene amplification in 8/9 medulloblastoma (Adesina et al., 1994) therefore excluding a major role for this gene in the biology of this series of tumours.

Other frequently found genetic abnormalities

Although it is abnormalities of chromosome 17 which occur most frequently in medulloblastomas, there are numerical and structural changes involving several other chromosomes which are nonrandomly associated with these tumours. Indeed, despite the fact that chromosome 17 displays the most frequently found specific alteration, chromosome 1 has the highest frequency of structural abnormalities found in medulloblastomas (Bigner et al., 1997; Griffin et al., 1988; Bigner et al., 1988; Biegel et al., 1989; Fujii et al., 1994). These changes are usually deletions or unbalanced translocations involving 1p. Instead of 1p being lost however, the abnormal chromosome 1 is usually represented as an extra copy so that there is a net gain of 1q. The observation that loss of 1p is uncommon in medulloblastomas is supported by the infrequent finding of LOH for 1p in allelotyping studies (Raffel et al., 1990; James et al., 1990; Bello et al., 1995; Blaeker et al., 1996).

An extra copy of chromosome 7, often seen in tumours with an i(17q), is the second most common cytogenetic abnormality found in medulloblastoma (Battacharjee et al., 1997.,

Biegel et al., 1989., Bigner et al., 1997., Griffin et al., 1988). The fact that trisomy 7 is seen in association with other structural chromosomal abnormalities in medulloblastoma implies that rather than being an initiating event it is a secondary change. However, trisomy 7 has also been detected in normal tissue adjacent to neoplastic cells which do not have the extra copy of this chromosome (Johansson et al., 1993) so the potential oncogenic contribution of this abnormality remains uncertain.

Loss of all or part of chromosome 6 has been noted as a single change in several medulloblastoma (Biegel et al., 1989; Griffin et al,., 1988) suggesting that this is another primary genetic event. LOH studies have confirmed the deletion of chromosome arm 6q in some medulloblastoma (Thomas and Raffel, 1991). Deletion of chromosome 6 is usually found in tumours that do not contain an i(17q), suggesting that it may define a subset of medulloblastomas that arises through a different initiating genetic event (Biegel, 1997).

Another cytogenetic abnormality in medulloblastoma is loss of chromosome 22 either alone or with additional chromosomal changes. In one series, monosomy 22 occurred in 4 of 17 cases which had additional cytogenetic abnormalities (Bigner et al., 1997). The highest incidence of monosomy 22 reported to date is 4 of 10 of medulloblastoma with chromosomal abnormalities in the series of Vagner-Capodano et al. (1988) who stressed the association of this finding with a desmoplastic histology. Biegel et al. (1990) noted frequent monosomy 22 in rhabdoid tumors of the CNS, which has been suggested as representing a variant of medulloblastoma when occurring in the cerebellum (Bigner et al., 1997). It has been suggested that chromosome 22 deletions may be a secondary change as they are usually seen in tumours with a variety of other abnormalities (Biegel et al., 1989). Moreover, in another study, LOH for chromosome 22 was not detected in 20 informative patients (Thomas and Raffel, 1991). It is possible that abnormalities of chromosome 22 define another subset of medulloblastomas.

LOH on chromosome 11p was seen in 3 of 11 medulloblastomas by James et al. (1990) and in 1 of 7 patients screened by Raffel et al (1990). In a study by Fults et al. (1992), 3 of 11 medulloblastomas had LOH on 11p with the region of common overlap being 11p15.4-pter, whilst additionally Blaeker et al. (1996) showed that 3/7 samples had partial deletions of the 11p arm with a minimum region of overlap at 11p15. However, microsatellite analysis of chromosome 11 did not reveal loss at the one locus in 15 informative medulloblastomas in a study reported by Albrecht et al.(1994). Unfortunately, all these studies were limited in numbers of patients examined and the number and location of the chromosome 11 markers used. Occasional examples of 11p and 11q deletions have been reported from cytogenetic studies (Griffin et al., 1988; Bigner et al., 1988; Vagner-Capodano et al., 1992). More commonly however, karyotypic studies have indicated loss of the entire copy of chromosome 11 (Karnes et al., 1992; Neumann et al., 1993). Additional molecular and cytogenetic studies are required for determining the frequency and nature of the chromosome 11 changes in medulloblastomas before their significance can be understood.

Loss of 9q may involve deletion/mutation of *PTC* (Raffel et al., 1997; Wolter et al., 1997; Vorechovsky et al, 1997; Zurawel et al., 2000) and may highlight a subset of tumours with poor prognosis (Scheurlen et al., 1998). Medulloblastomas with 9q deletions have been found not to have 17p deletions suggesting alternate pathways for tumour initiation (Biegel, 1999).

Unbalanced translocations or deletions of chromosomes 5q, 8, 10 and 16q have also been observed in small numbers of cases (Biegel et al., 1989; Bigner et al, 1988; Raffel et al., 1990; Thomas and Raffel, 1991; Albrecht et al., 1994; James et al., 1990) suggesting that

several different tumour suppressor genes are involved in the initiation or progression of medulloblastoma.

Amplification events and medulloblastomas (*MYCN* and *MYCC*)

There is a very low frequency of amplification events in medulloblastomas (Biegel, 1999). However, the genes which may be involved are 2 members of the *MYC* family, *MYCC* (on chromosome 8q24) and *MYCN* (on 2p24) as well as the epidermal growth factor receptor (*EGFR)* located on 7p12.

MYCN expression is not usually elevated (MacGregor and Ziff, 1990; Raffel et al., 1990) although some studies have found gene amplification by Southern blot analysis in cell lines derived from medulloblastomas and in several primary tumours (Rouah et al., 1989; Wasson et al., 1990; Badiali et al.,1991; Fuller and Bigner, 1992). Also, Bayani et al. (1995) described an example of a medulloblastoma with amplification at the *MYCN* site, which may suggest a role for these genes in medulloblastoma development since they found amplification of MYCN in 17% of cases; a higher frequency than previously published data.

Amplification of the *MYCC* oncogene and increased level of *MYCC* expression have been demonstrated in some medulloblastoma tumour samples (Badiali et al., 1991).Moreover, analyses of established cell lines have indicated a relatively high frequency of amplification of *MYCC* (Bigner et al.,1990). The latter authors found *MYCC* amplification in 3 cell lines and 4 xenografts obtained from 4 primary medulloblastomas containing double minutes (DMs) and subsequently hypothesised that amplification of this gene confers a growth advantage on medulloblastoma cells in vitro and in nude mice. Cytogenetically, another investigation (Badiali et al., 1991), although

finding *MYCC* amplification in only 1 of 32 medulloblastomas investigated, revealed the presence of numerous DMs in this one case. The authors assert that this observation supports the assumption that DM-bearing medulloblastomas contain *MYCC* gene amplification. Moreover, this tumour disseminated extensively via the CSF despite chemotherapy. Tomlinson et al. (1994) have reported that for both *MYCN* and *MYCC* a single case with amplification coincided with an unusually swift and aggressive progression of the disease with no response to chemotherapy, and similarly, Jay et al. (1999) described amplification of *MYCC* in a medulloblastoma with aggressive clinical behaviour. Furthermore, such particularly aggressive behaviour has also been reported in a patient whose tumour gave rise to a cell line with *MYCC* amplification (Friedman et al., 1988). These findings suggest that *MYCC* amplification may provide a growth advantage for medulloblastoma cells not only in vitro and in xenografts but also in the patient enhancing their swift dissemination. Another investigation however, found no *MYCN* amplification in 9 medulloblastomas examined but did see overexpresssion of this gene in 6 of 11 samples (Garson et al. 1989). Additionally, *MYCN* overexpression without amplification, as demonstrated by a high level of *MYCN* mRNA, was observed in 1 medulloblastoma in another study (Nisen et al., 1986). Moreover, Fujimoto et al. (1989) reported a recurrent medulloblastoma containing a high level of *MYCC* mRNA but without oncogene amplification. This suggests that in medulloblastomas the oncogenic mechanism is not restricted to gene amplification.

In a recent comparative genomic hybridisation (CGH) study, Reardon et al (1997) pinpointed amplified regions in only 3 of 27 tumours (at 5p15.3 and 11q22.3) and Feuerstein and Biegel (1999) observed only 2 amplified regions, 1 at the locus on 2p that contains *MYCN*, and another near the centromere of chromosome 7, in 32 medulloblastomas analysed by CGH. Conversely, Bigner et al. (1988; 1997) reported a high frequency of double minute chromosomes, a characteristic sign of gene amplification,

in medulloblastoma karyotypes. In their 1997 study, 7 of 17 medulloblastomas with abnormal karyotypes contained DMs, and 5 of these cases were seen in tumours with one or more copies of an i(17q). Furthermore, Schütz et al. (1996) found high-copy number amplifications in the regions where *MYCC* and *MYCN* are localised, a finding in contrast to previous studies where high-level amplification of these genes was rarely found. According to the CGH data of Schütz et al (1996), the frequency of such amplifications of *MYCC* in medulloblastoma was higher than one would expect from the previously published data, as *MYCC* was found to be amplified in 16.5% (3 of 18) of their cases. The authors suggest that the higher incidence found in their study was due to either statistical variations or a higher sensitivity of CGH in detecting high-copy-number amplifications as compared with Southern blot analysis. It is also interesting to note that in this study, in keeping with the Jay et al. (1999), Tomlinson et al. (1994) and Friedman et al. (1988) findings described above, all three patients with tumours demonstrating high-copy-number amplifications had an early relapse despite aggressive chemotherapy and radiotherapy. It seems that for further elucidation of the possible correlation of MYC amplification with poor response to therapy investigations of larger sample numbers are needed.

Comparative Genomic Hybridisation

Until recently, the role of classical cytogenetics in the analysis of solid tumours has been limited because of the difficulties of obtaining good quality metaphases and in interpreting often complex karyotypes. CGH uses genomic DNA directly extracted from tumour biopsies (>50% tumour cells) or from cultured cells. The technique allows genome-wide screening for gains and losses of chromosomal material on the basis of competitive binding of tumour and normal control DNA to normal metaphase chromosome spreads. Allelic imbalances can be detected and mapped by simultaneous hybridisation of

differentially labelled control DNA (red fluorescing) and tumour DNA (green fluorescing) to a normal human metaphase spread. Regions of increased or decreased copy number (gene amplifications or deletions) can be detected across the whole genome by the ratio of fluorescence intensities (increases in red or green fluorescence). CGH is currently the optimal method to determine sites of genetic gains in genome-wide screens of medulloblastoma although the sensitivity of the technique is limited. For example deletions within a chromosomal band may not be detected. However, CGH cannot be used to detect balanced rearrangements, such as inversions and translocations. The quantitative linear relation between DNA sequence copy number and the green to red ratio is only obtained when the size of the region affected is large enough; >10-20 Mb changes have so far been demonstrated to follow this rule (Kallioniemi et al., 1994). However, qualitative detection of copy number aberrations affecting much smaller regions is possible if the DNA sequences show high-level amplification. It is estimated that the total amount of amplified DNA (amplicon size times level of amplification) has to be at least 2Mb for it to become detectable (Piper et al., 1994).

CGH was developed around 8 years ago and was the first molecular cytogenetic tool that allowed comprehensive analysis of the entire genome. As both drug resistance genes and oncogenes are known to be upregulated by DNA amplifications it has been hypothesised that DNA amplification sites in cancer could reveal the loci of novel genes with important roles in cancer progression (Kallioniemi et al., 1992). A large number of chromosomal gains and DNA amplifications have been discovered by CGH in some cancers. For instance, in breast cancer, up to 30 different chromosomal regions can be involved in recurrent gains and amplifications (Kallioniemi et al., 1994). This is a much higher number than was previously estimated based on the results obtained from studies with specific DNA probes to known oncogenes. It has been suggested that in comparison with Southern blot analysis, CGH has a higher sensitivity in detecting high-copy-number

amplifications (Schutz et al., 1996). The androgen receptor (AR) at Xq 11-q12 was found to be amplified by CGH in prostate cancer that had recurred during androgen deprivation therapy (Visakorpi et al, 1995). Other CGH studies have implicated amplification of oncogenes whose activation in cancer was previously known to occur by chromosomal translocations alone. An example is amplification of the BCL2 gene at (18q21.3) in lymphomas (Monni et al., 1996). It is likely that many additional examples of novel gene amplifications in cancer are likely to emerge in future CGH studies including the present investigation.

Also, many of the losses of genetic material discovered in cancer by CGH coincide with regions previously reported with LOH. These include losses of chromosomes 8, 9p, 10q, 11p, 16q and 22q in medulloblastomas (Reardon et al., 1997). Conversely, novel small regions of loss discovered by CGH could then be examined using LOH for microsatellite markers mapping to those loci. Therefore, ultimately, CGH would be of use in the search for new tumour suppressor genes.

Chromosomal changes detected via CGH in medulloblastoma

The CGH approach is being used increasingly for the identification of chromosomal imbalances in a variety of tumours including medulloblastoma. The areas of chromosomal loss and gain identified by CGH can be used as starting points for more detailed cytogenetic and molecular genetic analyses.

In one study eighteen cases of PNET/medulloblastoma were examined (Schütz et al., 1996). Chromosomal imbalances were present in fifteen tumours and the most frequent of these involved chromosome 17. Loss of 17p was shown in four out of the eighteen tumours (22%) and, in accordance with previous published reports, the existence of an

i(17q) was suggested by CGH in 2 of these 4 cases. Except for loss of 17p, loss of material was found in more than one case on 9q only. Other imbalances identified in the study, such as gains of the terminal regions of 4p, 5q, 7q and 9p had not, or only infrequently, been found before.

In a second study a larger sample group of twenty seven tumours was analysed by CGH (Reardon, et al., 1997). Again, the majority of imbalances observed involved chromosome 17. Loss of 17p was seen in 10 of 27 (37%) of tumours, whilst gain of 17q was found in 13 of 27 (48%) of cases. There was a much greater frequency of chromosomal imbalance than found in the Schutz et al. (1996) study. In addition to the loss of 17p, non random deletions were demonstrated at high frequencies on chromosomes 10q (11 of 27 cases, 41%), 11 (11 of 27, 41%), 16q (10 of 27, 37%) and 8p (9 of 27, 33%). Genetic gains were also observed at high frequencies. Apart from chromosome 17q, overrepresentation of genomic material most frequently involved chromosome 7, wherein gain was seen in 12 of 27(44%) cases. Also, gains of 1q and 18q were observed in 7 of 27 (26%) tumours.

In a third study of a sample group of twenty three, multiple chromosomal imbalances were detected in twenty cases (Avet-Loiseau et al., 1999). Chromosome 17 was the most frequently involved chromosome once more. Loss of 17p was seen in eight (34%) cases, and gain of 17q was observed in eleven (47%) tumours; almost identical frequencies to those seen in the Reardon et al., (1997) study. Other losses found at high frequencies were those of chromosomes 11 in nine (39%) cases and 10q in eight (34%) cases. Gains were less frequent but extra copies of chromosome 7 were seen in nine (39%) cases whilst gains of 1q were detected in 6 (26%) tumours. Another study conducted last year used a very small number of samples, only six, and found gains of 7q and 17q in 4/6 (66%) cases (Nishizake et al., 1999).

AIMS

Identification of a putative tumour suppressor gene locus or loci on chromosome 17p

It is clear from previous cytogenetic and molecular genetic research that 17p deletions are the primary genetic abnormality observed in medulloblastomas. Therefore, it was decided that the initial emphasis of the present work would be to use LOH for polymorphic loci in an attempt to define the regions of allelic loss on chromosome 17p. It is hoped that by using a large range of microsatellite markers, loci at which any putative tumour suppressor genes may reside will be identified. Thus, markers which map from p13.1, at the centromeric end of 17p, up to p13.3, at the telomeric end, were used. As the minimum region of deletion found on 17p is distal to p53 on 17p.13.1, all of the markers chosen map to this region. However, one of the markers does map to the p53 locus, whilst another maps to D17S 34, the most telomeric locus on 17p13.3. It is hoped that the results will contribute to the overall deletion map of 17p.

Screening the entire genome via CGH to pinpoint other consistent genetic changes

The second aim of this research is to use the CGH technique to screen the entire genome in order to detect consistent gains and losses of chromosomal material. Hopefully the CGH will confirm the results found by the microsatellite analysis with regard to chromosome 17p, but the primary aim is to identify consistent chromosomal changes elsewhere. As well as finding abnormalities that concur with those seen in previous studies, it is hoped that the

CGH work will identify losses of genetic material in which putative tumour suppressor genes may reside as well as high level gains and amplifications where putative oncogenes may be located.

Further analysis of consistent areas of genetic loss identified by CGH

Any consistent areas of loss found will be used as a starting point for more detailed genetic analysis using appropriate polymorphic microsatellite markers which map to the areas of deletion discovered. The resolution obtained with microsatellite markers is much greater than that which CGH produces so it is hoped that these studies will allow the determination of the minimal overlapping critical regions of deletion.

Correlation of genetic changes found with clinical parameters

The clinical information regarding the patient used in this study was obtained from the neurosurgeons who provided the tumour samples. The correlation between clinical parameters such as length of survival and genetic gains and losses in each tumour detected by the techniques used will be determined. It is hoped that this will provide a starting point for the eventual identification of new molecular genetic markers which can be used as prognostic indicators.

Chapter 2
METHODS AND MATERIALS

SOURCE OF TISSUE AND WHOLE BLOOD SAMPLES

The paired whole blood and tumour biopsy samples used in this study were kindly donated by W. Harkness and R.Hayward at The Hospital for Sick Children, Great Ormond Street, D. Porter at Leeds General Hospital and a frozen biopsy sample with corresponding paired blood obtained was from A. Dean at the Department of Neuropathology, Institute of Psychiatry, between 1987 and 1999. Tumour samples were removed at the time of routine neurological operation. They were taken from material not required for diagnostic purposes and which would otherwise have been discarded. Ten mls of venous blood was taken from each patient. Whenever possible this was taken at the time a routine blood sample was being taken. These procedures, and the present study as a whole, were approved by the Joint Medical Ethics committee of the National Hospital for Neurology and Neurosurgery and the Institute of Neurology. A total of 29 paired samples were obtained, 23 from males and 6 from females. The age range of the patients at initial presentation with tumour was between 3 months and 13 years with a mean age of 5.6 years. Twenty six tumours were classical medulloblastoma whilst 3 were of the desmoplastic variant (See table 2.1 for all patient details).

DETAILS OF ALL REAGENTS INCLUDING BUFFERS

A list of all the reagents together with the names and addresses of the pharmaceutical companies which supplied them can be found in Appendix I. Any buffers used in the study, together with their components, are also listed in Appendix I.

PREPARATION OF SAMPLES

In the operating theatre, tumour specimens were immediately placed in a sterile 25 ml universal (Scientific Laboratory Supplies, Nottingham) containing Hams F10 nutrient media (Life Technologies, Inchinan, Renfrewshire) for transportation to the laboratory.

IN Number (a)	Sex (b)	Age (years unless stated)	Histology	Location	Therapy (c)	Survival (months)
1212	M	13	classical	posterior fossa	partial excision	no follow up
1241	M	8	classical	posterior fossa	partial excision, RT, chemo (PNET 3)	13 (dead)
1482	M	6	classical	cerebellar cortex	partial excision, RT, chemo (CCNU, Vinc)	15 (dead)
1545	F	8	classical	cerebellum	total resection, RT	9 (dead
2077	M	4	classical	posterior fossa	partial excision, chemo (PNET 3), RT	11 (dead)
2266	M	11 months	classical	posterior fossa	partial excision, chemo (Baby Brain)	75 (alive)
2289	M	21 months	classical	posterior fossa	total resection, chemo, RT (Baby Brain)	79 (alive)
2465	M	23 months	classical	posterior fossa	total resection, chemo, RT (Baby Brain)	71 (alive)
2529	M	10	classical	posterior fossa	partial excision, RT, chemo (PNET 3)	19 (dead)
2588	M	3	desmoplastic	posterior fossa	total resection, RT	63 (alive)
2590	F	3	classical	posterior fossa	partial excision, RT, chemo (CHP 455)	67 (alive)
2636	M	3	classical	posterior fossa	total resection, chemo (PNET 3), RT	64 (alive)
2637	M	12	classical	posterior fossa	partial excision, RT, chemo (CHP 455)	6 (alive)
2659	M	6	classical	posterior fossa	total resection, RT	55 (alive)
2697	M	9	classical	posterior fossa	not known	no follow up
2715	F	8	classical	posterior fossa	total resection, chemo, RT (PNET 3)	54 (alive)
2741	F	4	classical	posterior fossa	partial excision, RT	16 (alive)
2765	M	6	classical	posterior fossa	total resection, chemo, RT (PNET 3)	22 (dead)
2805	M	9	classical	posterior fossa	total resection, RT	14 (dead)
2823	M	10	classical	posterior fossa	unknown	no follow up
2832	M	7	desmoplastic	cerebellum	total resection, RT	43 (alive)
2894	M	2	classical	posterior fossa	total resection, RT, chemo (etoposide)	25 (dead)
2901	M	4	classical	cerebellum	total resection, RT, chemo (CHP 455)	30 (alive)
2912	F	3 months	classical	posterior fossa	partial excision, chemo (Baby Brain)	54 (dead)
2917	M	7	desmoplastic	posterior fossa	total resection, RT	20 (alive)
2920	F	3	classical	posterior fossa	total resection, chemo, RT (CHP 455)	18 (alive)
2933	M	6	classical	posterior fossa	unknown	no follow up
2966	M	18 months	classical	posterior fossa	total resection, chemo (New infant PNET)	8 (alive)
2996	M	5	classical	posterior fossa	total resection, RT, chemo (CHP 455)	5 (alive)

Table 2.1: patient details of the sample group on which microsatellite analysis and/or CGH was conducted. (a) Each tumour was assigned a number for identification purposes and each number was prefixed with 'IN' (Institute of Neurology; (b) M: male,F: female; (c) RT: radiotherapy, chemo: chemotherapy (details of treatment regimens are given in Appendix III)

The tumour tissue is viable for five days so samples could easily be sent in the post from Leeds General Hospital, whilst the samples from the other hospitals were collected immediately they became available. On receipt of a tumour sample it was removed from its storage universal tube in sterile conditions in a laminar air flow cabinet (Gelaire, ICN Biochemicals, Basingstoke, Hampshire) used solely for the preparation of fresh biopsy material and placed in a 60 x 15 mm petri dish. The tumour was then cut, using sterile carbon steel scalpels (size 10), into two pieces; a small specimen for tissue culture and the remainder for isolation of genomic DNA and storage in LN_2 for future use. Whole blood samples, which had been collected in sterile 25 ml universals containing EDTA, were stored at -70^{0}C. The procedures for the isolation of genomic DNA from the whole blood and tumour specimens obtained throughout the duration of this study (1996-1999) were carried out immediately on receipt of the latter, whilst samples received prior to 1996 were removed from frozen storage (at -70 ^{0} C in the case of whole blood samples and LN_2 for the tissue) and thawed as and when needed. DNA was also extracted from short term cultures when frozen biopsy material was unavailable.

TISSUE CULTURE PROCEDURES

Preparation of biopsy material for short term cultures

Each specimen was sliced using crossed carbon steel scalpels (size 10) until fine enough to be pipetted into a sterile 25 ml universal tube. Two mls Ham's F10 nutrient media with 10% foetal calf serum (FCS) (Life Technologies) and 1 ml collagenase stock (2000 units/ml) (Sigma Aldrich, Gillingham, Dorset) in Hanks Balanced Salt Solution (HBSS) (Life Technologies) were then added to the specimen which was incubated at 37^{o}C for at least one hour.

After incubation, 7 mls Ham's F10 was added to the universal which was centrifuged at 1000 rpm for 5 minutes. The supernatant was removed and the cell pellet resuspended in 10 mls Ham's F10 and transferred to a 25 cm^2 tissue culture flask (Falcon, Marathon Lab Supplies, London).

Passaging of Cells

When cells had reached confluence the media was aspirated and the confluent monolayer washed twice with 5 mls HBSS. After washing, the cells were rinsed with 1ml trypsin EDTA (Life Technologies) before adding a further 3mls of trypsin and incubating for 15-30 minutes at 37^{0}C.

Seven mls Ham's F10 was then added to the detached cells which were transferred to a 25 ml universal and centrifuged at 1000 rpm for 5 minutes. The supernatant was removed and the cells resuspended in 10 mls Ham's F10 before being divided into tissue culture flasks. Cells from 1x 25 cm^2 flask were transferred into 1x 75 cm^2 flask, cells from 1x 75 cm^2 flask were split into 3 x 75 cm^2 flasks and when confluent, cells from each of the 3 x 75 cm^2 flasks were put into 1x 150 cm^2 flask. Finally, cells from each 150 cm^2 flask were divided into 3 x 150 cm^2 flasks. Ham's F10 was then added to each flask - 10 mls (25 cm^2 flask), 20 mls (75 cm^2 flask) and 30 mls (150 cm^2 flask).

Each time trypsin EDTA was used, the passage number increased by one.

Freezing cells

Any surplus cells not needed for experimentation were frozen. The procedure for passaging (see above) was followed from washing with HBSS through to centrifugation at 1000 rpm

for 5 minutes. The supernatant was then removed and the cells resuspended in FCS containing 10% dimethyl sulphoxide (DMSO) (Sigma Aldrich) to a final concentration of 1 x 10^6 cells per ml. This was aliquotted into freezing vials (Life Technologies), 1 ml per vial, which were packed in polystyrene and frozen slowly down to -70^{0}C. After 24 hours, the freezing vials were transferred into LN_2 tanks where they were stored.

To revive cells from the LN_2 tanks, the vial was removed from the liquid phase and quickly transferred to a covered plastic container with water at 37^{0}C (a precaution to avoid liquid nitrogen (if any) trapped within an incompletely sealed vial). Once thawed, the contents were removed from the vial and then slowly diluted into 10 mls of Ham's F10 in a 25 cm^2 flask and incubated overnight. The following day the cells were re-fed.

Feeding Cells

Cells were fed once a week or when all the nutrients had been absorbed from the media, depending on which occurred first. On absorption of all the nutrients, only metabolic waste products remain and these turn the media acidic and bright yellow in colour. To feed the cells, the old media was aspirated and replaced with Ham's F10 - 10 mls (25cm^3 flask), 20 mls (75 cm^3 flask) or 30 mls (150 cm^2)

DNA EXTRACTION PROCEDURES

DNA was extracted from blood, biopsy and short term cell cultures (between passage 4 and passage 6), using commercially available extraction kits (Qiagen, Crawley, Sussex)

Procedure for isolation of genomic DNA from whole blood

Each sample of frozen blood was removed from -70^{0}C storage and left at room temperature to thaw completely. Subsequently, 3 mls of blood was removed from each sample and placed in a 50 ml polypropylene screw cap tube (Falcon, Marathon Lab Supplies). One volume of ice-cold buffer C1 and 3 volumes of ice-cold dH_20 was added and mixed by inverting several times until the suspension became translucent. This was then incubated for 10 minutes on ice. The lysed blood was centrifuged for 15 minutes at 1300 x g and the supernatant discarded. One ml of ice-cold buffer C1 and 3 ml of ice-cold dH_20 were added and the pelleted nuclei resuspended by vortexing. Centrifugation was carried out again for 15 minutes at 1300 x g and the supernatant discarded. After adding 5 mls of buffer G2, the nuclei were resuspended completely by vortexing for 20 seconds and 95 µl of proteinase K (20 mg/ml) (ICN Biochemicals) was added. The solution was then incubated at 50^{0}C for 60 minutes or until the lysate became clear. A Qiagen Genomic-tip was then placed over a 50 ml polypropylene screw cap tube using a tip holder and the tip equilibrated with 4 mls of buffer QBT. The tip was subsequently allowed to empty by gravity flow. Following incubation, the sample was vortexed for 10 seconds and applied to the Genomic-tip. The DNA binds to the resin of the tip and the residual QBT buffer passed through the tip into the screw cap tube. The genomic-tip was washed twice with 7.5 mls of buffer QC before being transferred to a clean screw cap tube. The DNA was then eluted with 5 mls of buffer QF. Three and a half mls of isopropanol (Sigma Aldrich) was added and mixed with the sample and the DNA spooled with a glass rod. The DNA was then transferred to a 1.5 ml eppendorf tube (Alpha Laboratory Supplies, Hampshire) containing 1 ml TE (pH 8.0) buffer and resuspended. Each DNA sample was sheared with a 1 ml syringe prior to use and any remaining blood not used for DNA extraction was returned to storage at -70^0 C.

Procedure for isolation of genomic DNA from tissue

Frozen biopsy samples were removed from LN_2 storage, placed in 25 ml universal tubes and allowed to thaw in a 37^{0}C water bath. Approximately 25 mg of tissue was cut from each sample, under sterile conditions. This was chopped into very small pieces using crossed carbon steel scalpel blades (size 10) and then pipetted into a 1.5 ml eppendorf tube with 180 µl of buffer ATL. Twenty µl of Proteinase K solution (20 mg/ml) was then added and mixed by vortexing for 10 seconds. The solution was then incubated at 55°C for 60 minutes or until the tissue was completely lysed. Two hundred µl of buffer AL was added, the sample was mixed by vortexing for 10 seconds and then incubated for a further 10 minutes at 70°C. Following this, 210 µl of ethanol was added and the sample mixed by vortexing again for 10 seconds. A QIAmp spin column (Qiagen tissue kit) was placed in a 2ml collection tube (Qiagen tissue kit) and the sample applied. The lid of the spin column was then closed and the column centrifuged at 6000 x g for 1 minute. The collection tube containing the filtrate was then discarded, the column placed in a clean tube and the DNA was washed with 500 µl of buffer AW. The column was then centrifuged again at 6000 x g for 1 minute. The collection tube containing the filtrate was again discarded and the wash procedure was repeated. The column was placed in a clean collection tube and the DNA was eluted with the addition of 200 µl of 10 mM Tris-HCl (preheated to 70^{0}C) followed by the centrifugation of the column at 6000 x g for 1 minute. This was repeated twice using the same collection tube. After elution, the DNA samples were stored at -20^{0}C until use.

Procedure for isolation of genomic DNA from cultured cells

One ml of trypsin EDTA was added to each 150 cm^2 flask, containing approximately 3 x 10^6 cells when the latter were confluent, and incubated at 37^{0}C until all of the cells had detached from the flask. The cell suspension was then transferred to a 50 ml polypropylene

screw cap tube. The empty flask was then washed with 2 ml Phosphate Buffered Saline (PBS) which was then added to the tube.

The cells were recovered by centrifugation at 1500 x g for 10 minutes after which they were resuspended in 4 ml PBS and centrifuged at 1500 x g for a further 10 minutes. The cells were then resuspended in PBS at a concentration of 10^7 cells /ml. One volume of ice-cold buffer C1 and 3 volumes of ice-cold dH_20 were added and mixed by inverting the tube several times until the suspension was translucent. The suspension was then incubated on ice for 10 minutes.

The remainder of the DNA isolation procedure is identical to that used for the isolation of DNA from whole blood from the lysing procedure through to the shearing of extracted DNA.

FLUOROMETRIC QUANTIFICATION OF DNA

Following DNA extraction, the quantity of DNA in each sample was measured using a DyNAQuant 200 fluorometer (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire) and a DQ 130 capillary cuvette kit (Amersham Pharmacia Biotech). The former is a filter fluorescence photometer designed specifically for the accurate quantitation of low DNA concentrations using Hoechst 33258 dye (Sigma, St. Louis, USA for Amersham Pharmacia Biotech) The DQ 130 kit includes a capillary cuvette and capillary tubes which hold 3 to 9 µl of solution.

As the DNA fluorescence assay is based on a relative measurement of emitted light, a calibration reference value must be established with a known DNA sample before the concentration of DNA in an unknown sample can be determined. A capillary tube was

filled with the standard of 100 ng/µl calf thymus DNA (Sigma Aldrich) in capillary assay solution inserted into the cuvette and the reference value was entered on the fluorometer.

In order to measure the quantity of DNA in the actual samples, a capillary tube was filled with the sample solution diluted in capillary assay solution and placed in the cuvette. The quantity of DNA measured is given in ng/µl.

MICROSATELLITE ANALYSIS

PCR amplification

Genetic loss was investigated using a variety of polymorphic microsatellite markers, identified from the Genetic map from the Marshfield www site (March 1998) (//research.marshfieldclinic.org/genetics/) in the case of markers mapping to chromosome 17p, and the Genethon data base (www.genethon.fr/genethon_en.html) or the genome data base (www.GDB.org) for markers mapping to chromosomes 9q, 10q, 12q, 16q and 22q. Forward and reverse primers were synthesised by Amersham Pharmacia Biotech. The cytogenetic location, level of heterozygosity and size range for each marker is shown in tables 2.2 to 2.8. Amplification of both tumour DNA and paired normal DNA was carried out for each of the markers in 0.5ml sterile eppendorf tubes using the polymerase chain reaction (PCR). Each of the markers was optimised for magnesium chloride (Mg Cl) concentration and annealing temperature and these conditions and the primer sequences are shown in tables 2.2 to 2.8. The PCR reactions were carried out in a final volume of 50 µl containing 50 ng of genomic DNA. The PCR reaction mix consisted of 50 mM Kcl, 10mM Tris-HCl pH 9.0, 200 µmol of each dNTP (Amersham Pharmacia Biotech) and 50 pmol of each forward and reverse primer. The PCR reaction mixtures were denatured by heating at 95^0C for 5 minutes in the thermal cycler (Techne, Duxford, Cambridge) before 1 unit of

Tables 2.2-2.8: showing names, cytogenetic locations, levels of heterozygosity and size ranges, primer sequences, optimum annealing temperatures and optimum MgCl concentrations of the polymorphic microsatellite markers used in mapping chromosomes 9q, 10q, 12q, 16q, 17p and 22. Information regarding levels of heterozygosity and product size came from the Genetic map from Marshfield www site (March 1998) (//research.marshfieldclinic.org/genetics/) in the case of the 17p markers and the Genethon database (www.genethon.fr/genethon_en.html) and the genome database (www.GDB.org) in the case of the remaining markers.

Marker	Cytogenetic location	Level of heterozygosity	Product size	Primer sequences	Optimum annealing temperature	Optimum MgCl concentration
D9S166	9q21.1	0.824	233-261	**F** 5' aaatcatgcaattcatttca 3' **R** 5' tcctaattcactgggaaaac 3'	57^{0}C	1.5μM
D9S287	9q22.3	0.6735	168-180	**F** 5' aggatgctcctcacgc 3' **R** 5' accactacattgttcaaggg 3'	58^{0}C	2μM
D9S176	9q22.3	0.8230	129-147	**F** 5' agctggctgttggagaaa 3' **R** 5' tgaccaatggcagggtat 3'	61^{0}C	1.5μM
D9S170	9q33	0.7526	108-126	**F** 5' caggcacactcatacac 3' **R** 5'acttcaggaatagcctttacc 3'	57^{0}C	1.5μM

Table 2.2: chromosome 9q

Marker	Cytogenetic location	heterozygosity	product size	primer sequences	optimum annealing temperature	optimum MgCl concentration
D10S581	10q21.3	0.8000	129-155	**F** 5'gctgctaaatgcaaccat 3' **R** 5'acaacttctccactcaaccc 3'	59^0C	1.5µM
D10S580	10q23.1	0.7300	91-105	**F** 5' aaaacatattgcgcgtgc 3' **R** 5' catatacgtcaggaacatctgc 3'	60^0C	1.5µM
D10S540	10q24	0.6200	255-259	**F** 5' ttcgttacagcacaaaccag 3' **R** 5' aatgaagcccctgctacac 3'	61^0C	3µM

Table 2.3: chromosome 10q

Marker	Cytogenetic location	heterozygosity	product size	primer sequences	optimum annealing temperature	optimum MgCl concentration
D12S327	12q22	0.8501	182-201	**F** 5' aaagtttctggatggtaatatcg 3' **R** 5' agagcaagaccttgtctcaa 3'	59^{0}C	2μM
D12S360	12q23	0.6004	187-223	**F** 5' ttagatggagtgctactgcc 3' **R** 5' ttcctaaagagcaactaactgg 3'	60^{0}C	2μM
D12S330	12q24.11-24.13	0.7315	156-198	**F** 5' caggggactataatcatgcc 3' **R** 5' agctccagacatgtgtattctatg 3'	60^{0}C	1.5μM
D12S366	12q24.2	0.8100	185-201	**F** 5' ctcctgtctgcgcttcag 3' **R** 5' gctctcaccaggcaattactat 3'	61^{0}C	2μM
D12S342	12q24.32-33	0.7400	217-237	**F** 5' cgctctcacagttctggagg 3' **R** 5' cgctctcacagttctggagg 3'	61^{0}C	1.5μM

Table 2.4: chromosome 12q

Marker	Cytogenetic location	heterozygosity	product size	primer sequences	optimum annealing temperature	optimum MgCl concentration
D16S409	16q12.1	0.7099	135-147	**F** 5' tgaatcttacatcccatccc 3' **R** 5' agtcagtctgtccagaggtg 3'	59^{0}C	1.5µM
D16S416	16q13	0.4258	217-223	**F** 5' agcagtttgggtaaacattg 3' **R** 5' aaatatgccttctggaggtg 3'	59^{0}C	1.5µM
D16S514	16q21	0.8200	117-133	**F** 5' ctatccactcactttccagg 3' **R** 5' tcccactgatcatcttctc 3'	57^{0}C	2µM
D16S512	16q22.1	0.7640	201-211	**F** 5' tgagagccaaataaataaatgg 3' **R** 5' tcacgttgtgaatgcaagt 3'	59^{0}C	1.5µM
D16S516	16q24.1	0.7330	164-176	**F** 5' cctccagaaaccgtgagat 3' **R** 5' ggtgccatcctgacaga 3'	60^{0}C	1.5µM

Table 2.5: chromosome 16q

Marker	Cytogenetic location	heterozygosity	product size (bp)	primer sequences	optimum annealing temperature	optimum MgCl concentration
D17S34	17p13.3	0.9229	370	**F** 5' tcccaaactggagactggtc 3' **R** 5' aggatgacctgggccaac 3'	64^{0}C	1.5µM
D17S849	17p13.3	0.6761	251-261	**F** 5' caattctgttctaagattattttgg 3' **R** 5' ctctggctgaggaggc 3'	58^{0}C	2µM
D17S926	17p13.3	0.8107	243-260	**F** 5' gcagtgggccatcatca 3' **R** 5' cctcagaaggctgttgt 3'	62^{0}C	3µM
D17S643	17p13.3	0.74	151	**F** 5' cttccttgtctctaaacagtccttt 3' **R** 5' gtagtcccagggagctggaagt 3'	65^{0}C	1.5µM
D17S695	17p13.3	0.91	201	**F** 5' ctgggcaacaagagcaaaattc 3' **R** 5' tgttgttcattgacttcagtct 3'	66^{0}C	2µM
D17S654	17p13.3	0.87	139	**F** 5' gacctaggccatgttcacagcc 3' **R** 5' gacatccattggcaccaccccaa 3'	75^{0}C	2.5µM
D17S675	17p13.2	0.67	193	**F** 5' ccaggtgtagtgacacatgct 3' **R** 5' gtaggcaggtcaaggtcttaggg 3'	65^{0}C	1.5µM
D17S619	17p13.2	0.51	770	**F** 5'aagagataggcacaaccaactgttt 3' **R** 5' ttttccttagagccctgattactct 3'	64^{0}C	1.5µM
D17S513	17p13.2	0.8800	193-203	**F** 5'ttcacttgtgggctgctgtc 3' **R** 5'taagaaaggctcccacaagca 3'	58^{0}C	1.5µM
D17S678	17p13.2	0.80	290	**F** 5' cagcttggcaacacagcgaaa 3' **R** 5'tattctgctcggcacatagtgcaa 3'	68^{0}C	1.5µM
D17S1149	17p13.2	0.92	276	**F** 5' aacaagagtgaactccatagagag 3' **R** 5' cgctgatctgtcaggcagcct 3'	73^{0}C	1.5µM

Table 2.6: chromosome 17p

Marker	Cytogenetic location	heterozygosity	product size (bp)	primer sequences	optimum annealing temperature	optimum $MgCl_2$ concentration
D17S796	17p13.2	0.8244	144-174	**F** 5'caatggaaccaaatgtggtc 3' **R** 5'agtccgataatgccaggatg 3'	62^0C	3μM
AKG2-1	17p13.2		225	**F** 5'tcctgctctgcaacagtgac 3' **R** 5'cagcccctgctatctgat 3'	62^0C	2μM
D17S720	17p13.1	0.76	221	**F** 5' ccagccttggcaacatagcaaga 3' **R** 5' gaattctgagcatattgtttgcctg 3'	70^0C	2.5μM
D17S952	17p13.1	0.6741	129-141	**F** 5'accttaccatgcacacagtt 3' **R** 5'tccccaggagacagca 3'	59^0C	3μM
D17S786	17p13.1	0.7732	135-157	**F** 5'tacagggataggtagccgag 3' **R** 5' ggatttgggctcttttgtaa3'	58^0C	3μM
D17S804	17p13.1	0.6243	156-170	**F** 5'gcctgtgctgctgataacc 3' **R** 5'cactgtgatgagatgtcattcc 3'	62^0C	2μM
D17S945	17p13.1	0.8642	186-208	**F** 5'aaccaatctggactcccc 3' **R** 5'cctgaagcctgacccc 3'	61^0C	2μM
D17S954	17p13.1	0.7300	218-234	**F** 5'acctccatcatgtggct 3' **R** 5'ataaaacgcccgattctt 3'	55^0C	1.5μM
D17S799	17p13.1	0.6942	186-200	**F** 5'attgccagccgtcagtt 3' **R** 5'gaccagcatatcattatagacaagc 3'	61^0C	2μM
D17S798	17p13.1	0.8	209-229	**F** 5'ccatgagaaagttgtttagtagtc 3' **R** 5'tgttcttgggagtgcag 3'	57^0C	2.5μM
TP53	17p13.1	0.4992	103-135	**F** 5'agggatactattcagcccgaggtg 3' **R** 5'actgccactccttgccccattc 3'	68^0C	2.5μM

Table 2.7: chromosome 17p (continued)

Marker	Cytogenetic location	heterozygosity	product size	primer sequences	optimum annealing temperature	optimum MgCl concentration
F8VWFP	22q11.2	0.6930	197-212	**F** 5'gctgctaaatgcaaccat 3' **R** 5'acaacttctccactcaaccc 3'	53^{0}C	3μM
D22S301	22q12-13	0.7970	198-214	**F** 5' aaaacatattgcgcgtgc 3' **R** 5' catatacgtcaggaacatctgc 3'	60^{0}C	3μM
D22S294	22q13	0.3300	124-146	**F** 5' ttcgttacagcacaaaccag 3' **R** 5' aatgaagcccctgctacac 3'	62^{0}C	1.5μM

Table 2.8: chromosome 22

Taq DNA polymerase (Amersham Pharmacia Biotech) was added. This was followed by 35 cycles of annealing at the optimum temperature for 1 minute and extension at 72^{0}C for 1 minute and a final extension at 72^{0}C for 10 minutes.

The efficiency of PCR amplification was confirmed by running 10 µls of each product on a 3% agarose (Bio-Rad, Hemel Hempstead, Herts) gel immersed in 1 x TAE buffer (Tris-acetate, EDTA) under electrophoresis at 100V for 1 hour with a GeneRuler size ladder (MBI, Helena Biosciences, Sunderland, Tyne and Wear). The products on the agarose gel were stained with ethidium bromide (0.5 µg/ml) (Sigma Aldrich) and visualised under UV light.

Polyacrylamide Gel Electrophoresis of amplified products

The two alleles in each sample were separated by horizontal polyacrylamide gel electrophoresis (PAGE) on a GenePhor Electrophoresis Unit (GEU) (Amersham Pharmacia Biotech) using GeneGel Excel 15/24 kits (Amersham Pharmacia Biotech). The precast PAGE gels (15% polyacrylamide) were rehydrated for 1 hour in 12 mls rehydration buffer containing an extra 24% urea. Six µl of each sample was mixed with 1µl of loading dye (see appendix I) and loaded on to the gel in normal/tumour pairings and run at 15^{0}C for 400V for 15 minutes followed by 600V for 45 minutes with a GeneRuler size ladder. For visualisation of the amplification products, the gel was fixed in a solution of 3% benzene sulphonic acid in 24% ethanol for 30 minutes and stained using a PlusOne DNA silver staining kit (Amersham Pharmacia Biotech). The fixing solution was replaced by silver solution in which the gel was left for 30 minutes. The silver solution was then poured off and the gel washed in water for 1 minute before being placed in developing solution for 6 minutes. Finally, the gel was soaked in stopping and preserving solution for 30 minutes

Data analysis

The gel data obtained by polyacrylamide electophoresis was analysed by Scan-It gel scanning software.

Scanning and digitising

Each gel was placed face down on a flat-bed scanner (Epson GT-5000) and Epson Scan software opened. A preview of the gel image was then seen on the computer monitor. The area of interest was highlighted using a drawing tool. Each lane corresponded to one half of each sample pair, e.g. in lane 1 are the alleles of a separated normal blood DNA sample whilst lane 2 will show the alleles of its paired tumour DNA. The image was then exported to the computer's hard disk and this was then opened using UnScan-it (Silk Scientific Corporation) software. The lanes were then digitised. During this process, the computer viewed the alleles in each lane, calculated the pixel total of each allele and stored this information. When digitisation was complete the amount of pixels in each allele was represented as peaks for each sample.

Calculation of allele ratios

The pixel density of the alleles were assigned according to the peaks of greatest area in order to rule out the assessment of the pixel density of shadow bands. The values given for peak area of the alleles in the paired normal and tumour samples were used to calculate allelic loss as described by Solomon et al (1987). The pixel ratio of alleles was calculated for each normal and tumour sample and subsequently the tumour ratio was divided by the normal ratio, i.e. T1:T2;/N1:N2 in which T1 and N1 are the area values of the lesser pixel

density allele product peak for the tumour and normal sample respectively, and T2 and N2 are the area values of the greater pixel density allele product peak for the tumour and normal sample respectively. An assigned ratio of less than or equal to 0.50 was deemed to be indicative of a loss of heterozygosity on the basis that tumours containing no normal contaminating cells and showing complete allele loss would theoretically give a ratio of 0.00, but that as the tumours may contain up to 50% normal cells then complete allele loss in the tumours would give an allele ratio of between 0.00 and 0.50 (Cawkwell et al., 1993). Similarly, an assigned ratio of between 0.51 and 0.75 was deemed to show allelic imbalance assuming a higher incidence of contamination by normal cells, without which LOH would be shown. A ratio of between 0.76 and 1.00 was taken to indicate preservation of both alleles.

Calculation of MIN

Each pair of normal blood and tumour samples on each polyacrylamide gel were examined for evidence of MIN. This was defined as the presence of addition alleles or shifts of electrophoretic mobility in the tumour samples at 2 or more independent loci when compared to the normal. When MIN was seen the chromosomal loci were recorded along with the type of MIN (i.e. additional allele or shift of electrophoretic mobility).

COMPARATIVE GENOMIC HYBRIDISATION

Labelling of tumour DNA

SpectrumGreen-dUTP (Vysis, Downers Grove, IL) was incorporated in to approximately 1mg of genomic DNA extracted from each tumour sample using a nick translation kit

(Vysis). The reaction was carried out in chilled sterile 0.5ml ependorf tube containing 1mg of tumour DNA, 2.5ml 0.2mM SpectrumGreen, 5ml 0.1mM dTTP, 10ml 0.1mM dNTP (dATP, dGTP, dCTP) 5ml 10X nick translation buffer and either 5 or 10ml of nick translation enzyme.

The final volume was adjusted to 50ml with nuclease-free water. Each tube was vortexed briefly and incubated at 15°C for 2 hours. The reaction was stopped by heating at 70°C for 10 minutes.

Determining the probe size

The optimum probe size for CGH analysis is in the 300-3000 bp range. As the amount of enzyme in the nick translation reaction is increased, the size distribution shifts to progressively smaller fragments. As the quality of the starting DNA varies greatly between different samples, two volumes of enzyme were used for each tumour in order to ensure that the appropriate sized smear was obtained.

The size of the labelled DNA probe was estimated by running 10 ml of the reaction mix on a 1% agarose gel immersed in 1X TAE buffer under electrophoresis at 100V for 1 hour with a 1 DNA/Eco 911 size ladder (MBI). The DNA smear was stained with ethidium bromide (0.5 mg/ml) and visualised under UV light.

Preparing the probe mix

In order to produce a hybridisation signal with equivalent intensities, a ratio of 2:1 SpectrumGreen to SpectrumRed labelled DNA was used.

For each sample 400 ng SpectrumGreen tumour probe was combined with 200 ng SpectrumRed total genomic reference DNA (Vysis) and 10 mg COT1 human DNA (Vysis) in a 1.5 ml eppendorf tube. 0.1 volume of 3M sodium acetate and 2.5 volumes of 100% ethanol were added to precipitate the DNA. The sample was vortexed briefly, placed on dry ice for 15 minutes and then centrifuged at 13,000 rpm for 30 minutes to pellet the DNA. The supernatant was removed and the DNA pellet was dried for 15 minutes under vacuum before being resuspended in3ml nuclease-free water and 7 ml CGH hybridisation buffer. The probe mix was denatured at 73°C for 5 minutes immediately prior to hybridisation.

Control experiments using SpectrumGreen labelled normal DNA were performed with each set of tumour samples.

Hybridisation to metaphase spreads

Normal metaphase target slides were obtained from Vysis. The slides had been prepared from phytohaemaglutin (PHA) stimulated lymphocytes derived from a karyotypically normal male donor. The lymphocytes were cultured for 48 – 72 hours prior to synchronisation with thymidine to produce chromosome lengths of 400 - 550 bands.

Hybridisation areas were marked on the slide using a diamondvscribe. The slide was immersed in denaturation solution at 73°C for 5 minutes before being dehydrated serially by immersion in 70%, 85% and 100% ethanol for 1 minute each. After air-drying, 10ml of denatured probe mix was applied to the slide and a coverslip was immediatley applied and sealed with cow gum. The slide was incubated in a sealed, humidified container at 37°C for 72 hours.

Post hybridisation washes

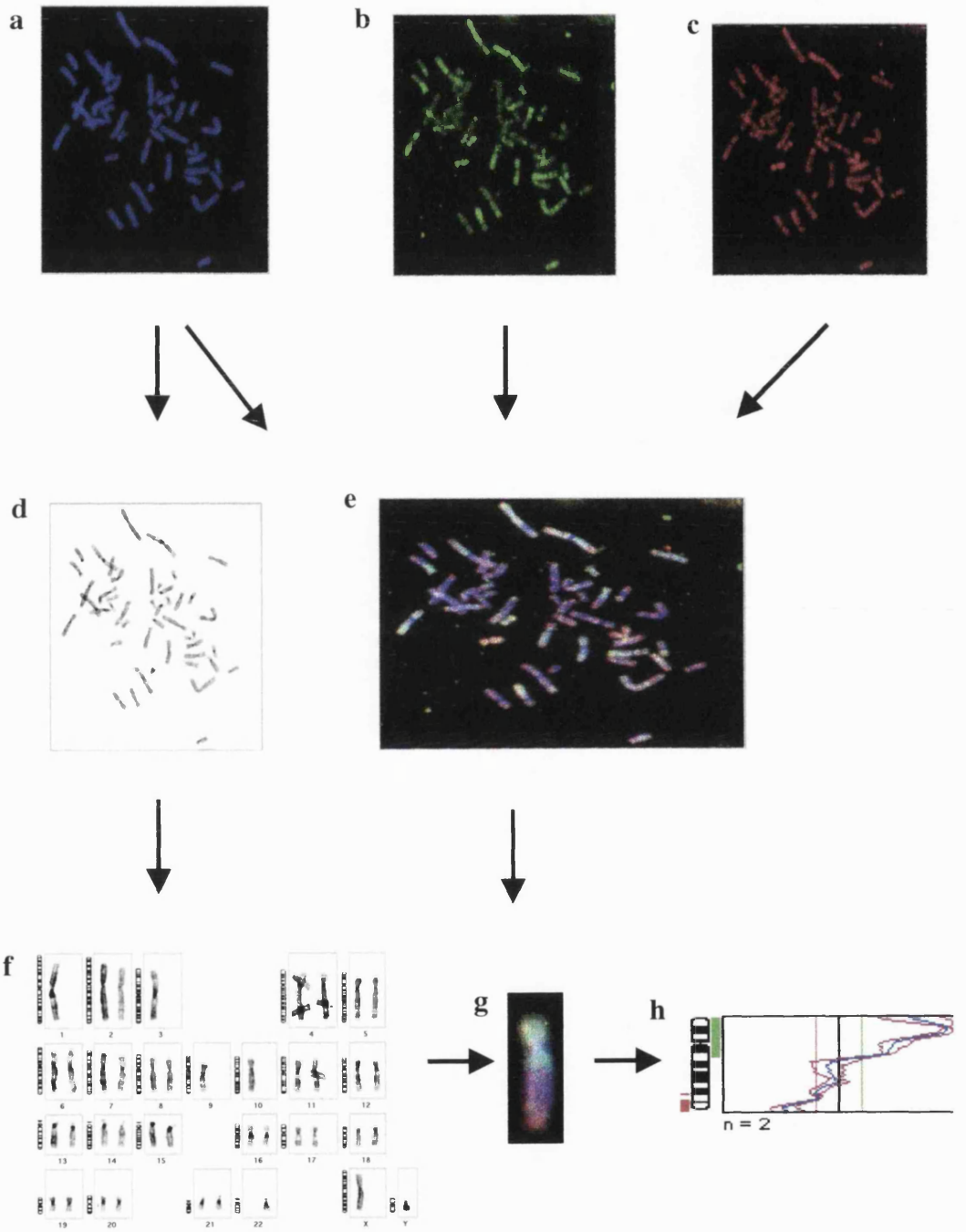
The cow gum seal and coverslip were removed and the slide was immersed in 0.4X SSC/0.3%NP-40 wash at 74°C for 2 minutes. It was then immersed in 2X SSC/0.1%NP-40 at room temperature for 1 minute before air-drying in darkness. Slides were counterstained with 4,6-diamidino-phenylindole (DAPI) in antifade solution (Vysis).

Digital imaging and analysis

Metaphase images with uniform hybridisation were acquired with a Zeiss Axioscope epifluorescent microscope equipped with a triple bandpass filter (Vysis) designed to simulataneously excite and emit light specific for DAPI, SpectrumGreen and SpectrumRed, and a cooled charge-coupled device camera (Photometrics, Tuscon, AZ). The hybridisations were evaluated by eye prior to digital image analysis as a high quality hybridisation ensures an accurate interpretation of the data within and across experiments. Metaphases used for the CGH analyses had background fluorescence that was low and uniform around each chromosome, had minimal surrounding cytoplasm and balanced red and green fluorescence. Mean ratios of SpectrumGreen to SpectrumRed signal intensities were calculated with SmartCapture software (Vysis). Between 5 and 15 metaphases were analysed and averaged to yield CGH profiles for each tumour. Upper and lower threshold values of >1.2 and <0.8 were used to interpret gain or loss of chromosomal material and high level amplifications were defined by a tumour/reference ratio of >1.4. For the normal blood controls, the mean green to red ratios remained between 1.2 and 0.8 along the whole length of all chromosomes.

The CGH procedure is illustrated in Figure 2.1 (page 79).

Figure 2.1: Comparative genomic hybridisation procedure



Digital images of DAPI counterstain (a), SpectrumGreen tumour DNA (b) and SpectrumRed normal DNA are combined to produce a composite image (e). The DAPI counterstain also produces a pseudo G-banding pattern to enable identification of each chromosome (d, f). A large region of amplification (green) can be easily seen in the composite image of chromosome 12 (g) and in the fluorescence ratio profile (h).

Statistical Anaylsis

In order to assess the effect of LOH of chromosomes 9q, 10q, 12q, 16q, 17p and 22q on survival times log rank tests were used since this is the standard statistical test to use when calculating survival curves. The same tests were used to assess the effect on survival of LOH at different marker loci, of different treatment regimens, MIN, and the most frequent losses and gains seen by CGH. In order to analyse age ($\leq$ 3 years and > 3 years) versus LOH the Fisher Exact test was used. The number of patients used in this study is small so it was felt that the latter test would be more reliable than a Chi Square test. The Fisher Exact test was also used to assess age versus LOH at particular chromosomal loci and age versus the most frequent losses and gains seen by CGH.

Chapter 3

RESULTS 1: MICROSATELLITE ANALYSIS

Introduction

LOH analysis is one of the most widely used molecular genetic techniques for determining loss of genetic material. These studies are based on the detection of differences between the normal maternal and paternal alleles in constitutional DNA. If a chromosomal locus has been lost in a tumour, one of the two normal alleles will be absent in the tumour DNA when compared with the constitutional DNA. In recent years, PCR technology has been combined with polymorphic systems to distinguish the two alleles. One such system is based on variable numbers of tandem repeat sequences, such as the dinucleotide repeat $(CA)_n$, where n varies from 10 to 60 copies (Weber and May, 1989; Litt and Luty, 1989). $(CA)_n$ repeats are plentiful throughout the human genome; approximately 50,000-100,000 separate $(CA)_n$ sites are estimated to exist (Louis et al., 1992). Because of the variability in n, these markers are highly polymorphic and have a high level of heterozygosity (Louis et al., 1992) . Differences in the number of $(CA)_n$ repeats between alleles enables them to be resolved on polyacrylamide sequencing gels. Thus, $(CA)_n$ repeats can be used for LOH analysis since by distinguishing the two alleles in constitutional tissue, they allow the detection of LOH in tumour tissue.

Rigorous criteria are used for scoring alleles. In informative cases, the two most intense bands are considered true alleles. A light band above a dark band is scored as allele 1 with the dark band allele 2, whereas a dark band above a light band is considered allele 1 with the light band an extra, shadow band, and not allele 2. Non informative cases have one

intense band above shadow bands of decreasing intensity (Weber and May, 1989; Litt and Luty, 1989: Burmeister et al., 1991; Louis et al., 1992).

Previous studies have used different criteria for assessing LOH and allelic imbalance. Allelic imbalance can be interpreted as LOH with residual allele signals from contaminating normal tissue (Tran et al., 1998). For example, two studies defined the LOH cut off point as a reduction in allele intensity of one third or more in tumour DNA compared to the constitutional DNA (Frank et al., 1997; Nakamura, et al, 1998), i.e. a ratio of 0.66 or less when 1 is preservation of both alleles. However, two other investigations assumed LOH if densitometry showed a reduction in intensity of 50% (or ratio of 0.5) in one tumour allele (Cawkwell et al., 1993; Chughtai et al., 1999; Tran et al., 1998). In a further study, analysis was scored as allelic imbalance ratios of intensity reduced by 40-50%, i.e. a ratio of 0.5-0.6 (Tran et al., 1998). One further study was even more stringent in that LOH was defined as greater than a 75% reduction in band intensity in comparison to the constitutional sample, i.e. a ratio of 0.25 (Bose et al., 1998). In the present study, a ratio of 0.5 (or a reduction in intensity of 50%) has been used to define LOH, whilst a ratio of between 0.51 and 0.75 (or a reduction in intensity of between 25 and 50%) was used to define allelic imbalance.

A range of polymorphic microsatellite markers mapping to chromosomes 9q, 10q, 12q, 16q, 17p and 22q were used to assess LOH, allelic imbalance and microsatellite instability (MIN) in a series of paired medulloblastoma and normal blood samples. MIN reflects expansion or decrease of the original length of the microsatellite (Thibodeau et al., 1993). The cytogenetic locations of all these markers are given in tables 2.2-2.8 (pages 66 to 72)

Sample details

Twenty seven patients with medulloblastomas were assessed for LOH of tumour DNA in comparison to paired normal blood controls. Twenty one samples were from males and six were from females and age at diagnosis ranged from 3 months to 13 years. Twenty four cases were classical medulloblastomas whilst three were of the desmoplastic type. One tumour, IN1212, was used in the 17p analysis only.

Data analysis

Appendix II shows the scanning densitometry data calculated subsequent to PCR amplification and polyacrylamide gel electrophoresis of all the samples with each primer. Non informative (homozygous) samples were not scanned and hence do not appear on these data sheets. Proceeding from left to right, the columns shows the pixel ratio calculated for each normal (N) and each tumour (T) sample from the peak representation of pixels in each allele. Computer software divided the tumour ratio by the normal ratio, i.e. T1:T2;/N1:N2, where T1 and N1 were the lesser pixel density products and T2 and N2 had the greatest pixel densities respectively, as described by Cawkwell et al. (1993). The results are shown in the last two columns on the right where each column shows a ratio for each allele. A ratio of less than or equal to 0.50 is indicative of a loss of heterozygosity for a particular tumour, a ratio of between 0.51 and 0.75 shows is scored as allelic imbalance, whilst a ratio of between 0.76 and 1.00 indicates preservation of both alleles. Figures 3.1-3.2 (pages 108-109) show examples of scanned gels illustrating band shifts and the presence of extra alleles at several independent loci.

CHROMOSOME 17p

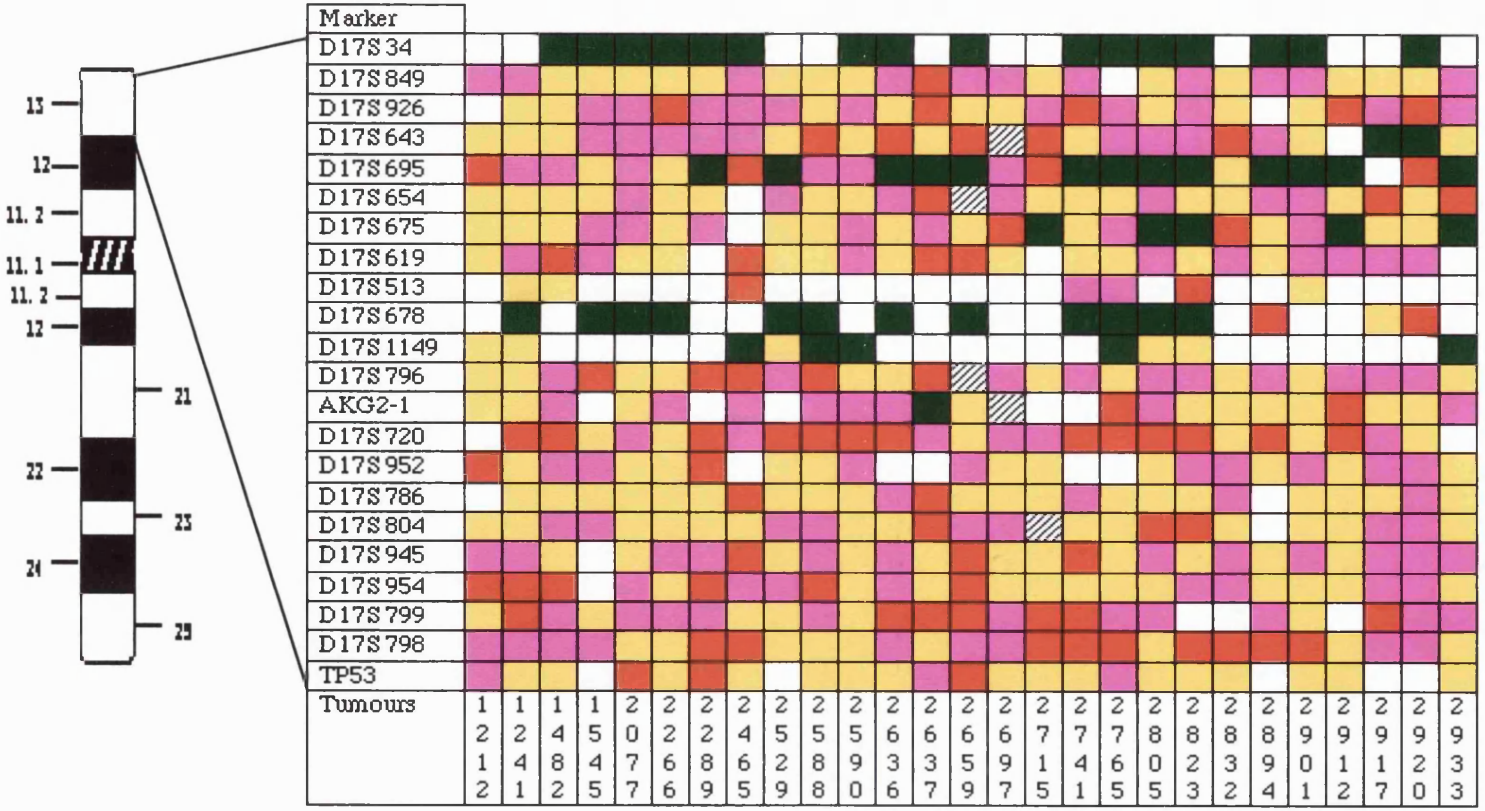
The initial, and most comprehensive, of the microsatellite work was conducted on chromosome 17p. Twenty seven tumours were analysed with twenty two polymorphic markers mapping from 17p13.1 to 17p13.3. All of the tumours studied were informative for at least fourteen markers and two tumours were informative at all twenty two loci.

Frequency and extent of loss of heterozygosity

There was loss of one allele for at least one marker in twenty seven cases (100%) and the positions and extent of deletions are summarised in Table 3.1, page 85. Eight tumours had LOH for one marker only, whilst nine tumours showed LOH of adjacent loci and one tumour had deletions at eight markers.

One tumour (IN2077) showed LOH at the most proximal marker TP53 (mapping to 17p13.1) only. No case showed LOH at the most distal marker D17S34 (mapping to 17p13.3) in all sixteen tumours that were analysed. The remaining eleven samples were not analysed using this marker to conserve DNA for other studies. IN1212 showed LOH at three markers, D17S695 (mapping to 17p13.3) and two non-adjacent markers on 17p13.1 (D17S952 and D17S954). IN1241 revealed loss at three markers on 17p13.1 only. Two of these (D17S799 and D17S954) were adjacent, whilst the third (D17S720) was more distal. IN1482 had LOH at two loci on 17p13.1 (D17S954 and D17S720) and at one marker on 17p13.2 (D17S619). The one marker for which IN1545 showed LOH was also on 17p13.2; D17S796, whilst the one marker for which IN2266 had LOH was one of the more distal ones on 17p13.3 (D17S926). IN2289 demonstrated LOH for six markers and all except D17S796 mapped to 17p13.1 LOH was seen at the adjacent markers D17S720 and D17S952 and also at the most proximal adjacent markers D17S798

Table 3.1 Allelic loss on chromosome 17p in the direction of centromeric (TP53) to telomeric (D17S678)



Key for explaining colour scheme of above table

Preserved		LOH	
Allelic imbalance		Non informative	
No information		Band shift	

and TP53. Loss was also seen at an intermediate marker locus, D17S954. LOH was observed at markers mapping to 17p13.1 to 17p13.3 in IN2465. The most distal marker lost was D17S695 on 17p13.3. There was loss at three markers on 17p13.2, the adjacent D17S619 and D17S513 and the more proximal D17S 796. LOH was seen at three markers on 17p13.1 (D17S786, D17S945 and D17S798). The only marker at which there was LOH in IN2529 was D17S720 (mapping to 17p13.1). Tumour IN2588 showed LOH at four markers. One was on 17p13.3 (D17S643), one was on 17p13.2 (D17S796) and two were on 17p13.1 (D17S720 and D17S954). D17S720 was also the locus for the only loss seen in IN2590. IN2636 had LOH at one marker on 17p13.3 (D17S643) and two markers on 17p13.1 (D17S720 and D17S799). Case IN2637 showed LOH at a distal marker on 17p13.3 (D17S643) and two markers on 17p13.1 (D17S720 and D17S799). IN2637 demonstrated LOH at the most markers, eight in all. Two were adjacent markers on 17p13.3 (D17S849 and D17S926) and one was a more proximal marker on 17p13.3 (D17S654). There was LOH at two markers on 17p13.2 (D17S619 and D17S796) and at three markers on 17p13.1, the adjacent D17S786 and D17S804 loci and the more proximal D17S799 locus. Like IN2637, IN2659 showed LOH at markers spanning the whole region looked at. One marker on 17p13.3 showed LOH (D17S643), LOH was seen at one marker, D17S619, on 17p13.2 and the remaining four loci at which LOH was seen were on 17p13.1. Three of these were adjacent markers (D17S945, D17S954 and D17S799) and the other was the most proximal marker, TP53. LOH was seen at the D17S675 marker only in IN2697. IN2715 showed LOH at two adjacent markers on 17p13.3 (D17S643 and D17S695) and also at two adjacent markers on 17p13.1 (D17S799 and D17S798). Tumour IN2741 also showed LOH at the latter two adjacent loci as well as at two other 17p13.1 markers (D17S945 and D17S720) and at one 17p13.3 marker (D17S926). Case IN2765 showed LOH at two adjacent loci, AKG2-1 (on 17p13.2) and D17S720 as well as at D17S798. Loss was seen at two 17p13.1 loci only in IN2805 (D17S720 and D17S804). IN2823 also showed LOH at the latter two markers as well as

at D17S798 and D17S513. LOH was seen at three loci in IN2832 (D17S643, D17S675 and D17S798) and also at three markers in IN2894 (D17S678, D17S720 and D17S798). D17S798 was also the only marker at which IN2901 showed LOH. IN2912 showed LOH at D17S926 and also at two adjacent loci (AKG2-1 and D17S720). LOH was observed at only two loci in IN2917 (D17S654 and D17S799), at three loci in IN2920 (D17S926, D17S695 and D17S678) and at only one locus in IN2933 (D17S654).

Incidence of allelic imbalance

Allelic imbalance was observed much more frequently than LOH. It was present in twenty seven tumours (100%) at a greater number of loci. The highest frequency was in tumour IN2917 in which imbalance was seen at nine markers, and in the lowest frequency in tumour IN2715 where it was seen at only two markers. Allelic imbalance was distributed across the entire region analysed (17p13.1-17p13.3) and markers which showed allelic imbalance were often adjacent to those at which LOH was seen. IN1212 showed imbalance at the distal D17S849 locus and at the two most proximal markers (TP53 and D17S 798). It was also seen at D17S945 adjacent to LOH at D17S954 in both this case and IN1241 where imbalance was also seen at D17S798 adjacent to LOH at D17S799. Imbalance was seen at three 17p13.3 markers in this tumour (D17S849, D17S695 and D17S619). Two markers which showed allelic imbalance were adjacent to markers which showed of LOH in IN1482. Imbalance was seen at D17S799 (adjacent to LOH at D17S954) and also at AKG2-1 and D17S952 which flank a marker showing LOH, D17S720. Imbalance was also shown at D17S798, D17S804, D17S796 and D17S695. Imbalance was seen at two adjacent 17p13.3 markers (D17S926 and D17S643), two adjacent 17p13.2 markers (D17S675 and D17S619) and three 17p13.1 markers (D17S952, D17S804 and D17S798) in IN1545. IN2077 showed allelic imbalance at five adjacent distal markers (D17S926, D17S643, D17S695, D17S654 and D17S675) as well as at two

adjacent proximal markers (D17S954 and D17S799). This tumour also demonstrated imbalance at D17S720. A marker showing allelic imbalance (D17S643) was adjacent to a marker showing LOH (D17S926) on 17p13.3 in IN2266. This tumour also had imbalance at the AKG2-1, D17S945 and D17S799 loci. IN2289 showed imbalance at the D17S945 and D17S799 markers which flank a marker showing LOH, D17S954. D17S799 is also adjacent to D17S798 where LOH was also seen. This suggests a sizeable region of loss in this tumour. Imbalance was also seen at three more distal markers (D17S675 and the adjacent D17S643 and D17S926). Three markers showing allelic imbalance were adjacent to markers showing LOH in IN2465. Imbalance was seen at D17S954 (adjacent to D17S945), AKG2-1 (adjacent to D17S796) and D17S643 (adjacent to D17S695). Imbalance was also seen at the D17S720, D17S926 and D17S849 markers. IN2529 showed imbalance at five loci (D17S954, D17S804, D17S796, D17S654 and D17S926). Markers showing allelic balance were observed adjacent to markers showing LOH at three regions in IN2588. A marker showing LOH, D17S954, was flanked by markers showing imbalance at D17S945 and D17S799, the AKG2-1 marker showing imbalance was flanked by two markers showing LOH, at D17S796 and D17S720, and imbalance was seen at D17S695 adjacent to LOH at D17S643. Imbalance was also seen at D17S804 in this tumour. A marker showing LOH (D17S720) was flanked by markers showing imbalance (D17S952 and AKG2-1) in IN2590. Imbalance was also seen at four other loci in this tumour (D17S619, D17S675, D17S695 and D17S926). In IN2636, a marker of LOH, D17S799, was flanked by markers showing imbalance (D17S798 and D17S654). More distally, a marker showing imbalance (AKG2-1) is adjacent to D17S720 where LOH was seen. Imbalance was also seen at D17S945, D17S786, D17S654 and D17S849. Allelic imbalance was only seen at three markers in IN2637; at the most proximal marker TP53, at AKG2-1 and at D17S675. IN2659 showed allelic imbalance at D17S804 and D17S798. In between these two loci were three LOH markers and D17S798 is adjacent to a further LOH marker, TP53. This suggests a sizeable region of loss of 17p13.1 in this tumour.

Imbalance was also observed at D17S952 and D17S849.. Allelic imbalance was seen at the D17S675 marker in IN2697 adjacent to a marker of LOH, D17S675. Imbalance was also found at the D17S798, D17S799, D17S804, D17S720, D17S796, D17S695 and D17S849 loci,. IN2715 showed the least amount of allelic imbalance. It was seen at only two markers, D17S926 (adjacent to the LOH marker D17S643) and D17S720. IN2741 showed imbalance at D17S849, adjacent to loss at D17S926 as well as at D17S513, D17S796 and D17S786. In IN2765 the LOH marker D17S798 was flanked by two markers of imbalance (TP53 and D17S799). Imbalance was also found at D17S513, D17S675, D17S643 and D17S926). IN2805 showed a sporadic distribution of allelic imbalance over the whole region analysed. It was seen at AKG2-1 and D17S945 adjacent to markers of LOH, as well as at D17S799, D17S796D17S619, D17S654 and D17S643. Allelic imbalance was found at three adjacent 17p13.3 markers in IN2823 (D17S849, D17S926 and D17S643) as well as at a 17p13.2 marker (D17S796) and two 17p13.1 markers (D17S952, adjacent to a marker of LOH, D17S720, and D17S954, whilst in IN2894 it was again seen in three 17p13.3 markers (D17S849, D17S643 and D17S654) as well as the 17p13.2 marker D17S796 and the 17p13.1 marker D17S799, adjacent to loss at D17S798. IN2901 showed imbalance at six loci (D17S849, D17S654, D17S675, D17S619, D17S720 and D17S945) and IN2912 at only two (D17S619 and D17S796, adjacent to loss at AKG2-1). IN2917 showed the highest frequency of allelic imbalance at nine markers. With the exception of three markers (D17S926, D17S619 and D17S796) the imbalance was seen at markers mapping to 17p13.1 (D17S720, D17S952, D17S804, D17S945, D17S954 and D17S798). A similar pattern was seen with IN2920 where imbalance was seen at all the latter 17p13.1 markers, with the exception of D17S720, as well as D17S786, also in this region, and D17S796 on 17p13.2. IN2933 showed allelic imbalance at five loci. Two were adjacent on 17p13.3 (D17S849 and D17S926), one was on 17p13.2 (AKG2-1) and the remainder were on 17p13.1 (D17S954 and D17S799).

Determination of minimum region of loss

Of the twenty seven tumours with LOH, fourteen (51%) showed loss at either D17S799 or D17S798 and two cases had LOH at both these markers. An additional ten tumours showed allelic imbalance at one or both of these loci. No information was available for one of these markers for one further tumour. This suggests that a putative tumour suppressor gene is located at 17p13.1, marginally distal to p53. However, thirteen of the twenty seven tumours (48%) showed LOH at D17S720 with a further six showing allelic imbalance at this locus. There is thus the possibility of a second putative tumour suppressor gene located more distally on 17p13.1. Six of twenty six tumours (19%) showed LOH at D17S643 on 17p13.3, so it is possible that there is a third putative tumour suppressor gene at this locus.

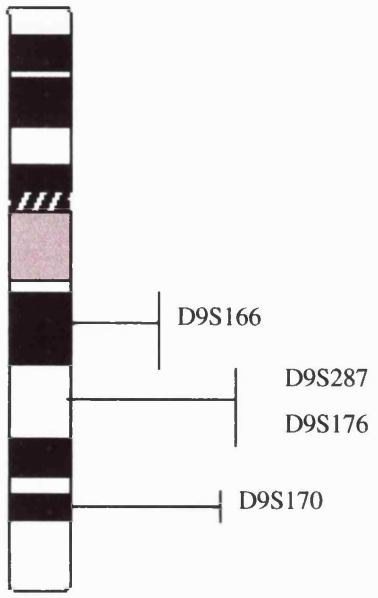
CHROMOSOME 9q

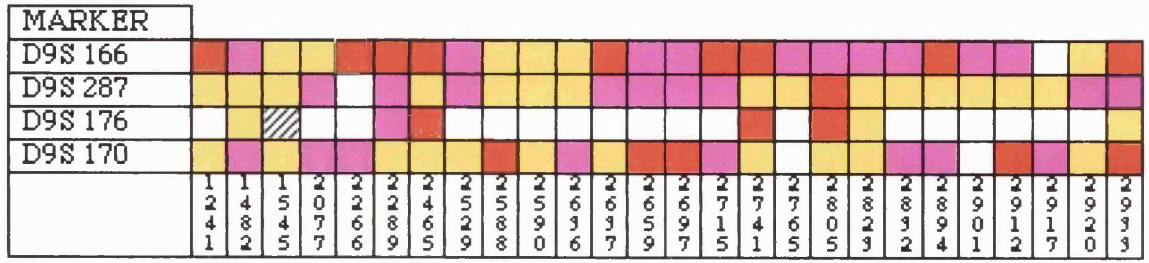
Twenty six tumours were analysed with four polymorphic markers mapping from 9q21.1 to 9q33. All of the tumours studied were informative for at least two markers and seven tumours were informative at all four loci.

Frequency and extent of loss of heterozygosity

There was loss of one allele for at least one marker in fourteen cases (54%) and the positions and extent of deletions are summarised in Table 3.2, page 91. Ten tumours had LOH for one marker only, whilst one tumour showed LOH of adjacent loci.

Table 3.2 Allelic loss on chromosome 9q in the direction of centromeric (D9S166) to telomeric (D9S170)





Key for explaining colour scheme of the above table

Preserved	
Allelic imbalance	
No information	
Band Shift	
LOH	

Six tumours (IN1241, IN2266, IN2289, IN2637, IN2715 and IN2894) showed LOH at the most proximal marker D9S166 (mapping to 9q21.1) only. A further four cases (IN2588, IN2659, IN2697 and IN2912) had loss at the most distal marker D9S170 (mapping to 9q33) only. Tumours IN2465 and IN2741 both showed loss at D9S166 and D9S176 (which maps to 9q22.3). IN2933 showed loss at the most proximal marker and the most distal marker. Although heterozygosity was preserved at D9S176 which is in between the deleted regions, allelic imbalance was also seen at the D9S287 marker (mapping to 9q22.3) so it is possible that the whole region between 9q21.1 and 9q33 is lost in this tumour or that there are two independent targets on this chromosome. IN2805 also has a sizeable region of loss with LOH seen at two adjacent markers, D9S287 and D9S176.

Incidence of allelic imbalance

Allelic imbalance was observed much more frequently than LOH alone. It was present in twenty five tumours (96%). In eleven of these cases (IN1482, IN1545, IN2077, IN2529, IN2636, IN2765, IN2823, IN2832, IN2901, IN2917 and IN2920) there was no evidence of LOH. Three of these tumours (IN2765, IN2823 and 2901) showed imbalance at the most proximal marker, D9S166, only, whilst one (IN2529) showed imbalance both at this marker and its adjacent marker, D9S287, and two others (IN2636 and IN2917) showed imbalance at the most distal marker, D9S170, only. IN1482 and IN2832 showed imbalance at both the most proximal and the most distal markers only. Tumour IN2077 showed imbalance at both the most distal marker and at D9S287 whilst IN2920 showed imbalance at the latter marker only.

Of the nine tumours with both allelic imbalance and LOH, five tumours (IN2289, IN2637, IN2715, IN2805 and IN2933) had markers showing allelic balance adjacent to markers

showing LOH. Furthermore, with all these tumours this was found at the two most proximal markers. Tumours IN2659 and IN2697 both showed imbalance at these two proximal markers. IN2912 showed imbalance at the most proximal marker whilst IN2266 and IN2894 showed imbalance at the most distal marker. Tumours IN1241, IN2465, IN2588 and IN2741 showed LOH ONLY whilst all informative alleles were preserved in IN2590.

Determination of minimum region of loss

Of the fourteen tumours with LOH, ten (71%) showed loss at either D9S166 or D9S287. An additional eleven tumours showed allelic imbalance at one or both of these loci. There was no information for one of these markers in two further tumours. However, eight (57%) of the fourteen tumours with LOH showed loss at either D9S176 or D9S170. An additional ten tumours showed allelic imbalance at one of these loci. There was no information for one or both of these markers in a further seven tumours. This suggests that there are at least two putative tumour suppressor genes, one located at 9q21.1 - 9q22.3 which is flanked by the D9S166 and D9S287 markers, and one located at 9q22.3 - 9q33 which is flanked by the D9S176 and D9S170 markers.

CHROMOSOME 10q

Twenty six tumours were analysed with three polymorphic markers mapping to 10q21.3-10q24. Twenty three of the tumours studied were informative for at least two markers and one tumour was informative at all three loci.

Frequency and extent of loss of heterozygosity

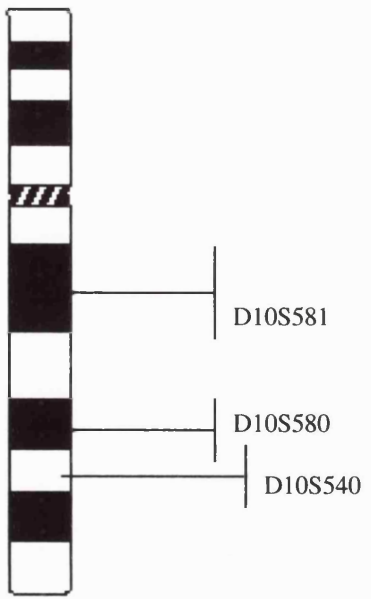
There was loss of one allele for at least one marker in six cases (23%) and the positions and extent of deletions are summarised in Table 3.3, page 95. Five tumours had LOH for one marker only, whilst one tumour showed LOH of adjacent loci.

Three tumours (IN1482, IN2266 and IN2933) showed LOH at the most proximal marker D10S581 (mapping to 10q21.3) only. A further two cases (IN2637 and IN2823) had loss at the most distal marker D10S540 (mapping to 10q24) only. In tumour IN2289 LOH was shown at D10S540 and its adjacent marker D10S580 (mapping to 10q23.1). The most proximal marker, D10S581 showed allelic imbalance so it is possible that the whole region between 10q21.3 and 10q23.1 has been deleted in this tumour. There were no interstitial deletions.

Incidence of allelic imbalance

Allelic imbalance was seen at a much higher frequency than LOH alone. It was present in twelve tumours (46%). In six of these cases (IN1545, IN2077, IN2465, IN2529, IN2636 and IN2659) there was no evidence of LOH. Tumours IN1545, IN2465, IN2529 and IN2659 showed imbalance at the most proximal marker, D10S581, only, whilst IN2077 and IN2636 showed imbalance at both this marker and the most distal marker, D10S540. With both these cases, there was no information for the intermediate marker so it is possible that there may be allelic imbalance or loss of the whole region 10q21.3 - 10q24 in these tumours. Of the three cases where both LOH and allelic imbalance were seen, IN2289 showed imbalance at the most proximal D10S 581 marker and LOH at the adjacent D10S580 marker. It is possible that this tumour has lost the entire region studied. Similarly, IN2823 showed imbalance at D10S581, LOH at the D10S540 distal marker and

Table 3.3 Allelic loss on chromosome 10q in the direction of centromeric (D10S581) to telomeric (D10S 540)



MARKER																												
D10S 581																												
D10S 580																												
D10S 540																												
	1241	1482	1545	2077	2266	2289	2465	2529	2588	2590	2636	2637	2659	2697	2715	2741	2765	2805	2823	2832	2894	2901	2912	2917	2920	2933

Key for explaining colour scheme of the above table

Preserved		Non informative	
Allelic imbalance		No information	
LOH			

no information was available for the intermediate D10S580 marker. IN2266 showed imbalance at D10S540 and the adjacent D10S580 marker was non informative. Tumours IN1482, IN2637 and IN2933 showed LOH with no other imbalance whilst in IN1241, IN2588, IN2590, IN2697, IN2715, IN2741, IN2765, IN2805, IN2832, IN2894, IN2901, IN2912, IN2912 and IN2920 all alleles were preserved.

Determination of minimum region of loss

Of the six tumours with LOH, four (66%) showed loss at either D10S581 or D10S580. An additional seven tumours showed allelic imbalance at one or both of these loci. There was no information available for a further eleven tumours for one or both markers. This suggests that a putative tumour suppressor gene is located at 10q21 to 10q23.1 which is flanked by these two markers.

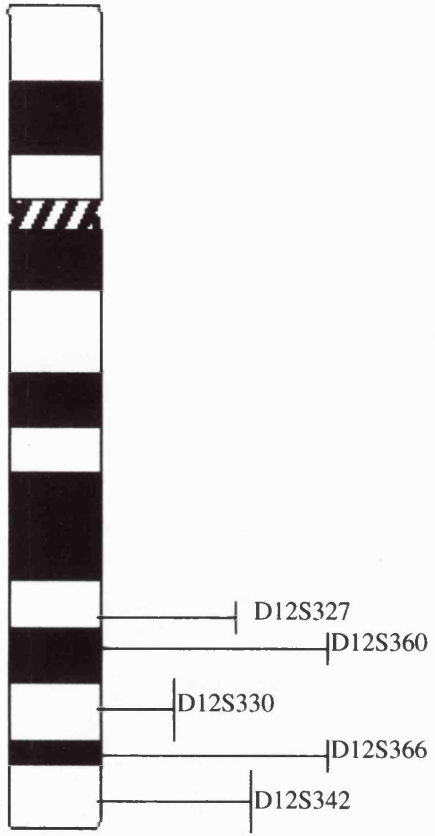
CHROMOSOME 12q

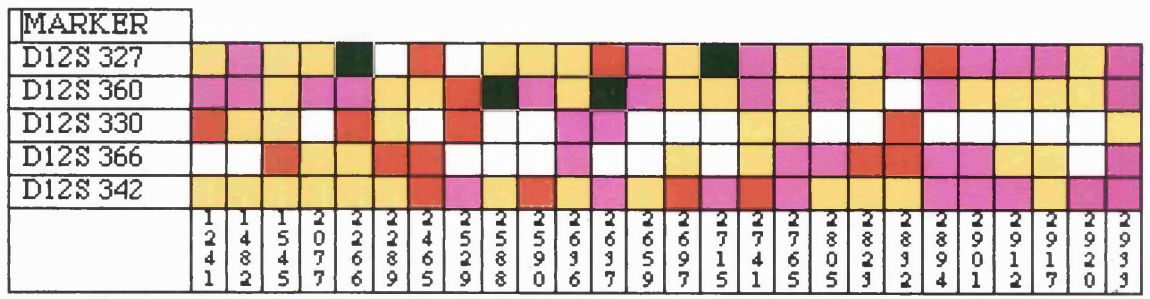
Twenty six tumours were analysed with five polymorphic markers spanning 12q22 to 12qter. All of the tumours studied were informative for at least two markers and six tumours were informative at all five loci.

Frequency and extent of loss of heterozygosity

There was loss of one allele for at least one marker in thirteen cases (50%) and the positions and extent of deletions are summarised in Table 3.4, page 97. Ten tumours had LOH for one marker only, whilst three tumours showed LOH of adjacent loci, one of which had deletions at three markers.

Table 3.4 Allelic loss on chromosome 12q in the direction of centromeric (D12S327)) to telomeric (D12S342)





Key for explaining colour scheme of the above table

Preserved		Non informative	
Allelic imbalance		No information	
LOH			

Two tumours (IN2637 and IN2894) showed LOH at the most proximal marker D12S327 (mapping to 12q22) only. A further three cases (IN2590, IN2697 and IN2741) had loss at the most distal marker D12S342 (mapping to 12q24.3) only. Tumour IN2465 showed LOH at D12S327 and at the two distal markers D12S366 and D12S342, although heterozygosity was preserved at D12S360 which maps to 12q23 in between the deleted regions. It is possible that the whole of 12q22-ter is lost in this tumour or that there are two independent targets on this chromosome. In tumour IN2529, LOH was observed at D12S360 and D12S330. However, no information was available for D12S327 and D12S366, although the most distal marker showed allelic imbalance and thus, the 12q22-ter region may be completely deleted in this tumour also. In the remaining cases, there was evidence of interstitial deletions. Tumours IN1241 and IN2266 showed LOH only at D12S330 which maps to 12q24.1. In three cases (IN1545, IN2289 and IN2823) there was loss at D12S366 only. In tumour IN2832, there was LOH at D12S330 and D12S366.

Incidence of allelic imbalance

Allelic imbalance was observed much more frequently than LOH alone. It was present in twenty five tumours (96%). In twelve of these cases (IN1482, IN2077, IN2636, IN2659, IN2715, IN2765, IN2805, IN2901, IN2912, IN2917, IN2920 and IN2933) there was no evidence of LOH. In two of these cases, IN1482 and IN2659, imbalance was found at the two most proximal markers, D12S327 and D12S360 (mapping to 12q22 and 12q23 respectively) only and in one case, IN2917 imbalance was found only at the most proximal marker, D12S327. Two cases, IN2920 and IN2715 showed imbalance at the most distal marker D12S342 (mapping to 12q24.3) only, whilst another, IN2765, showed imbalance at the two most distal markers; D12S342 and the more proximal D12S366. Tumour IN2912 demonstrated imbalance at both the most proximal (D12S327) and the most distal

(D12S342) markers, whilst IN2901 had imbalance at the two latter markers and the second most distal D12S366. IN2933 had imbalance at all markers except the intermediate D12S330. It is possible that the whole of 12q22-ter has allelic imbalance in this tumour. Similarly, in IN2805 imbalance was seen at three of four loci for which there is information including the two most proximal markers. The remaining tumours in this group, IN2077 and IN2636, showed imbalance at intermediate markers.

Of the cases in which both LOH and allelic imbalance was seen, in two tumours, IN1241 and IN2266, allelic imbalance was seen at the D12S360 marker and LOH was shown at the adjacent D12S330 marker. In tumour IN2894, imbalance is again seen at the D12S360 marker and LOH at its other adjacent marker, the most proximal D12S327. Whilst information is not available for all five markers, IN2894 and IN2529 showed allelic imbalance or LOH at all markers for which there was data, indicating that the entire 12q22-ter region may be deleted in these tumours. Tumour IN2832 showed imbalance at the most distal marker, D132S327, only. However, whilst there is no information for the adjacent marker, D12S360 LOH was seen at the D12S330 and D12S366 markers indicating an uninterrupted region of LOH/imbalance. Similarly, tumour IN2590 showed imbalance at the D12S360 marker only but there is no information for the markers between the latter and the most distal D12S342 marker where LOH was seen. IN2741 showed imbalance at the two most proximal markers, D12S327 and D12S360 only, whilst IN2637 had imbalance at the most distal marker, D12S342. Tumours IN1545, IN2289, IN2465, IN2697 and IN2823 showed LOH without any other imbalance, whilst IN2588 showed no allelic imbalance in any form.

Determination of minimum region of loss

Of the thirteen tumours with LOH, eight (61%) showed loss at either D12S330 or D12S366, of which one (IN2832) had LOH at both these markers. An additional seven tumours showed allelic imbalance at one or both of these loci. For ten further tumours no information was available for one or both markers. This suggests that a putative tumour suppressor gene is located at 12q24.1-24.2 which is flanked by these two markers. There is also the possibility of a second locus, at 12q24.3, in a subset of cases.

CHROMOSOME 16q

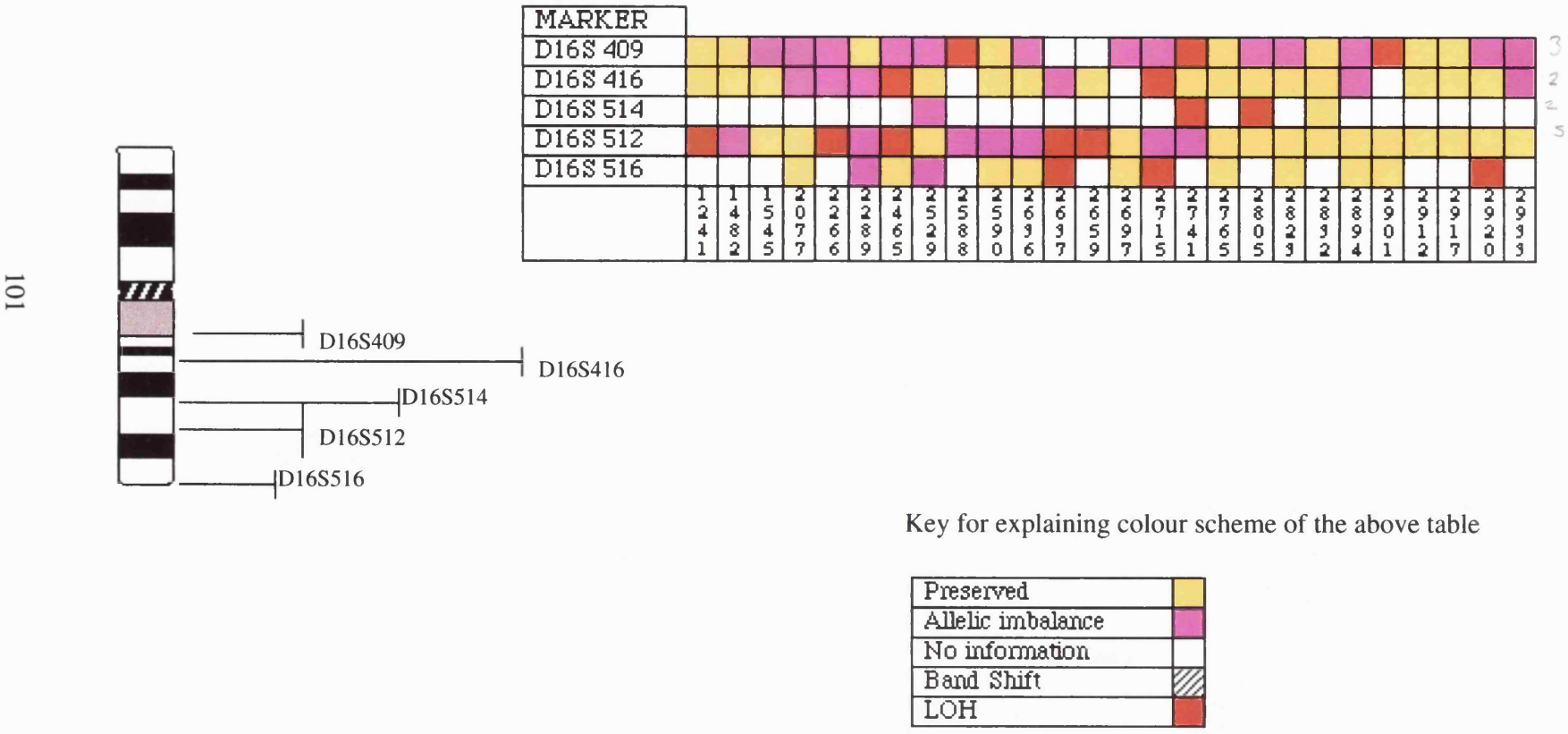
Twenty six tumours were analysed with five polymorphic markers mapping to 16q12.1 to 16q24.1. All of the tumours studied were informative for at least two markers and one of the tumours was informative at all five loci.

Frequency and extent of loss of heterozygosity

There was loss of one allele for at least one marker in eleven cases (42%) and the positions and extent of deletions are summarised in Table 3.5, page 101. Seven tumours had LOH for one marker only, whilst one tumour showed LOH of adjacent loci.

Two tumours (IN2588 and IN2901) showed LOH at the most proximal marker D16S409 (mapping to 16q12.1) only. One further case (IN2920) had loss at the most distal marker D16S516 (mapping to 16q24.1) only. Tumours IN1241, IN2266 and IN2659 all showed LOH at the D16S512 marker (mapping to 16q22.1) only. There was no information for the adjacent D16S516 marker for any of these three cases. IN2637, however, showed LOH for both these distal markers. There was no information for the D16S409 and

Table 3.5 Allelic loss on chromosome 16q in the direction of centromeric (D16S409) to telomeric (D16S 516)



D16S514 (mapping to 16q21) markers for this tumour but there was allelic imbalance at D16S416 (mapping to 16q13) so it is possible that the whole region between 16q12.1 and 16q24.1 is lost in this tumour. Similarly, tumour IN2465 had LOH at the D16S416 and D16S512 markers, IN2715 had LOH at the D16S416 and D16S516 markers and IN2741 had LOH at the D16S409 and D16S514 markers. In the only other case showing LOH (IN2805), loss was seen at D16S514 only.

Incidence of allelic imbalance

Allelic imbalance was observed much more frequently than LOH alone. It was present in twenty two tumours (84%). In eleven of these cases (IN1482, IN1545, IN2077, IN2289, IN2529, IN2590, IN2636, IN2697, IN2823, IN2894 and IN2933) there was no evidence of LOH. Three tumours (IN1545, IN2697 and IN2823) showed imbalance at the most proximal marker, D16S409, only, whilst IN2077 and IN2933 revealed imbalance at the two proximal markers only. No tumours demonstrated imbalance at the most distal marker alone. Three tumours (IN1482, IN2588 and IN2590) had imbalance at the D16S512 marker only, and IN2636 showed imbalance at this marker and at the D16S409 locus. IN2289 revealed imbalance at three out of four markers for which there was information (D16S416, D16S512 and D16S516), whilst for IN2529 there was information for all five markers, three of which demonstrated imbalance (D16S409, D16S514 and D16S516).

For the tumours in which LOH and allelic imbalance were seen, three tumours (IN2465, IN2715 and IN2741) had markers showing allelic balance next to markers showing LOH. In the first two of these cases, this was seen at the two proximal markers and with IN2715 it was seen at these markers as well as at the distal markers. There was no information for the marker in between. It is possible that the whole 16q12.1 to 16q24.1 region is lost in this tumour. Similarly, IN2266 showed imbalance at the two proximal markers, LOH at the

D16S512 locus and there was no information for the remaining two markers, whilst IN2637 showed imbalance at the D16S416 locus, LOH at the two distal markers and no information for the other two. For IN2741, imbalance was seen at the D16S512 marker with LOH at the adjacent D16S514 marker. No information was available for the most distal marker so this tumour may also have a sizeable region of loss. Two tumours (IN2805 and IN2920) showed allelic imbalance at the most proximal marker only with LOH at the D16S514 (IN2805) and D16S516 (IN2920) loci suggesting two independent targets on this chromosome. Similarly, IN2588 showed imbalance at D16S512 and LOH at D16S409 although no information was available for the other markers. Tumours IN1241, IN2659 and IN2901 showed LOH only, and tumours IN2765, IN2832, IN2912 and IN2917 had preservation of both alleles at all informative markers.

Determination of minimum region of loss

Of the eleven tumours with LOH, seven (63%) showed loss at either D16S514 or D16S512. An additional seven tumours showed allelic imbalance at one of these loci. In eleven further tumours there was no information for one of these markers. This suggests that a putative tumour suppressor gene is located at 16q21 - 16q22.1 which is flanked by these two markers. However, the same proportion of tumours (63%) showed loss at either D16S512 or D16S516 and one of these cases had LOH at both these markers. An additional seven tumours showed allelic imbalance at one or both of these loci, and there was no information for a further six tumours. Also, five tumours (45%) showed LOH at either the D16S409 or D16S416 loci with a further 13 showing allelic imbalance. This suggests that there may be multiple putative tumour suppressor genes located on chromosome 16q.

CHROMOSOME 22q

Twenty six tumours were analysed with three polymorphic markers spanning 22q11.2 to 22q13. All of the tumours studied were informative for at least one marker and thirteen tumours were informative at all three loci.

Frequency and extent of heterozygosity

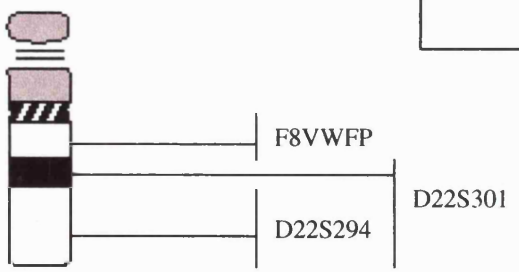
There was loss of one allele for at least one marker in twelve cases (46%) and the positions and extent of deletions are summarised in Table 3.6, page 105. Ten tumours had LOH for one marker only, whilst one tumour showed LOH of adjacent loci.

Seven tumours (IN1241, IN2637, IN2697, IN2741, IN2765, IN2832 and IN2917) showed LOH at the most proximal marker F8VWFP (mapping to 22q11.2) only. One case (IN2588) had loss at the most distal marker D22S294 (mapping to 22q13) only. IN2823 showed loss at D22S294 and the adjacent D22S301 marker (mapping to 22q12-13) whilst the remaining F8VWFP marker showed allelic imbalance. It is possible that the whole 22q11.2 to 22q13 region is lost in this tumour. Similarly, in IN2465 both the most proximal and the most distal markers showed LOH. Two tumours (IN2266 and IN2894) both showed LOH at the D22S301 marker only.

Incidence of allelic imbalance

Allelic imbalance was observed at a much higher frequency than LOH alone. It was present in twenty three tumours (88%). In eleven of these cases (IN1545, IN2077 IN2289, IN2529, IN2636, IN2659, IN2715, IN2805, IN2912, IN2920 and IN2933) there was no evidence of LOH. In three of these tumours (IN2289, IN2659 and IN2912)

Table 3.6 Allelic loss on chromosome 22q in the direction of centromeric (F8VWFP) to telomeric (D22S294)



Key for explaining colour scheme of the above table

Preserved		Non informative	
Allelic imbalance		No information	
LOH		Band shift	

imbalance was seen at the most proximal F8VWFP only, whilst in IN2077 it was seen at this marker and the adjacent D22S301. IN1545 and IN2529 showed imbalance at the most distal D22S294 only, whilst in IN2636, IN2805 and IN2933 it was seen at this marker and the adjacent D22S301. Cases IN2715 and IN2920 both showed imbalance at the D22S301 marker only.

Of those tumours which showed LOH and allelic imbalance, two (IN2588 and IN2823) had markers showing allelic imbalance adjacent to markers showing LOH. IN2588 showed imbalance at D22S301 and LOH at D22S294, whilst IN2823 showed LOH at D22S301 and imbalance at F8VWFP. Three tumours (IN2741, IN2765 and IN2832) all showed imbalance at the distal marker and LOH at the proximal marker. Cases IN2266, IN2465, IN2637, IN2894 and IN2917 showed LOH alone. IN1482, IN2590 and IN2901 showed preservation of both alleles.

Determination of minimum region of loss

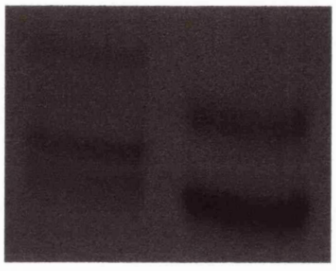
Of the twelve tumours with LOH, eleven (91%) showed loss at either F8VWFP or D22S301. An additional ten tumours showed allelic imbalance at one or both of these loci. There was no information for one of these markers in a further two tumours. This suggests that a putative tumour suppressor gene is located at 22q11.2 - 22q12-13 which is flanked by these two markers.

Microsatellite instability

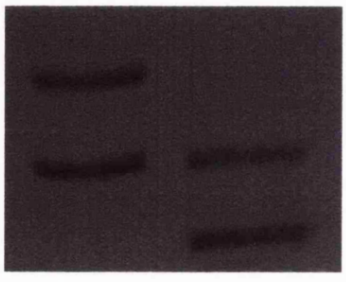
The stringent criteria established by Aaltonen et al. (1993) and used in other studies of MIN (Liu et al., 1995; Kim et al., 1994) that requires alterations in microsatellite size at two independent loci to classify a tumour as MIN+ was used. The frequency was low; two of

twenty seven tumour samples (IN2659 and IN2697) showed microsatellite size alterations at two loci. These loci were both on chromosome 17p; IN2659 showed changes in electrophoretic mobility (band shifts) at the D17S654 (17p13.3) and D17S796 (17p13.2) loci, whilst IN2697 revealed the presence of an extra allele at the D17S643 (17p13.3) and AKG2-1(17p13.1) loci. Several other tumour samples, showed band shifts or the presence of extra alleles at one locus only and therefore were not classified as MIN+. IN2715 had a band shift at the D17S804 locus on 17p13.1, whilst IN1241 had a band shift at D22S301 (22q12-13) and IN1545 showed this change at the D9S176 locus (9q22.3). Sample IN2912 revealed the presence of an extra band at the D16S512 locus (16q22.1). The tumours showing MIN and electrophoretic motility/an extra allele are shown in figures 3.1 and 3.2 (pages 108 and 109)

Figure 3.1 Examples of band shifts in 4 tumours at various loci

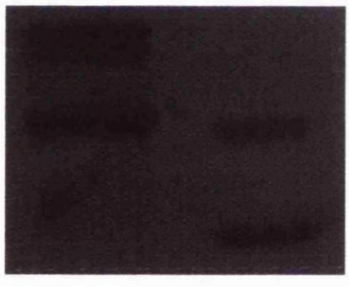


IN2659 (D17S654)

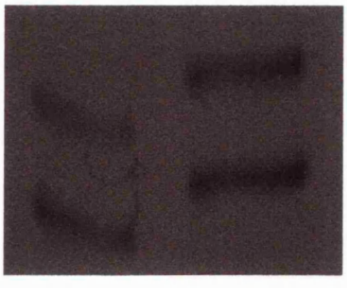


IN2659 (D17S643)

IN1545 (D9S176)

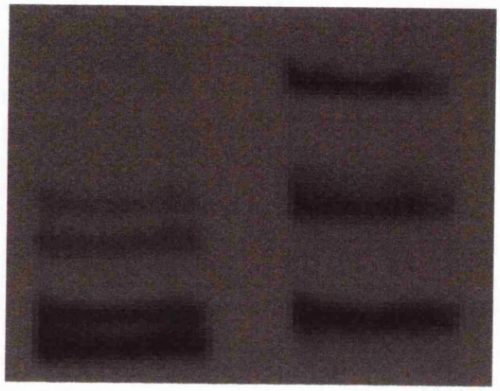


IN2715 (D17S804)

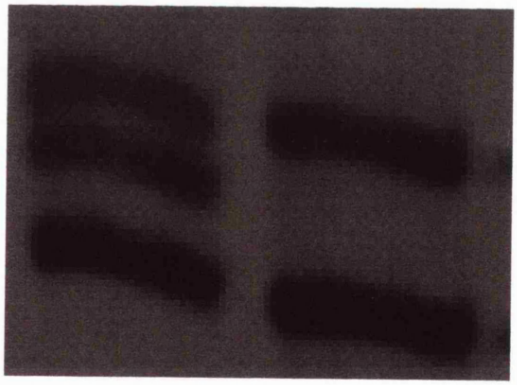


IN1241 (D22S301)

Figure 3.2 Examples of extra bands in two tumours at two loci



IN2912 (D16S512)



IN2697 (D17S643)

Chapter 4

RESULTS 2: CGH ANALYSIS

Introduction

Pages 114 to 128 show the ratio profiles of each tumour analysed with a description of the major changes seen. Each profile is composed of an average of individual ratio profiles from between 5 and 16 metaphase spreads. Each chromosome is represented as a small graph. The black line in the middle represents a ratio to the value of 1; i.e. there is an equal amount of tumour DNA and normal DNA. A threshold value of 1± 0.2 was used to determine loss or gain and this is indicated by the red or green lines on either side of the black one. The portions of the ratio profiles that are outside this range represent areas of change in the DNA sequence copy number. A bold green line to the left of the chromosome ideogram shows regions of gain in tumour DNA compared to normal, whilst a bold red line on the right of the ideogram shows where there is a loss of tumour DNA in comparison to normal. High copy number amplification is represented by a threshold of 1+ 0.4.

Peri-centromeric and heterochromatic repeat regions cannot be reliably evaluated by CGH as these areas are blocked to varying degrees in different chromosomes by the unlabelled Cot-1 DNA in the hybridisation reaction. These DNA sequences are highly polymorphic in copy number between individuals (Kallioniemi et al., 1994). Ratio changes at or near these regions have been interpreted cautiously in the present study, and have not been included as valid if the change is confined to these regions only in a chromosome and is not part of a larger region of loss or gain.

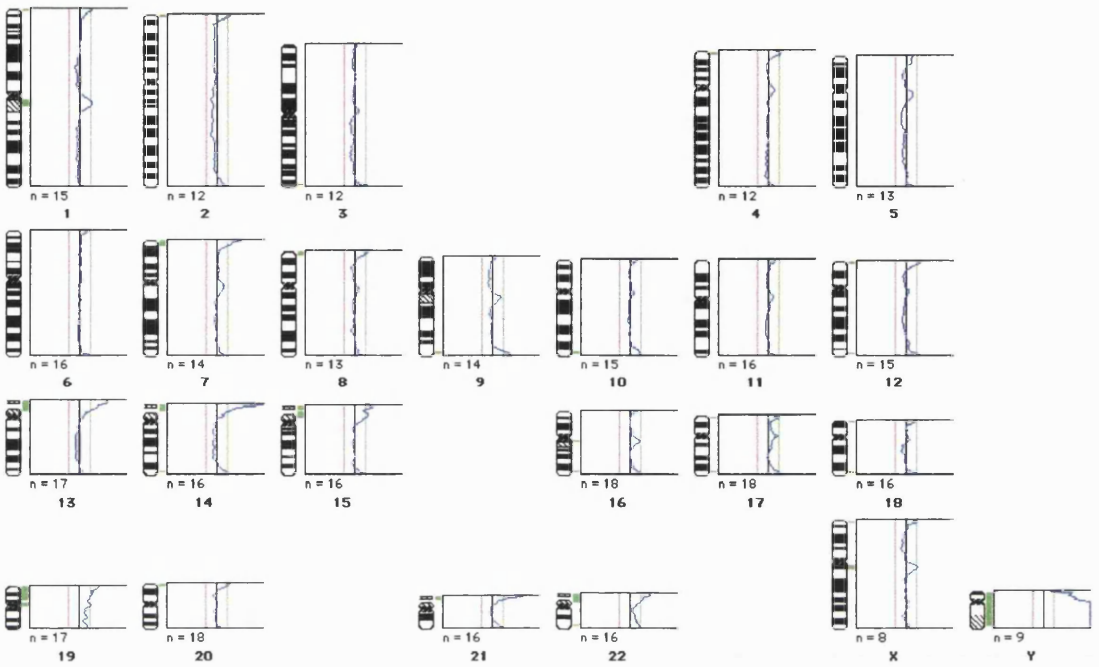
Similarly, the green and red fluorescence intensities gradually decrease at the chromosome telomeres. When the absolute intensities start to approach the background fluorescence levels, unreliable ratio changes may appear (Kallioniemi et al., 1994). Again, in the present study, changes in ratio at chromosome telomeres have been interpreted cautiously and have not been included as valid if the change is confined to the telomeres only in a chromosome and is not part of a larger region of loss or gain.

Recent evidence has indicated that in control experiments using normal DNA as both test and reference samples, the green to red fluorescence ratios at 1p32-pter, 16p, 19 and 22 may occasionally be substantially below the average ratio (Kallioniemi et al., 1994). The consequence would be a false positive interpretation of a deletion. These considerations were borne in mind when interpreting the results in the present study.

Sample details

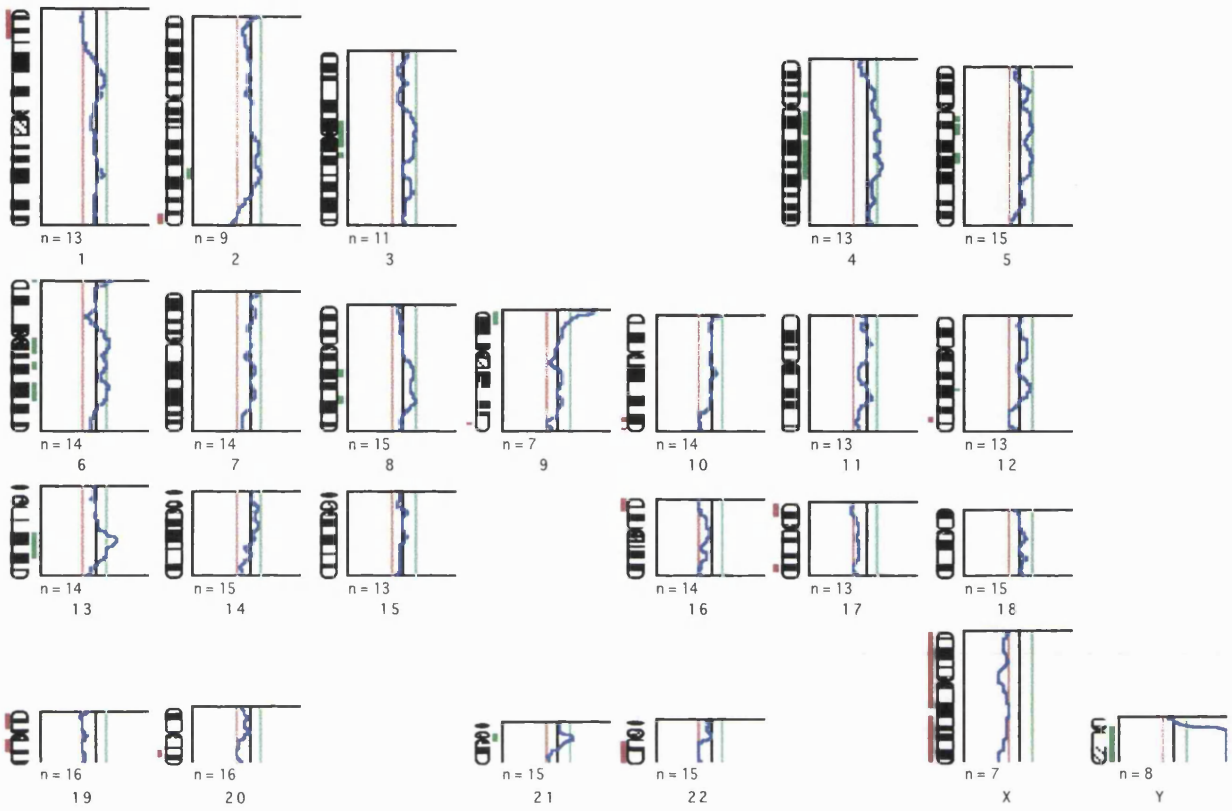
CGH analysis was performed on fifteen tumours. The age at diagnosis ranged from three months to ten years, with a male : female ratio of 13 : 2. Two samples were from desmoplastic medulloblastomas whilst the remainder were all of the classical type. In thirteen cases analysis was performed using DNA extracted from biopsy samples whilst in two cases (IN2077 and IN2266) analysis was carried out using DNA extracted from short term cultures at passage 4 and passage 3 respectively.

Control and reference normal DNA profile



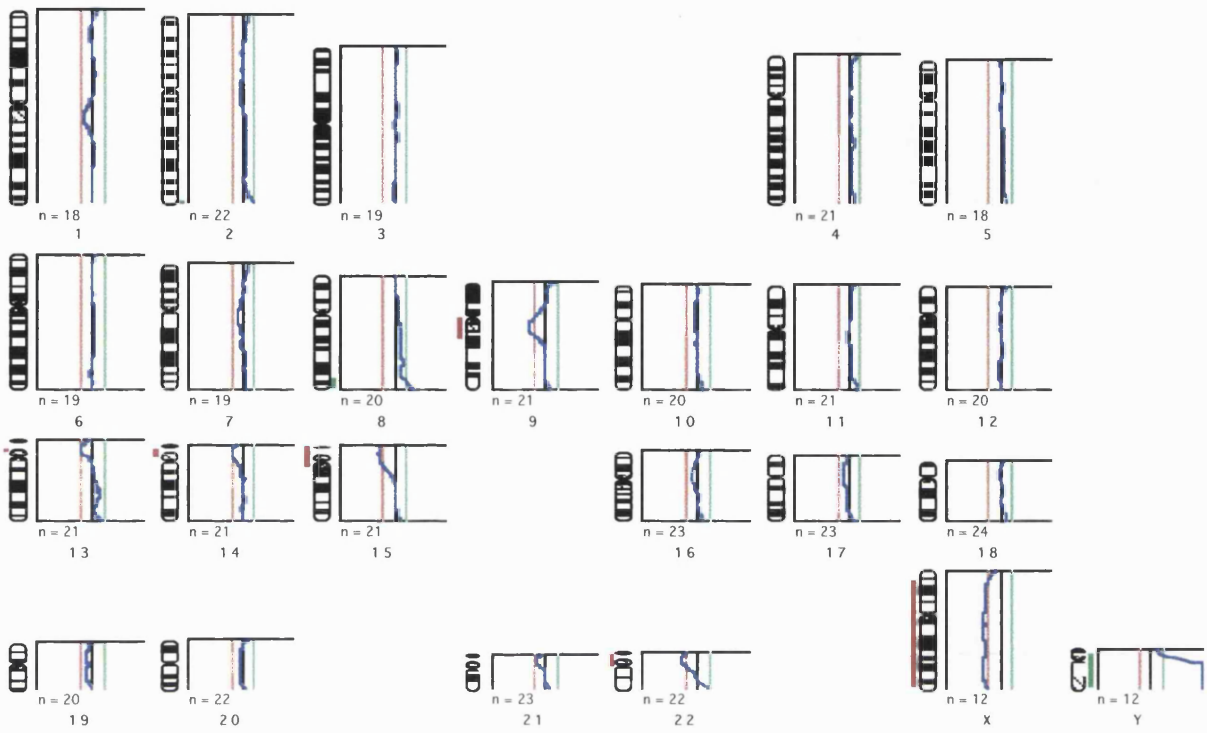
This is an example of a profile of a control hybridisation in which normal DNA from female donors was used as both test and reference. A control hybridisation was carried out with each set of tumour samples. The artefactual changes at some of the telomeres and centromeres are easy to see in the example above.

Tumour IN2077



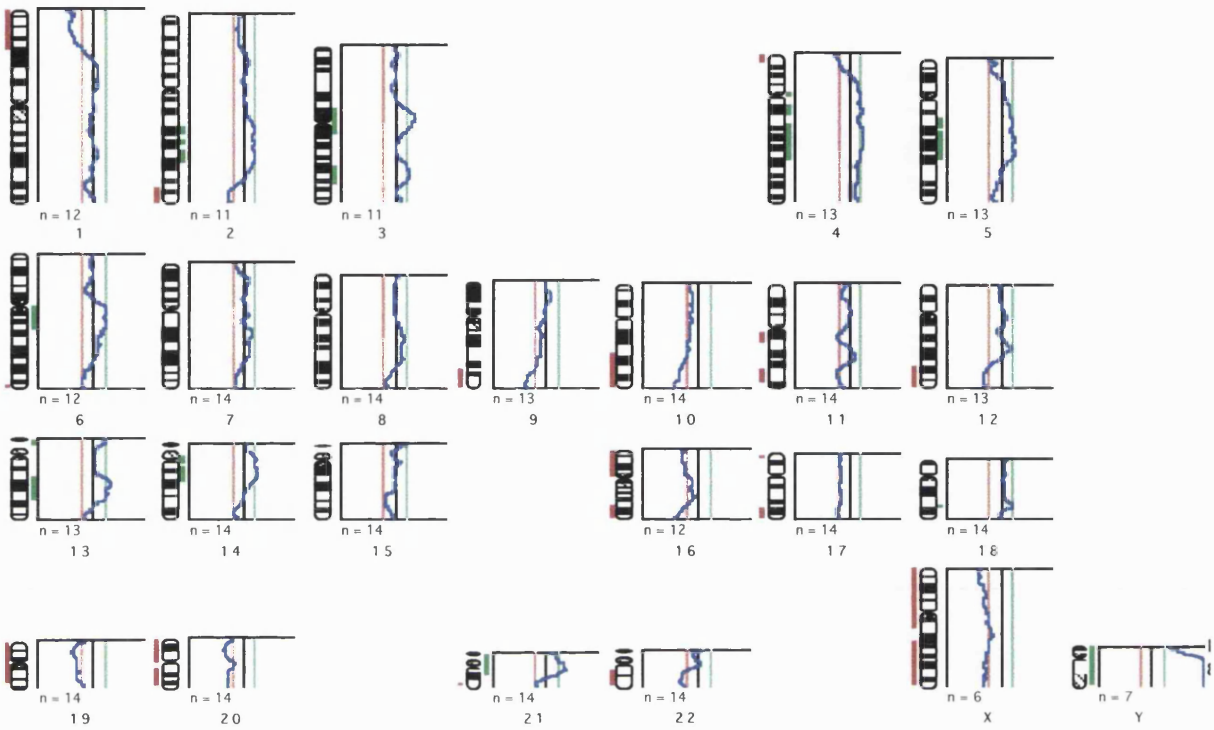
The profile of this tumour was composed from eight metaphase spreads and showed twenty five copy number aberrations (CNAs). However, many of these were located at pericentromeric (3q, 21) or telomeric (1p, 2q, 9q, 10q, 12q, 16p, 17q) regions and therefore should be interpreted carefully. However, there were sizeable regions of gain at 4q12-28, 5q11-21, 6q12-22 and 13q21-31 and loss at 17p12-13 and monosomy 19 and 22. Discreet regions of gain were found on 8q and 12q. Chromosomes 7, 11, 14, 15,18 and the sex chromosomes were unaffected by any changes.

Tumour IN 2266



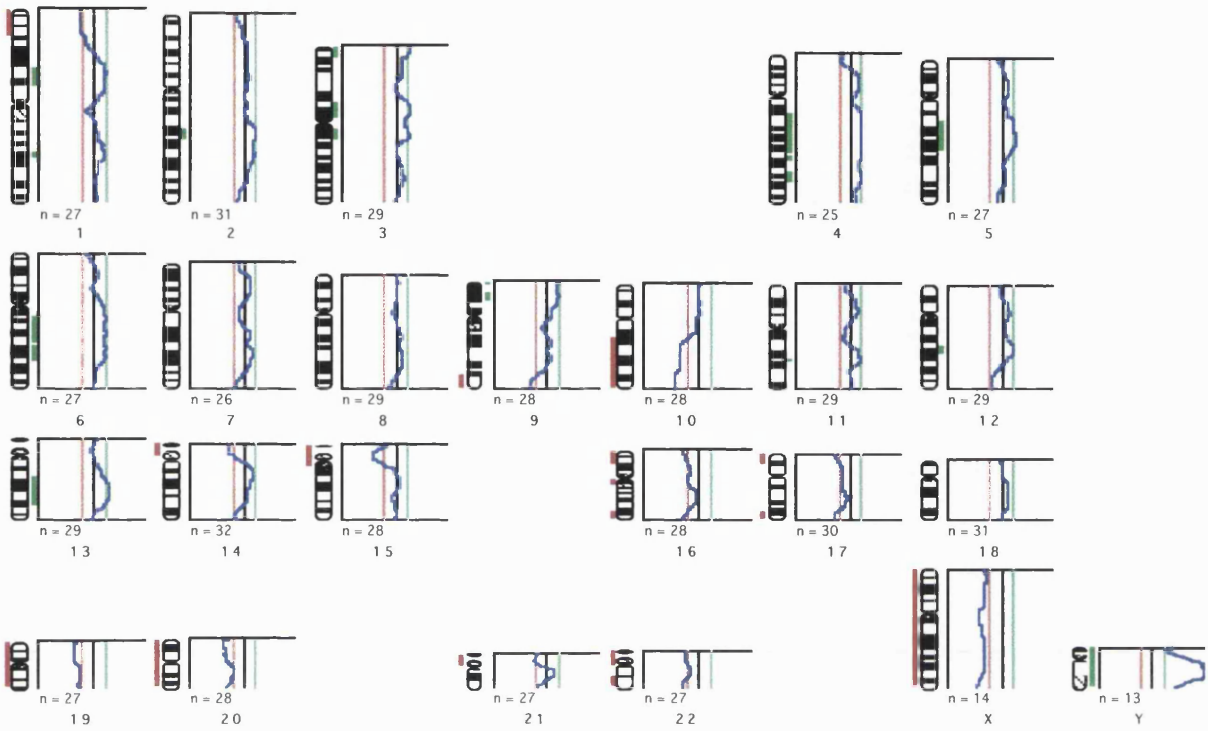
The profile of this tumour was composed from twelve metaphases. It demonstrated no CNAs except loss at the pericentric regions of 9, 13, 14, 15 and 22.

Tumour IN2588



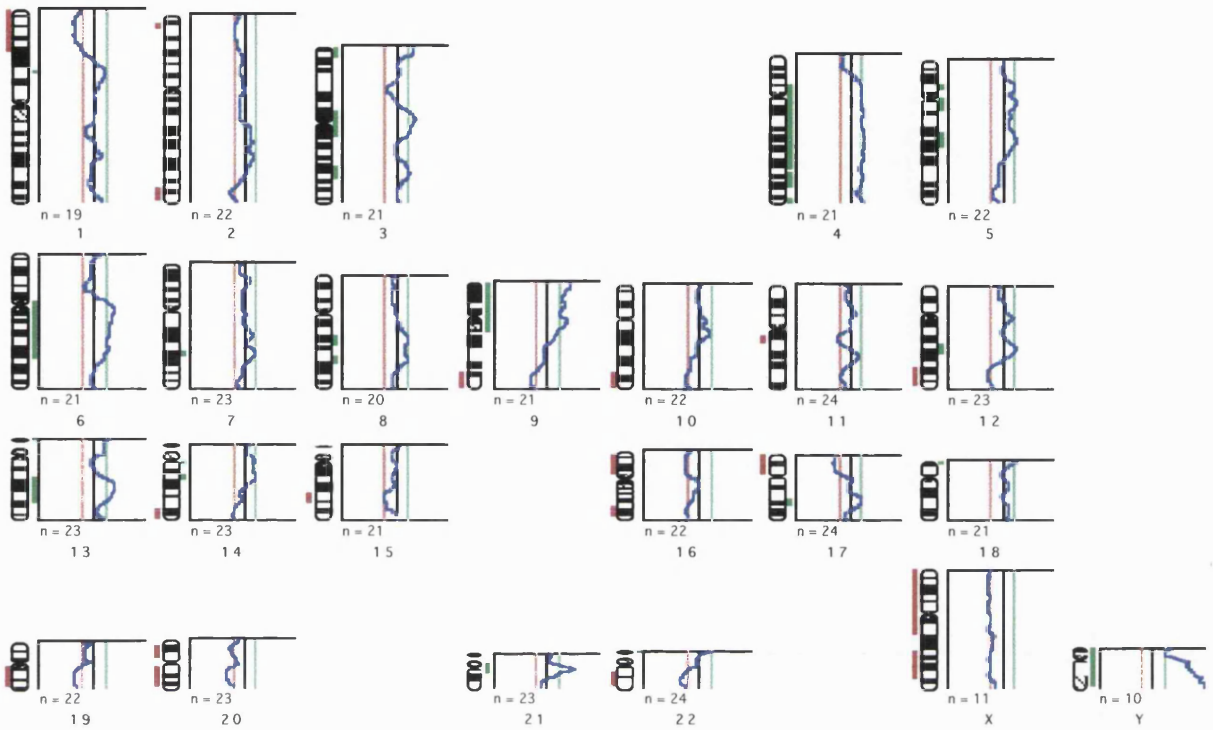
The profile of this tumour showed twenty seven CNAs and the profile was composed of an average of the individual ratio profiles from seven metaphase spreads. Chromosomes 7, 8, 15 and the sex chromosomes were unaffected. Although many copy number changes were found, some were located at pericentromeric (3p, 4p, 6q, 13p, 14p and 21p) or telomeric (4p, 6q, and 17p) regions and must be interpreted carefully. There were sizeable regions of gain at 2q22-32.3, 4q13-27, 5q13-23, 13q21-22 and 14q11.2-22, and loss at 1p32-36, 9q31-ter, 10q22-ter, 12q23-ter, 16p11.2-ter and 16q22-ter. Monosomy of chromosomes 19, 20 and 22 were seen. Discreet regions of gain were observed on chromosome 18p, and discreet regions of loss found on 2q, 11q and 17q.

Tumour IN2636



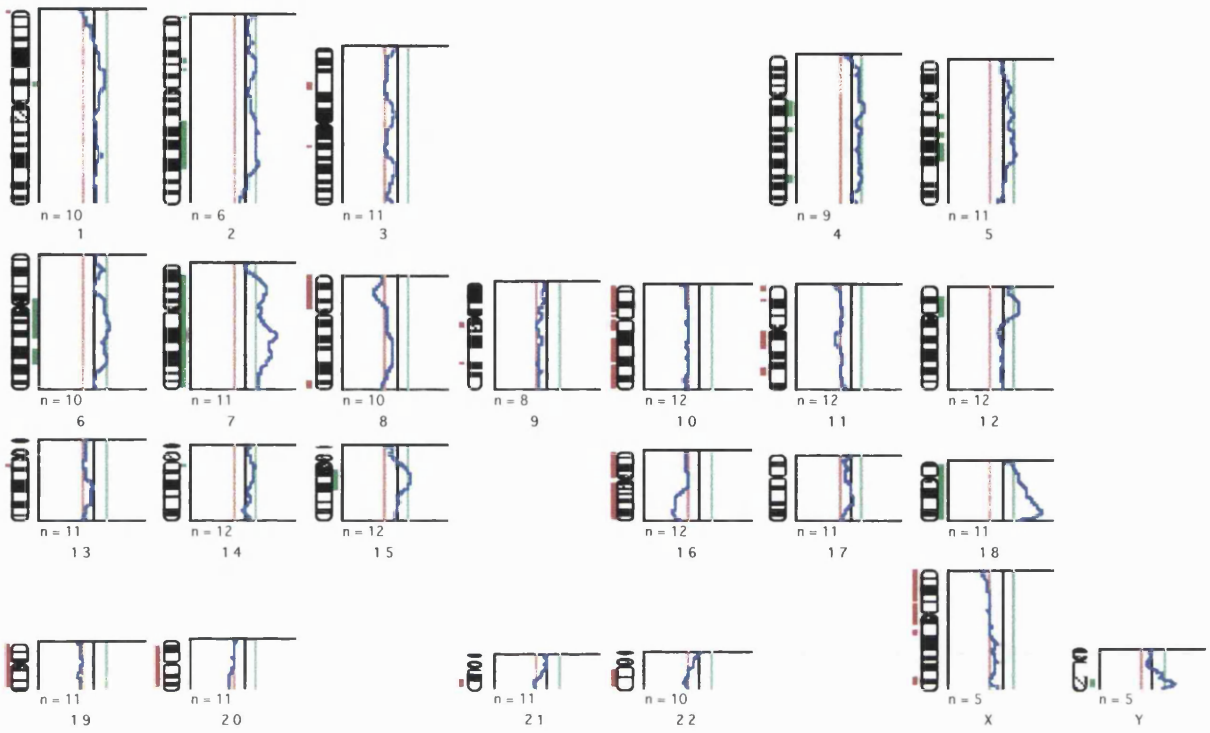
A total of thirty one CNAs were observed and the profile of the tumour was composed from sixteen metaphase spreads. Chromosomes 7, 8, 18 and the sex chromosomes were unaffected. Some of the CNAs were located at pericentromeric (3q, 14p, 15p, 16q, 21p) or telomeric (9p) regions and should be interpreted cautiously. Sizeable regions of gain were found at 4q21-31.3, 5q13-21, 6q13-23 and 13q14-31 whilst sizeable regions of loss were observed at 1p34.3-ter, 9q33-ter, 10q21-ter and 16p13.1-ter. Monosomy of chromosomes 19, 20 and 22 was found. Discreet regions of gain were seen on chromosomes 1p, 1q, 2q, 9p, 11q and 12q and discreet regions of loss were located on 16q, 17p and 17q. Figure 4.1 (page 131) shows loss of 10q in this tumour.

Tumour IN2659



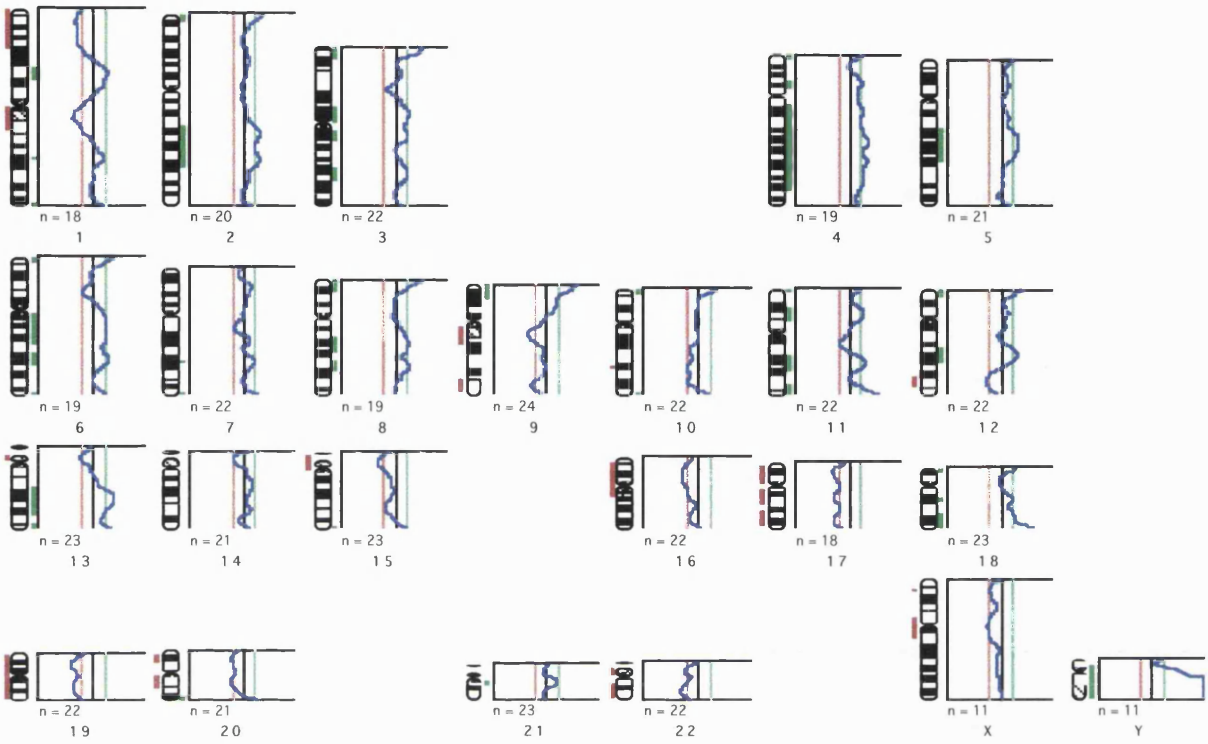
The profile was composed of an average of the individual ratio profiles from twelve metaphase spreads and a total of thirty six CNAs were seen; the highest amount of changes amongst the tumours studied. Every chromosome, with the exception of 18 and the sex chromosomes, was affected. A small number of the changes were located at pericentromeric (3q, 13p, 14p, 21p, 22p) and telomeric (3p, 18p) regions and should be interpreted carefully. Substantial losses were seen on chromosomes 1p31-ter, 9q32-ter, 10q25-ter, 12q23-ter, 16p11.2-ter, 16q22-23, 17p11.2-ter, 19q12-ter. Monosomy of chromosomes 20 and 22 was seen. Large areas of gain were observed on 4q12-ter, 5q11.2-21, 6q11.1-22, 9p11.1-ter and 13q14-22. As can be seen, loss of 9q was accompanied by a corresponding gain of 9p, and loss of 17p was shown alongside gain of 17q. This was the only tumour that showed gain of material on 17q. Also of note is that there was both loss and gain of regions on 12q.

Tumour IN2741



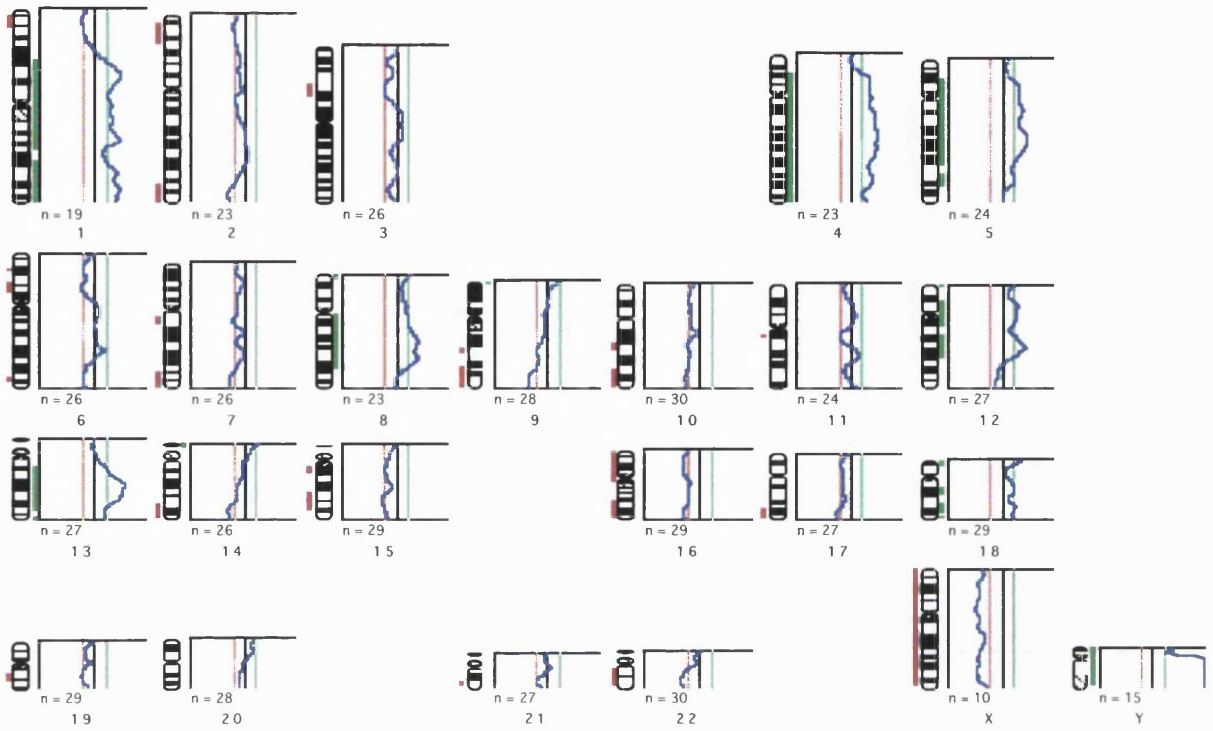
The profile was composed of an average of the individual ratio profiles from six metaphase spreads and a total of thirty three CNAs were seen. Every chromosome was affected. A small number of changes were at telomeric (1p, 2p, 11p) regions and should be interpreted with care. Most of the CNAs involved very large regions. For example, monosomy of chromosomes 10, 16, 19, 20 and 22 was seen as well as trisomy 7 and 18. In addition, high copy number amplification was observed at 18q21-22. Substantial regions of gain were also seen on chromosomes 2q23-31, 4q12-31.1, 5q13-23, 6q11.1-23, 12p11.2-12 and 15q14-21, whilst areas of loss were also found on chromosomes 8p11.2-ter and 11q11.12-23. Also of note is that whilst there was a discreet region of loss on 9q, there was no corresponding gain of 9p.

Tumour IN2765



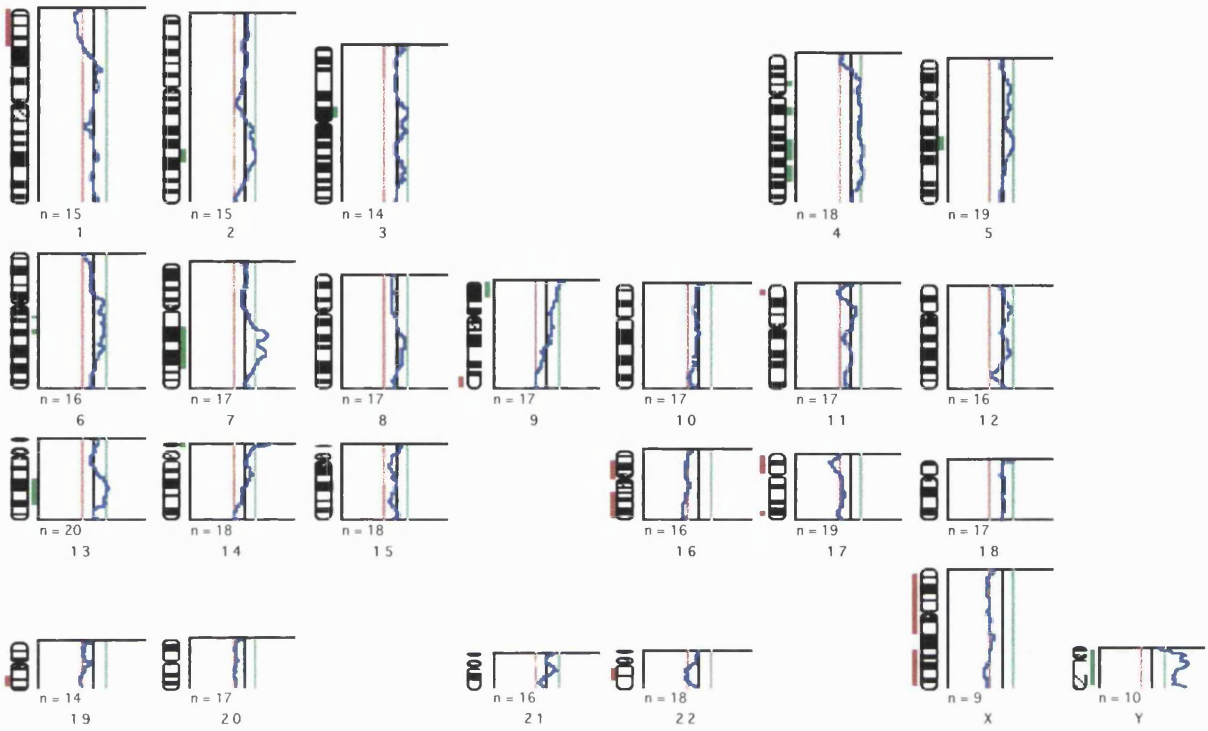
A total of thirty five copy number changes were seen and the profile was composed of an average of the individual ratio profiles from twelve metaphase spreads. Some of the changes were located at pericentromeric (3q, 13p and 21p) or telomeric (1q, 2p, 4p, 6p, 6q, 10p, 11p, 12p and 18p) regions and should be interpreted carefully. The changes affected every chromosome with the exception of the sex chromosomes and chromosomes 14, 15 and 21. Monosomy of chromosomes 17, 19 and 22 were seen as well as substantial regions of loss on 1p32-ter, 9q12-ter and 16p11.2-ter. There was gain of almost the entire q arm of chromosome 4, as well as large areas of gain of chromosomes 2q21-32.2, 3, 5q14-23, 6q12-22, 8q13-22, 9p21-ter, 11q13-ter, 13q21-ter and 18q12-ter. As with IN2659 both loss and gain of material on 12q was seen, as well as loss of 9q with a corresponding gain of 9p. In addition high copy number amplification at 9p21-23 was observed.

Tumour IN2805



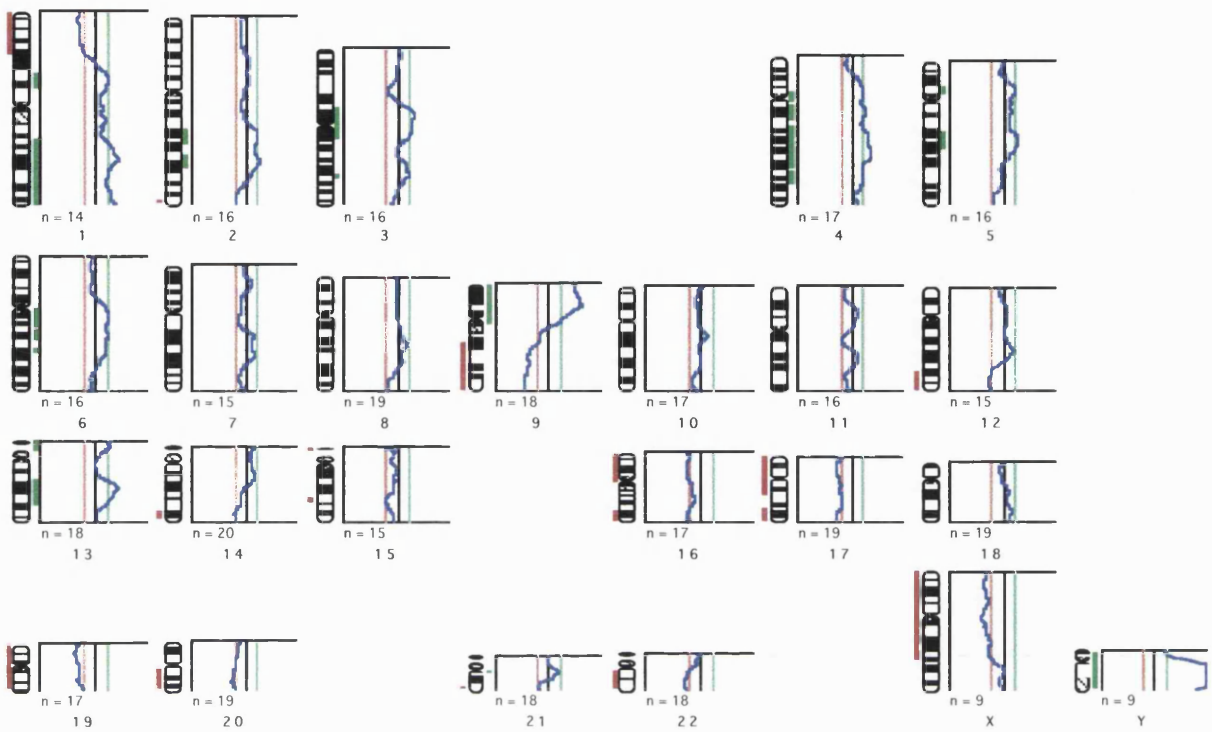
A total of twenty six CNAs were seen and the profile was composed of an average of the individual ratio profiles from fifteen metaphase spreads. A few changes wre located at telomeric (8p, 12p, 18p) regions and should be carefully interpreted. This tumour showed changes affecting every chromosome apart from the sex chromosomes and chromosomes 11 and 20. Monosomy of chromosome 16 and 22 was observed, as well as substantial regions of loss on 2, 7q, 9q, 10q, 14q, 15q and 22. All of the regions of gain seen were very large, no discreet gains were observed. There was gains of almost the whole of 4 and 5 as well as sizeable gains of material on chromosomes 1q, 8q, 12, 13q and 18q. High copy number amplification was seen at 13q21-31. However, although there was loss of 9q, there was no corresponding gain of 9p.

Tumour IN2823



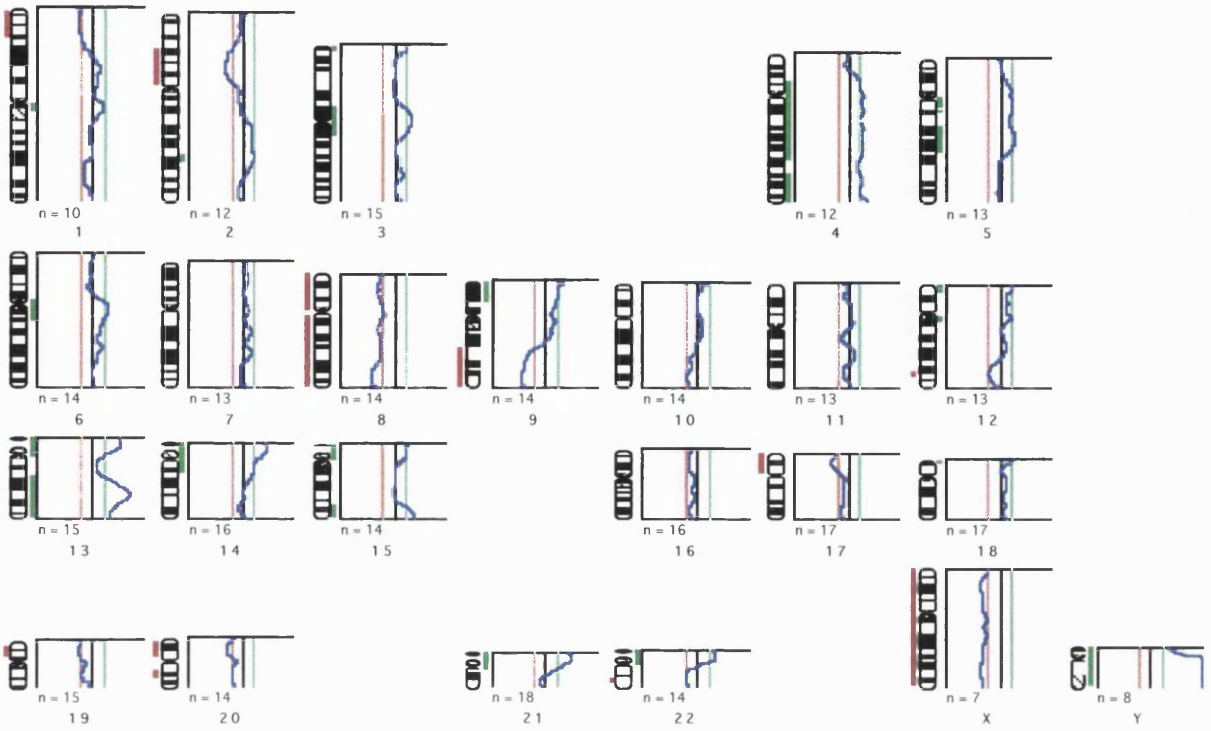
This tumour revealed a total of eighteen CNAs and the profile was composed of an average of the individual ratio profiles from ten metaphase spreads. Most of the regions of loss and gain observed were discreet and several chromosomes, 8, 10, 12, 15, 18, 20 and 21 as well as the sex chromosomes, were unaffected. The most substantial region of loss was monosomy of chromosome 16, whilst sizeable deletions were also seen on 1p, 17p, 19q and 22. The largest regions of gain were observed on chromosomes 4q and 13q. This tumour showed a loss of material on 9q with a corresponding gain of material on 9p.

Tumour IN2832



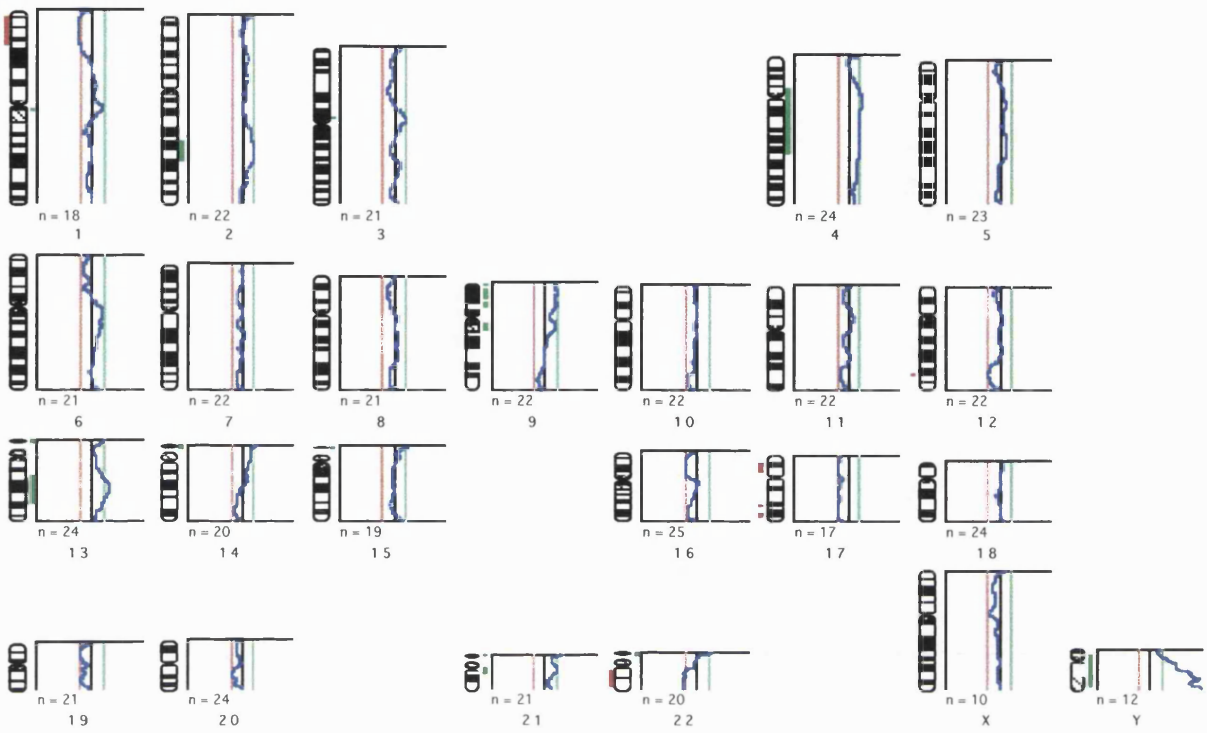
This tumour revealed a total of twenty seven CNAs and the profile was composed of an average of the individual ratio profiles from ten metaphase spreads. Changes located at pericentromeric (13p, 15p and 21p) regions should again be interpreted carefully. Most of the areas of loss and gain seen in this tumour were fairly large. For example, on chromosome 9, there was a gain of the entire p arm with a corresponding loss of almost the entire q arm. As with IN2077 and IN2765, there was high copy number amplification at 9p21-23. Furthermore, chromosomes 17, 19 and 22 were monosomic and there were large deletions of 1p, 12q, 16, 20 and 22. As well as the gain of the 9p arm, sizeable regions of gain were also observed on chromosomes 1q23-ter, 4q12-31.3, 6q12-22 and 13q14-31.

Tumour IN2894



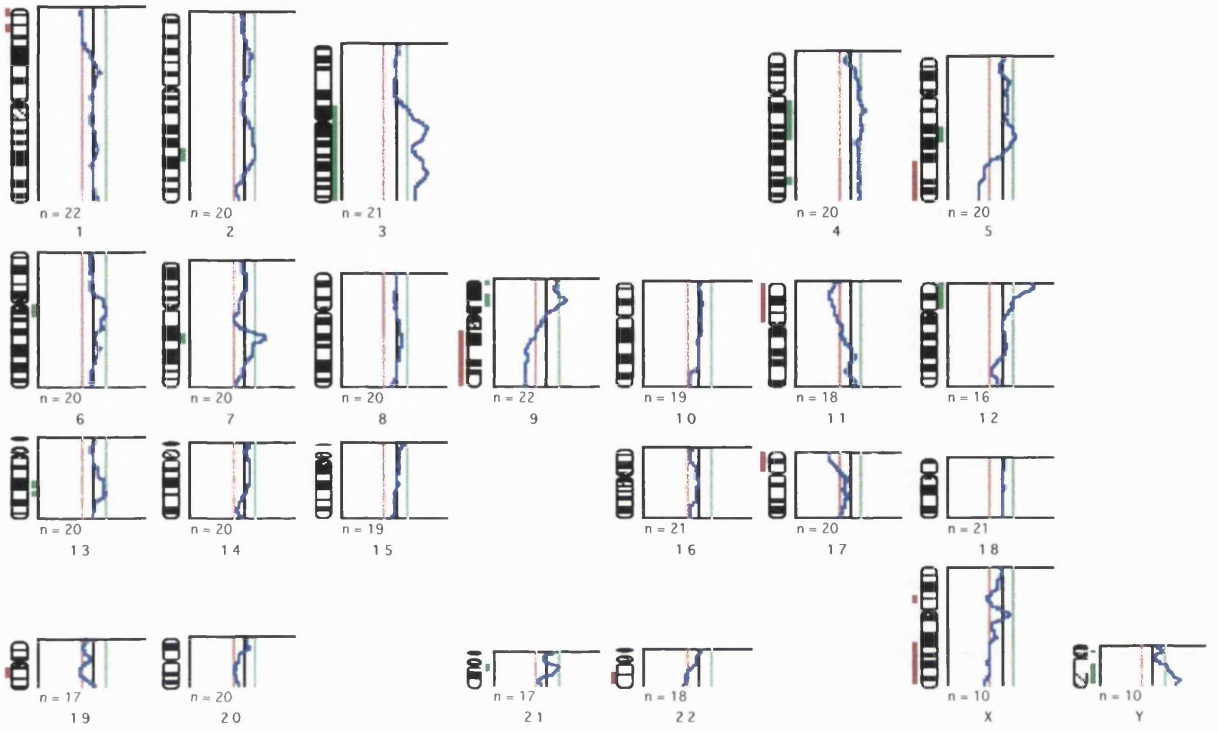
This tumour showed twenty nine copy number changes, and the profile was composed of an average of the individual ratio profiles from nine metaphase spreads. Although a large number of changes were seen chromosomes 7, 10, 11, 16 and the sex chromosomes were unaffected. Some of the changes were located at pericentric (1q, 3q, 6q, 12q, 13p, 14p, 15p, 21p and 22p) or telomeric (3p, 12p and 18p) regions and therefore should be interpreted cautiously. There was monosomy of chromosomes 8 and 20 as well as large areas of loss of chromosomes 1p34.1-ter, 2p11.2-22, 9q22-ter, 17p11.2-ter and 19p13.1-ter. Discreet regions of loss were found at 12q24.1 and 22q12. There was almost trisomy of chromosome 4 and sizeable regions of gain on 5q, 9p and 13q. As can be seen, loss of 9q with a corresponding gain of 9p was found. and a discreet region of gain was found on 2q31. High copy number amplification was found on 13q21-31

Tumour IN2912



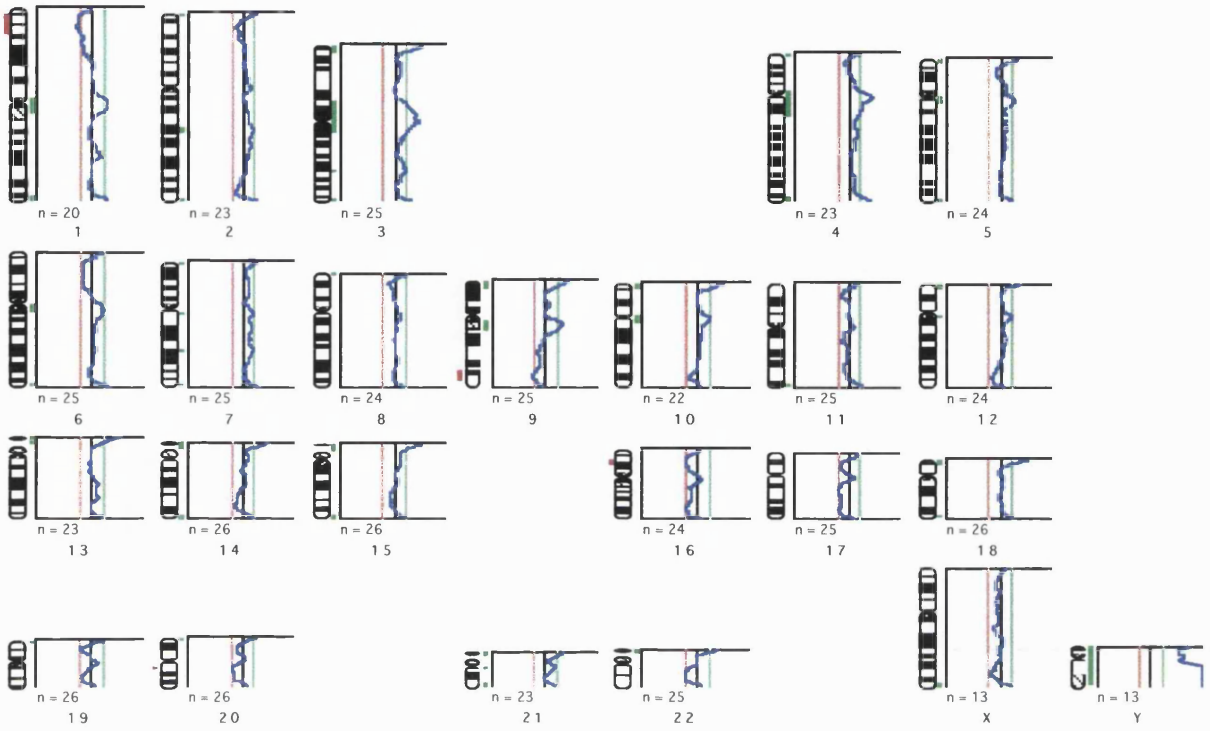
A total of twelve CNAs were seen in this tumour and the profile was composed of an average of the individual ratio profiles from thirteen metaphase spreads. Loss of material was found on only four chromosomes. The ratio almost reached the threshold of loss for the whole of chromosome 17, there was a sizeable losses of 1p, chromosome 22 was monosomic, whilst there was a small region of loss at 12q. Substantial regions of gain were seen on chromosomes 2q24-32.2, 4q13-26, 9p21-ter and 13q14-31. As can be seen, gain of 9p was not accompanied by loss of 9p.

Tumour IN2933



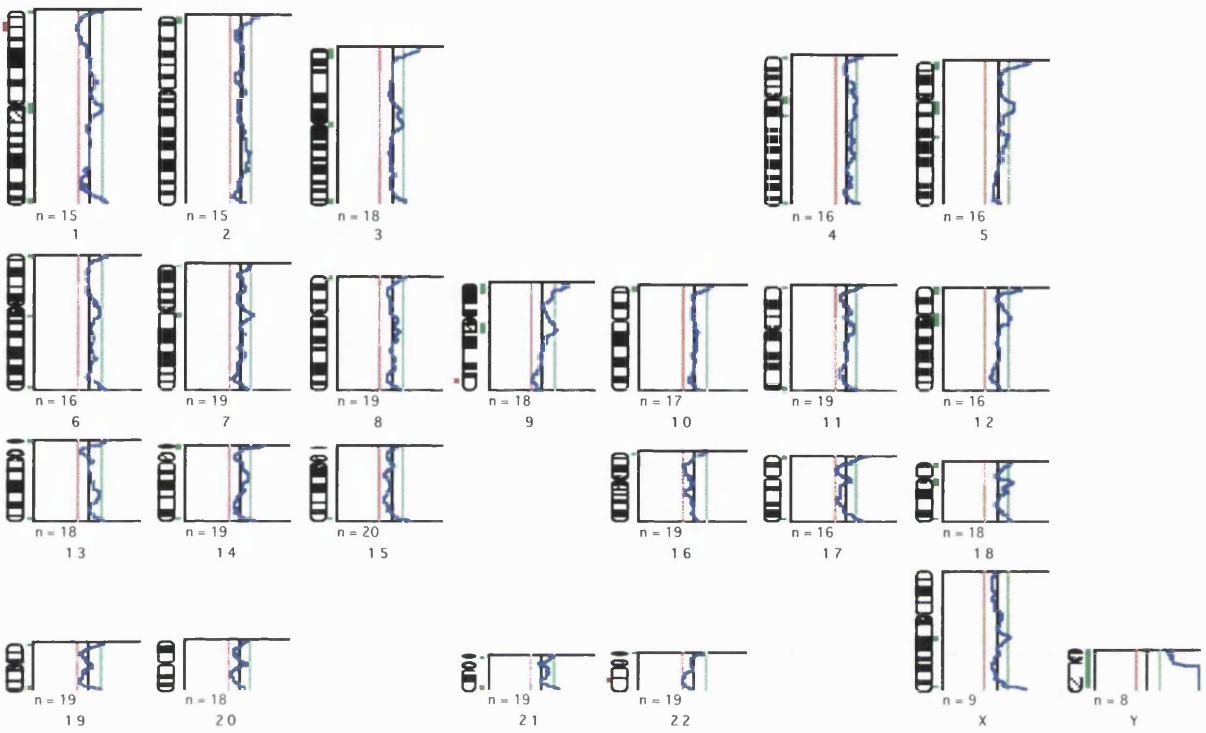
The profile was composed of an average of the individual ratio profiles from eleven metaphase spreads and a total of seventeen CNAs were seen in this tumour. Several large areas of loss were seen; most notably loss of almost the entire arms of chromosomes 9q, 11p and 17p. Sizeable deletions of 1p35-ter, 5q31-ter, and 22q12-ter were also seen. Of the gains, most noticeable was the gain of the entire arm of chromosome 3q , with a high copy number amplification at 3q24-26. Other large regions of gain were seen on chromosomes 4q, 9p and 12p. A small region of loss was seen on 19q and small regions of gain were detected on 2q31, 7q21 and 13q21-22.

Tumour IN2966



The profile was composed of an average of the individual ratio profiles from thirteen metaphase spreads and a total of eight CNAs were seen in this tumour. Only four chromosomes showed loss of material in this tumour; 1p, 9q, 16p and 20. Low level gains were seen on chromosomes 2, 3, 4 and 7.

Tumour IN2996



Only two copy number changes were seen in this tumour from a profile composed of an average of the individual ratio profiles from ten metaphase spreads. The two changes observed were loss on chromosome 9q and gain on 9p.

Summary of most prevalent changes observed

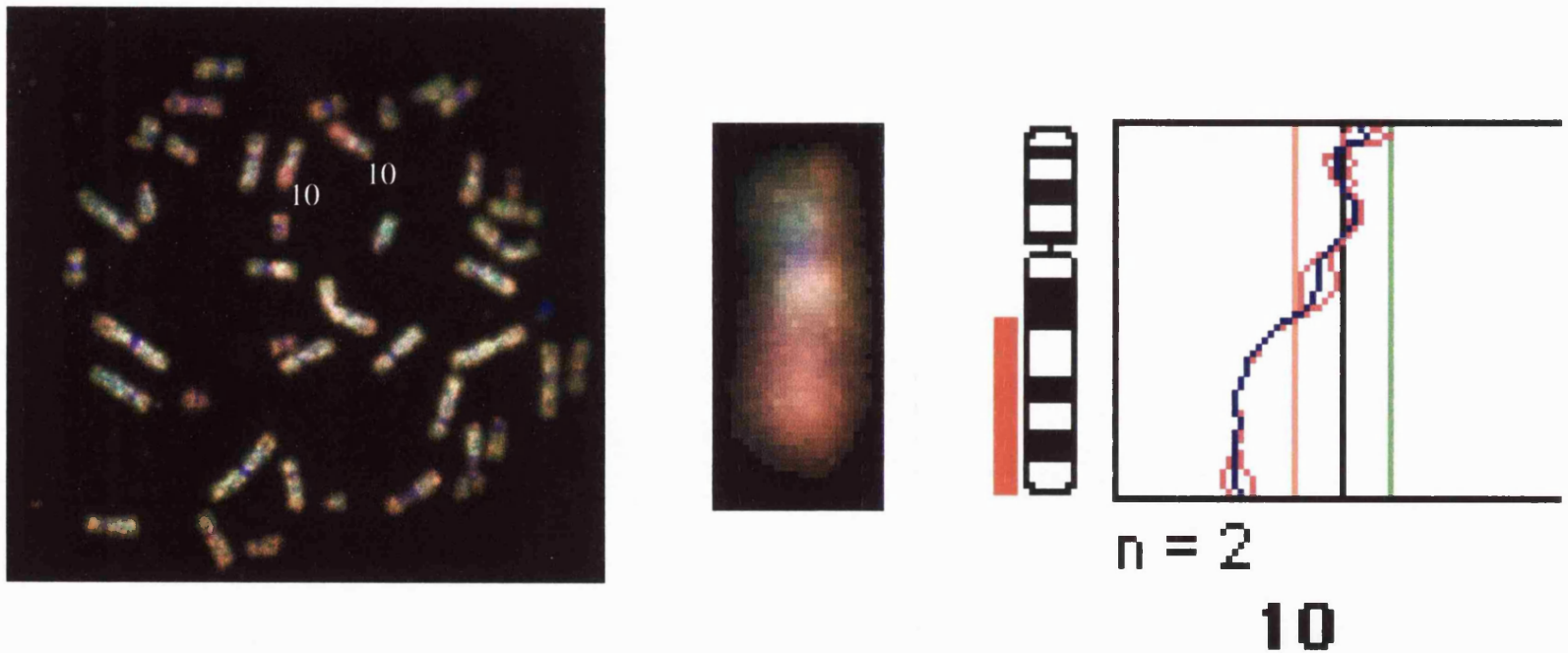
A summary of the cumulative chromosomal gains and losses found can be seen in figure 4.2, page 131 and table 4.1, page 132 . Regions of genomic imbalance were detected in 14 of 15 tumours. These tumours showed multiple copy number changes ranging from 6 to 39 per tumour (mean 22.2). Apart from the sex chromosomes, all chromosomes were involved in copy number alterations. A number of common areas of losses and gains were observed in several of the tumours. The most prevalent changes were gain at 4q in 14 of 15 (93%) cases, 5q in 12 of 15 (80%), 2q in 11 in of 15 (73%), 13q in 11 of 15 (73%), 6q in 10 of 15 (66%), 9p in 10 of 15 (66%), 3q in 9 of 15 (60%), and 4p in 8 of 15 (53%), and loss at 1p in 13 of 15 (86%), 9q in 13 of 15 (86%), 22q in 13 of 15 (86%), 19 in 11 of 15 (73%), 16p in 10 of 15 (66%), 17p in 9 of 15 (60%), 20 in 9 of 15 (60%), 17q in 8 of 15 (53%), 16q in 8 of 15 (53%) 12q in 7 of 15 (46%), and 10q in 7 of 15 (46%) cases. High copy number amplification was observed at 3q24-26 (tumour IN2933), 9p21-23 (tumours IN2765 and IN2832), 13q21-31 (tumours IN2894 and IN2805) and 18q21-22 (tumour IN2741) . Loss of material on 9q was accompanied by gains of 9p in 10 of 15 (66%) cases. Examples of high copy number amplification are shown in Figure 4.3.

COMPARISON OF CGH RESULTS WITH MICROSATELLITE DATA

CGH was performed on thirteen of the twenty seven samples used for microsatellite analysis. The remaining fourteen samples could not be analysed by CGH as there were insufficient amounts of DNA available for this procedure.

Figure 4.1

Loss of 10q in IN2636



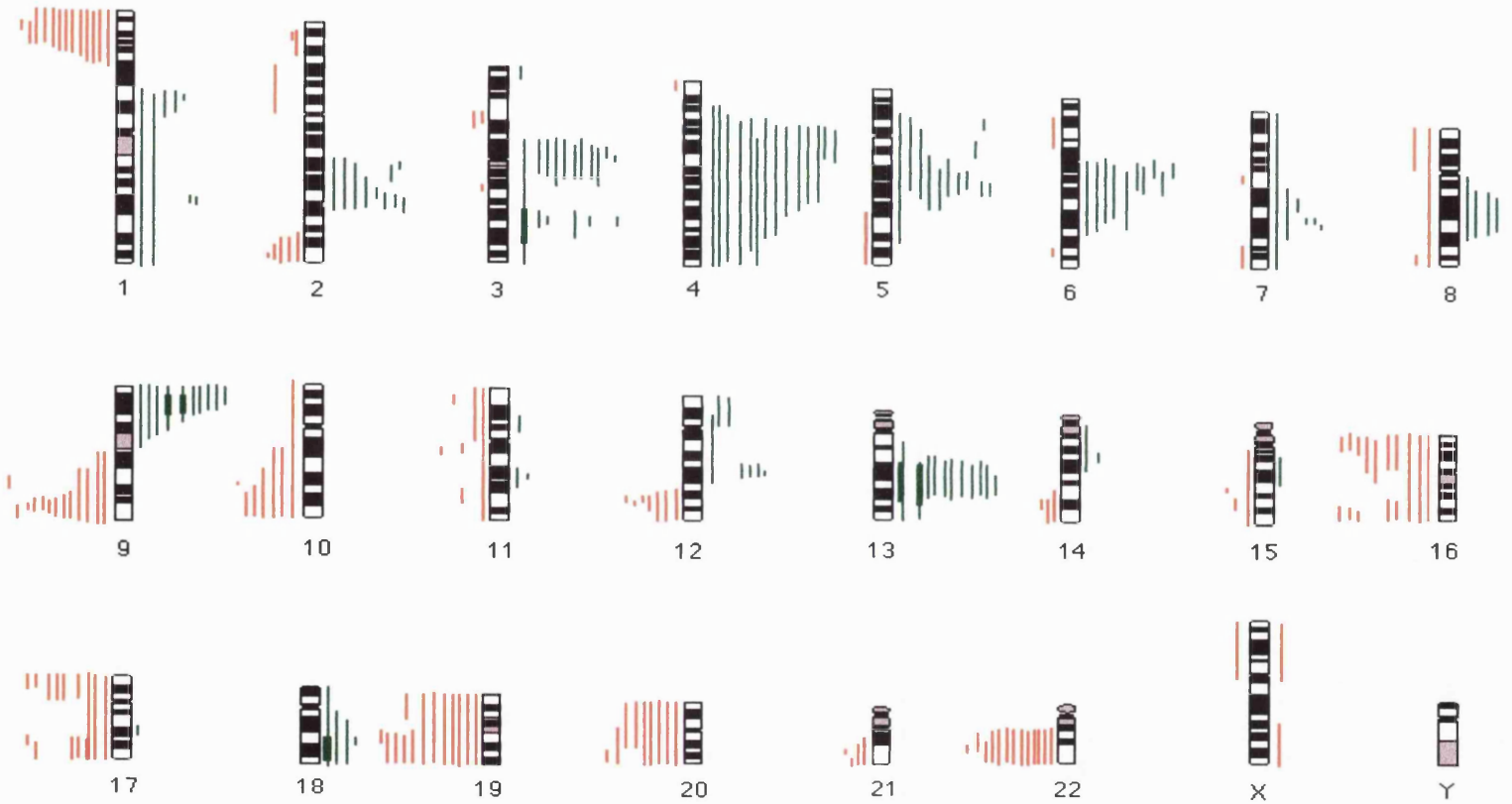
composite metaphase

ratio profile

IN	Age[a]	Sex[b]	CGH Results		
			Amplification	Gains	Losses
2077	4	M		3q 4q 5q 6q 8q 12q 13q 21	1p 2q 9q 10q 12q 16q 17 19
2266	11 mths	M			
2588	3	M		2q 3p 4 5q 6q 13 14q 18p 21p	1p 2q 4p 6q 9q 10q 11q 12q 16 17 19 20 22
2636	3	M		1 2q 3q 4q 5q 6q 9p 11q 12q 13q	1p 9q 10q 14p 15p 16 17 19 20 21q 22
2659	6	M		3 4q 5q 6q 9p 13 14q 17q18p 21q 22p	1p 9q 10q 12q 16 17p 19q 20 22
2741	4	F	18q	2 4q 5q 6q 7 9p 12p 15q 18	1p 8p 9q 10 11 16 19 20 22
2765	6	M	9p	1q 2 3 4 5q 6 8q 9p 10p 11 12 13q 18 21q	1p 9q 12q 13q 16p 17 19 22
2805	9	M	13q	1q 4 5 8 12 13q 18	2 7q 9q 10q 14q 15q 16 22
2823	10	M		4q 9p 13q	1p 9q 16 17p 19q 22
2832	7	M	9p	1q 4q 6q 9p 13q 21q	1p 9q 12q 15p 17 16 19 20 22
2894	2	M	13q	1q 2q 3 4 5q 9p 12q 13q 14q 15q 18p 21q 22q	1p 2p 8 9q 12q 17p 19p 20 22q
2912	3mths	F		2q 4q 9p 13q	1p 12q 17 22
2933	6	M	3q	2q 3q 4q 7q 9p 12p 13q	1p 5q 9q 11p 17p 19q 22q
2966	18mths	M		2 3 4 7	1p 9q 16p 20
2996	5	M		9p	9q

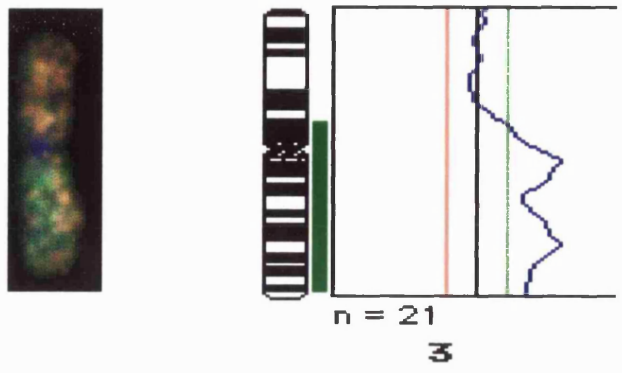
Table 4.1: A Summary of the cumulative chromosomal gains and losses found. a: years unless stated, b: M= male, F= female

Figure 4.2 Summary of CNAs detected by CGH in 15 medulloblastoma

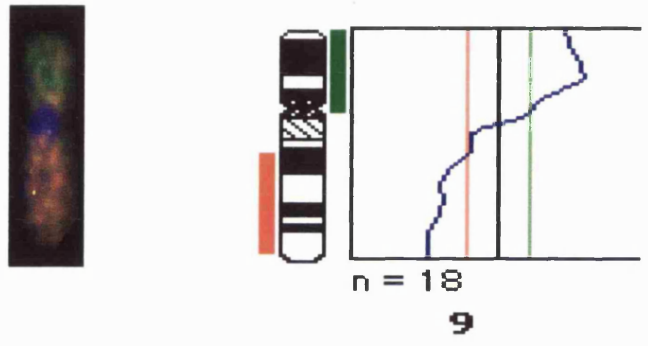


Vertical lines to the right of the chromosomes represent gains; vertical lines to the left represent losses; bold lines represent high copy number amplification.

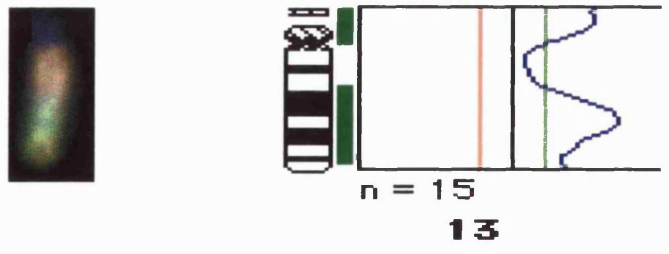
Figure 4.3 Examples of high copy number amplification detected by CGH



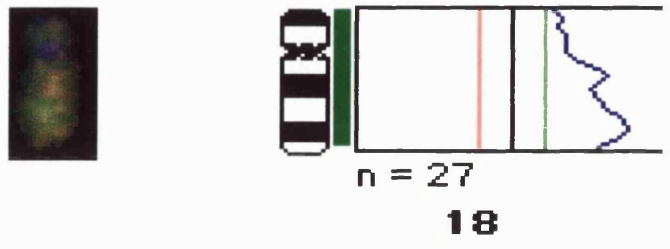
3q24-26 in IN2933



9p21-23 in IN2832



13q21-31 in IN2894



18q21-22 in IN2741

Chromosome 9q

Overall, the microsatellite analysis located areas of loss not seen by CGH. As expected, a correlation occurred in that 54% of cases (14 of 26) showed LOH and 92% (24 of 26) of the tumours showed LOH or allelic imbalance on chromosome 9q, just as 80% (12 of 15) tumours showed loss of this area by CGH. However, with seven tumours on which both sets of analyses had been conducted, the markers used revealed areas of loss or imbalance that was not visible on the corresponding CGH profiles. For example, with tumour IN2659 there was no information for one of the intermediate markers (D9S176 mapping to 9q22.3) but for the remaining three, LOH or allelic imbalance is shown indicating a loss of most of the chromosome 9q arm. However, the corresponding CGH data revealed a loss of only the 9q31-ter region (see profile, page 117). The two sets of data show only a very small overlapping region of loss. Nevertheless, the LOH study suggests a putative tumour suppressor gene between 9q22.3 and 9q33, so with regard to case IN2659, the CGH data supports this hypothesis. With tumour IN2266, a loss at 9q12-13 only is shown by CGH, whereas for the three of 4 microsatellite markers for which information is available, loss or imbalance is shown at 9q21.2, q22.3 and q33 indicating a large area of deletion. Again, with sample IN2912, no loss at all was found with CGH whereas with the microsatellite anlaysis allelic imbalance was seen at 9q21.1 and LOH at 9q33, and with IN2077 a small area of deletion was seen by CGH at 9q34 whilst the LOH data revealed allelic imbalance at 9q22 and 9q33. These differences are probably due to the fact that CGH is less sensitive than PCR based methods in detecting deletions (Weiss et al., 1999). With one tumour however, IN2823, although allelic imbalance was seen at 9q21.1, no actual LOH was found. This matches the CGH data in that only a small region of loss was found, at 9q34, although it is possible that this is CGH artefact. Similarly, with IN2636, the LOH data shows allelic imbalance at 9q33 and CGH also revealed loss at 9q33-34. If microsatellite markers mapping to 9q34 had been used, it is possible that they would also have shown

loss in this region. Similarly, other small regions of deletion were found by CGH on other tumours; at 9q31 (IN2741), as well as 9q12, q13, and q34 (IN2765). Three tumours showed large areas of deletion by CGH; IN2894, IN2832 and IN2933 showed unbroken regions of deletion from 9q22-ter, 9q21-ter and 9q13-ter respectively. In the corresponding LOH data, loss and imbalance was seen consistently in these regions with these tumours supporting the hypothesis that there is at least one putative tumour suppressor gene on 9q.

Chromosome 10q

The CGH data revealed loss of chromosome 10q in 46% (7 of 15) of samples studied whilst the microsatellite analysis showed LOH in 23% of tumours (6 of 26) and allelic imbalance or LOH in 46% of samples (12 of 26). For three of the tumours on which both microsatellite analysis and CGH had been carried out (IN2832, IN2894 and IN2912), both sets of data revealed no loss of genetic material. With three other tumours (IN2823, IN2266, IN2933) there was no loss by CGH, but allelic imbalance and/or LOH at one or two loci. Again, this may be due to the greater sensitivity which is obtained from the microsatellite markers. The IN2636 CGH data showed a large unbroken region of loss from 10q21-ter and this was represented by areas of allelic imbalance at 10q21.3 and 10q24 in the LOH data. No information was available for the remaining marker with this tumour, but it is possible that 10q21-10q24 region was lost. The microsatellite analysis suggested a putative tumour suppressor gene at 10q21 to 10q23.1 and the CGH data for IN2636 supports this. Furthermore, CGH analysis showed a very small area of deletion mapping to 10q23 in tumour IN2765 within this putative region. The LOH data showed no LOH or imbalance for this tumour, but unfortunately, there is no information for the marker which mapped to 10q23 and which may of course have shown a corresponding loss. Three of the samples for which both LOH and CGH data are available (IN 2588,

IN2741 and IN2805) reveal large areas of loss as shown by CGH and yet no LOH or allelic imbalance as shown by microsatellite analysis.

Chromosome 12q

The CGH data showed loss of material in 46% (7 of 15) of samples, whilst the microsatellite analysis showed a corresponding LOH in 50% of cases (13 of 26) and allelic imbalance or LOH in 96% (25 of 26) of samples. In six tumours that were studied using both methods of analysis (IN2266, IN2636, IN2741, IN2805, IN2823 and IN2933), no loss was found using CGH but areas of allelic imbalance and/or LOH were found at at least one marker locus using microsatellite analysis. In one tumour (IN2894), the area of loss seen with CGH appears to fall inside a bigger region found by the LOH studies; loss is observed at markers mapping to the 12q22-24.3 region by the microsatellite analysis, an area which encompasses 12q23-24.1 where loss was seen using CGH. Thus the CGH data supports the hypothesis that a putative tumour suppressor gene is located at 12q24.1 to 12q24.2. Similarly, the area from 12q23-ter was found to be lost in IN2832 by CGH whilst the corresponding microsatellite analysis showed loss from between 12q22 and q24.2, and furthermore, three tumours, IN2588, IN2659 and IN2765 showed small overlapping regions of loss a revealed by the two different methods of analysis, sharing regions of loss at 12q24.2, 12q23 and 12q24.2 respectively. The remaining tumour, IN2912, showed a very small region of loss at 12q23 by CGH which is located in between bands 12q22 and 12q24.32-33 which showed allelic imbalance by LOH.

Chromosome 16q

Loss of genetic material was seen in 61% (8 of 13) of samples by CGH, and LOH was seen in 42% of cases (11 of 26). LOH and allelic imbalance was seen in 84% (22 of 26) of samples examined by microsatellite analysis.

Four tumours that were studied using both methods (IN2077, IN2266, IN2894 and IN2933) revealed no loss by CGH yet microsatellite analysis showed allelic imbalance and/or LOH at at least one marker locus for each tumour. One tumour (IN2912) showed no loss with either the CGH or the microsatellite analyses. IN2741 shows a large area of loss by CGH, which extends from the centromere to 16q24. This loss is reflected in the microsatellite analysis which shows LOH or allelic imbalance at all informative markers except that mapping to 16q13. Loss of the 16q22-16q24 region was seen by CGH with tumour IN2588. The microsatellite analysis of this tumour gives information for only two loci; LOH at 16q12.1 and allelic imbalance at 16q22.1. As these loci are quite a distance apart on 16q, it is possible that were information available for the other marker loci, it would show loss of most of the 16q arm encompassing the region shown as lost by CGH. Tumour samples IN2805 and IN2659 showed small overlapping regions of loss by the two different methods of analysis, sharing regions of loss at 16q21.1 and 16q22.1 respectively. Loss at 16q11.2 was the only region to be deleted in IN2765 by CGH. However, as none of the microsatellite markers used mapped to this area, it is not possible to compare the results of the two analyses. The losses seen by CGH with tumours IN2823 and IN2636 were actually in different regions to those found with the LOH studies, whilst with tumour IN2832, loss was observed at the region of 16q22-24 in the CGH analysis but no loss was found via microsatellite analysis, although there was no information for the marker that mapped to 16q24. Thus the CGH data supports the suggestion that there are multiple putative tumour suppressor genes on chromosome 16q.

Chromosome 17p

The microsatellite analyses of chromosome 17p was the largest and most comprehensive, using 22 markers in all, which spanned 17p13.1-p13.3. Genetic loss as measured by LOH alone was seen in 100% (27 of 27) of samples. In comparison, loss was seen in 60% (9 of 15) of samples by CGH.

With six tumour samples (IN2659, IN2765, IN2823, IN2894, IN2832 and IN2933) the entire 17p arm was lost by CGH. Loss (LOH and allelic imbalance) was seen across the p13.1-p13.3 region from the microsatellite analysis, although this was not continuous and tumours showed preservation of both alleles at some intermediate loci. It is possible that if markers mapping to the other loci on 17p (11.2 and 12) had been used, they too would have shown genetic loss. There were different patterns and different frequencies of loss with these six tumours. With IN2933, although loss was seen over such a broad region, LOH or imbalance was only actually seen at 6 of 14 (43%) of marker loci and there are no 'clusters' of loss, i.e. loss at several markers which map to adjacent loci. Similarly, with IN2894, where LOH or imbalance was seen at 8 of 14 (57%) of informative markers, there is no clustering pattern, and the same is true of IN2832 where LOH or imbalance was observed at 8 of 17 (47%) of informative marker loci and IN2823 (loss or imbalance at 10 of 17, or 59%, of informative loci). The continuous losses seen by CGH could be due to some artefact; previous investigators have described artefacts in the CGH ratio on 17p (Kallioniemi et al., 1994; Kim et al., 1995) or, because it is a less sensitive technique than microsatellite analysis, it may not have been able to identify small interstitial deletions within a band as separate entities. However, with the two remaining tumours of these six, a definite pattern is seen. IN2659 showed loss, imbalance or a change in electrophoretic

mobility (or band shift) at 12 of 17 (70%) of informative marker loci and several of these map to adjacent areas on 17p13.1.

Similar clustering patterns were seen in other tumours on which both CGH and microsatellite analyses had been conducted. With IN2588, the only loss seen via CGH was a very small deletion at the telomere which is probably due to an artefact. However, with the LOH studies, loss or imbalance was seen at 50% (9 of 18) of informative marker loci, again probably due to the greater sensitivity of microsatellite analysis versus CGH. Several of these loci map to adjacent areas on p13.1 although probably not sufficiently telomeric to support the CGH analysis. The same was true of IN2741 where no loss was found by CGH but where 9 of 16 (56%) of informatve markers revealed loss, several of which were at adjacent loci at virtually the most telomeric region of p13.1. The CGH data for IN2636 showed loss of the entire 17p13 area whilst the remainder of 17p was preserved, and again, whilst LOH and allelic imbalance were observed over the length of the same region (at 10 of 16 or 62% of informative loci), a consistent area of loss was found at several adjacent marker loci on p13.1.

The remaining tumours showed yet more variations across the two sets of data. IN2077 showed a loss by CGH in the region of 17p12-ter, whilst the microsatellite studies demonstrated loss and allelic imbalance was seen over the entire 17p13 region. However, loss or imbalance was only seen at 9 of 18 (50%) of informative marker loci and there was no pattern of loss. The CGH data for IN2912 showed loss of the 17p12-13.1 region, and with the LOH data loss was observed at one marker locus mapping to p13.1 (D17S720). Loss or imbalance was shown at another four informative marker loci but these all mapped to p13.2-p13.3. With IN2266 and IN2805 no loss was found by CGH, but the microsatellite data shows loss or imbalance from 17p13.1-p13.3. However, actual LOH was only seen at 2 of 17 (12%) of informative marker loci for IN2805 and 1 of 18 (5%) of

loci for IN2266, i.e. discreet interstitial deletions that were identified due to the sensitivity of the PCR technique. Overall, the large regions of loss of 17p seen in most tumours by CGH support the microsatellite analysis which suggests two putative tumour suppressor genes are located on 17p13.1 distal to p53.

Chromosome 22q

Loss of genetic material was seen in 80% (12 of 15) cases as shown by the CGH data, whilst the microsatellite analysis revealed LOH in 46% of cases (12 of 26) and allelic imbalance or LOH in 85% (22 of 26) of samples. The frequency of the deletions found by the two techniques were therefore almost identical.

The only tumour studied using both methods of analysis which did not show loss by CGH was IN2266. The LOH studies revealed one area of loss, at the 22q12-13 locus whilst both alleles were preserved at the only other locus for which information is available. Again, the greater sensitivity of the microsatellite markers when compared to CGH may have allowed the discovery of a very discreet deletion. Conversely, a large region of loss was observed by CGH in tumour IN2659, but in the microsatellite analysis allelic imbalance was found at only one of the three loci used. This anomaly could be the result of CGH artefact. Previous investigators have described artefacts in the CGH ratio on chromosome 22 (Kallioniemi et al., 1994; Kim et al., 1995).

However in other tumours (IN2077, IN2588, IN2741, IN2765 and IN2805) loss of the entire 22q arm was revealed in the CGH studies, and the LOH analyses demonstrated LOH or allelic imbalance at either one or two locus/loci. For IN2588, there was LOH/imbalance at two loci and no information for the remaining locus. In tumour IN2823 a small region of loss (from 22q11.2-12) was observed by CGH, but the LOH analyses indicate that the

entire 22q arm was lost. With the remaining five samples, the CGH and the LOH data matched in that regions of loss overlapped completely. The high frequency of loss seen by CGH supports the LOH data both in terms of the degree of loss seen and the hypothesis that a putative tumour suppressor gene may reside at 22q11.2 to 22q12-13.

Summary of CGH versus microsatellite analysis

With all the chromosomal regions investigated, loss was found at a greater frequency with the microsatellite analysis than with CGH due to the greater sensitivity of the former technique. Similarly, in some cases the microsatellite data showed preservation at all marker loci which confirmed the lack of deletions found by CGH. In instances where loss was found by the CGH studies but not by microsatellite analysis, the loss of alleles was probably due to unreliable changes in the CGH ratio at the telomeric regions. Overall, the CGH data supports the LOH data both in terms of the amount of loss seen and also in terms of the locations of putative tumour suppressor genes on the regions investigated.

Chapter 5

RESULTS 3: PATIENT FOLLOW UP AND CLINICAL CORRELATIONS

Follow up information

Samples were obtained from a total of twenty nine patients and of these, twenty eight were from primary medulloblastomas whilst one (case IN2912) was from a recurrent tumour. Of the samples, 23 were taken from males and 6 from females. Males are traditionally more affected than female children and the overall male to female ratio for medulloblastoma has been shown to be 1:8:1 (Lannering et al., 1990). Thus, in the present study, where the male to female ratio is 3.8:1, there is a significant male bias in the sample group. Additionally, the samples were donated at the discretion of the neurosurgeons and this was partially deppendent on the degree of resection and location of the tumour so the sample group may not be representative of medulloblastomas as a whole. A summary of follow up details is shown in Table 2.1 (page 58), which includes information of length of survival times, extent of surgical resection and radiotherapy and chemotherapy treatment regimens. Of the four remaining cases, three (IN2697, IN2823 and IN2933) were completely lost to follow up, whilst nothing is known post discharge, following a partial excision, of the other (IN1212). The histology of each tumour sample is also stated in table 2.1. This was verified by the examining neuropathologist for each case and detailed in the neuropathology report obtained to accompany each tumour sample. All of the samples have been obtained since 1987, with twenty of these being subsequent to 1994, and thus long term survival rates are impossible to assess. Furthermore, with two cases (IN2637 and IN2741) follow up was not maintained for more than sixteen months subsequent to initial diagnosis. Thus, with the twenty five samples for which follow up is available, the 5 year survival rate was calculated and six of twenty five (24%) patients were still alive 5 years from initial diagnosis. Table 5.1 (overleaf) shows a summary of the clinical data.

Number of patients	29
Age at first presentation: range (years)	3/12-13
mean (years)	5.6
median (years)	5
Sex	23M 6F
Chemotherapy after first operation *	5/25
Radiotherapy after first operation *	10/25
Radiotherapy and chemotherapy after first operation *	10/25
Survival times: range (months)	5-79
mean (months)	34
median (months)	22

Table 5.1 Summary of clinical data

* It is unknown as to whether 4 patients had chemotherapy and/or radiotherapy since they were lost to follow up.

Correlation of therapy with length of survival

Of the twenty five cases for whom clinical follow up was available, seven patients had surgery followed by radiotherapy only, three patients had surgery followed by chemotherapy only, whilst the remaining fifteen patients had both radiotherapy and chemotherapy following surgery (see Table 2.1, page 58 for details). There was no correlation between treatment regimen and survival (standard log rank test, p=0.95).

Influence of age on survival in children with medulloblastoma

A standard log rank test was performed to assess the correlation of age with survival. Irrespective of the extent of surgical resection and the chemotherapy/radiotherapy treatment

regime received, the patients ≤ 3 years of age had a significantly better prognosis than those > 3 years of age (p= 0.05). The mean and median survival for ≤ 3 years of age are 48 months and 57 months respectively, whilst mean and median survival for those > 3 years are 23 months and 16 months respectively. This data is summarised in figure 5.1, page 146

Correlation of length of survival with LOH data

The relationships between survival times and LOH data (for patients with both LOH at ratio 0 - 0.5 and LOH and imbalance taken at the 0 - 0.75 ratio from the corresponding tumour samples from these patients for chromosomes 9q, 10q, 12q, 16q, 17p and 22 are described below.

Chromosome 9q

Of twenty three patients for whom clinical follow up and LOH data was available, twelve had LOH at one or more loci (ratio 0 - 0.5) and twenty one had LOH/imbalance at one or more loci (ratio 0 - 0.75). LOH 9q was not associated with survival in this group of patients (p=0.41, Log Rank Test).

The association of survival and LOH at each marker locus was assessed individually. Of twenty two patients for whom data was available, eight had LOH (0 - 0.5 ratio) and eight had LOH/imbalance (0 - 0.75) at D9S166. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.48 Log Rank Test) or the 0 - 0.75 ratio (p=9.8 Log Rank Test). Of twenty two patients for whom data was available, one had LOH (0 - 0.5 ratio) and eight had LOH/imbalance (0 - 0.75) at D9S287. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.17 Log Rank Test) or the 0 - 0.75 ratio (p=0.69 Log Rank Test).). Of twenty one patients for whom data was

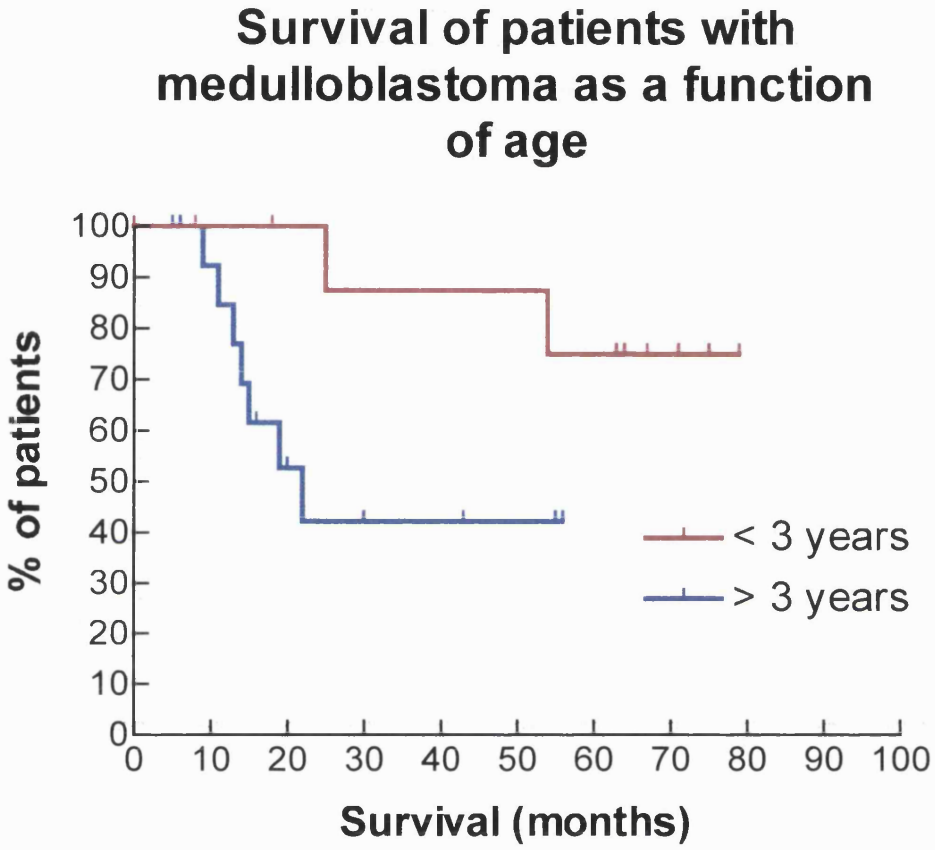


Figure 5.1 Association of survival with age. Patients aged 3 years or less survived significantly longer than those patients older than 3 years (Log-rank test, P = 0.0496)

available, three had LOH (0 - 0.5 ratio) and eleven had LOH/imbalance (0 - 0.75) at D9S170. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.67 Log Rank Test) or the 0 - 0.75 ratio (p=0.82 Log Rank Test).

The LOH data was also analysed to assess whether there was a relationship between LOH and age ≤3 years versus age >3 years. With each marker there was no association with age at either the 0 - 0.5 ratio or the 0 - 0.75 ratio (see Table 5.2, page 153, for p values).

Chromosome 10q

Of twenty three patients for whom clinical follow up and LOH data was available, four had LOH at one or more loci (ratio 0 - 0.5) and ten had LOH/imbalance at one or more loci (ratio 0 - 0.75). LOH 10q was not associated with survival in this group of patients (p=0.69, Log Rank Test).

The association of survival and LOH at each marker locus was assessed individually. Of twenty one patients for whom data was available, two had LOH (0 - 0.5 ratio) and nine had LOH/imbalance (0 - 0.75) at D10S581. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.64 Log Rank Test) or the 0 - 0.75 ratio (p=0.69 Log Rank Test). Of twenty one patients for whom data was available, two had LOH (0 - 0.5 ratio) and five had LOH/imbalance (0 - 0.75) at D10S540. Length of survival was not associated with loss at this locus for either the 0 - 0.5 ratio (p=0.63 Log Rank Test) or the 0 - 0.75 ratio (p=0.48 Log Rank Test).

The LOH data was also analysed to assess whether there was a relationship between LOH and age ≤3 years versus age >3 years. With each marker there was no association with age at either the 0 - 0.5 ratio or the 0 - 0.75 ratio (see Table 5.3 page 153, for p values)

Chromosome 12q

Of twenty three patients for whom clinical follow up and LOH data was available, eleven had LOH at one or more loci (ratio 0 - 0.5) and twenty two had LOH/imbalance at one or more loci (ratio 0 - 0.75). LOH 12q was not associated with survival in this group of patients (p=0.96, Log Rank Test).

The association of survival and LOH at each marker locus was assessed individually. Of twenty one patients for whom data was available, three had LOH (0 - 0.5 ratio) and eleven had LOH/imbalance (0 - 0.75) at D12S327. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.35 Log Rank Test) or the 0 - 0.75 ratio (p=0.69 Log Rank Test). Of twenty two patients for whom data was available, one had LOH (0 - 0.5 ratio) and nine had LOH/imbalance (0 - 0.75) at D12S360. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.59 Log Rank Test) or the 0 - 0.75 ratio (p=0.31 Log Rank Test). Of eleven patients for whom data was available, four had LOH (0 - 0.5 ratio) and six had LOH/imbalance (0 - 0.75) at D12S330. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.53 Log Rank Test) or the 0 - 0.75 ratio (p=0.24 Log Rank Test). Of fourteen patients for whom data was available, four had LOH (0 - 0.5 ratio) and nine had LOH/imbalance (0 - 0.75) at D12S366. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.44 Log Rank Test) or the 0 - 0.75 ratio (p=0.96 Log Rank Test). Of twenty three patients for whom data was available, three had LOH (0 - 0.5 ratio) and eleven had LOH/imbalance (0 - 0.75) at D12S342. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.21 Log Rank Test) or the 0 - 0.75 ratio (p=0.48 Log Rank Test).

The LOH data was also analysed to assess whether there was a relationship between LOH and age ≤3 years versus age >3 years. With each marker there was no association with age at either the 0 - 0.5 ratio or the 0 - 0.75 ratio (see Tables 5.4. page 154, for p values).

Chromosome 16q

Of twenty three patients for whom clinical follow up and LOH data was available, eleven had LOH at one or more loci (ratio 0 - 0.5) and eighteen had LOH/imbalance at one or more loci (ratio 0 - 0.75). LOH 16q was not associated with survival in this group of patients (p=0.13, Log Rank Test).

The association of survival and LOH at each marker locus was assessed individually. Of twenty one patients for whom data was available, three had LOH (0 - 0.5 ratio) and thirteen had LOH/imbalance (0 - 0.75) at D16S409. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.12, Log Rank Test) or the 0 - 0.75 ratio (p=0.66 Log Rank Test). Of twenty one patients for whom data was available, two had LOH (0 - 0.5 ratio) and seven had LOH/imbalance (0 - 0.75) at D16S416. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.58 Log Rank Test) or the 0 - 0.75 ratio (p=0.42 Log Rank Test). Of twenty three patients for whom data was available, five had LOH (0 - 0.5 ratio) and twelve had LOH/imbalance (0 - 0.75) at D16S512. Length of survival was not asociated with loss at this locus for the 0 - 0.5 ratio (p=0.54 Log Rank Test) but it was significantly associated with loss at the 0 - 0.75 ratio (p=0.01 Log Rank Test). Of twelve patients for whom data was available, three had LOH (0 - 0.5 ratio) and five had LOH/imbalance (0 - 0.75) at D16S516. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.33 Log Rank Test) or the 0 - 0.75 ratio (p= 0.46 Log Rank Test). Figures 5.2 and 5.3 (pages 157 and 158) show summaries of the data for the chromosome 16q markers.

The LOH data was also analysed to assess whether there was a relationship between LOH and age ≤3 years versus age >3 years. With each marker there was no association with age at either the 0 - 0.5 ratio or the 0 - 0.75 ratio (see Table 5.5 page 154, for p values).

Chromosome 17p

Of twenty three patients for whom clinical follow up and LOH data was available, all had LOH at one or more loci (ratio 0 - 0.5) and all had LOH/imbalance at one or more loci (ratio 0 - 0.75). As the extent of LOH seen is so frequent and so widespread a statistical analysis assessing association with overall survival not been performed.

Since many markers were used in the microsatellite analysis of chromosome 17p, the association of LOH and LOH/allelic imbalance on survival was assessed at only four markers. These were the D17S720, D17S799, D17S798 and TP53 loci. Of twenty three patients for whom data was available, twelve had LOH (0 - 0.5 ratio) and seventeen had LOH/imbalance (0 - 0.75) at D17S720. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.27 Log Rank Test) or the 0 - 0.75 ratio (p=0.35 Log Rank Test). Of twenty one patients for whom data was available, seven had LOH (0 - 0.5 ratio) and sixteen had LOH/imbalance (0 - 0.75) at D17S799. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.31 Log Rank Test) or the 0 - 0.75 ratio (p=0.93 Log Rank Test). Of twenty three patients for whom data was available, eight had LOH (0 - 0.5 ratio) and fifteen had LOH/imbalance (0 - 0.75) at D17S798. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.32 Log Rank Test) or the 0 - 0.75 ratio (p=0.78 Log Rank Test). Of eighteen patients for whom data was available, three had LOH (0 - 0.5 ratio) and five had

LOH/imbalance (0 - 0.75) at TP53. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.97 Log Rank Test) or the 0 - 0.75 ratio (p= 0.59 Log Rank Test).

The LOH data was also analysed to assess whether there was a relationship between LOH and age ≤3 years versus age >3 years. With each marker there was no association with age at either the 0 - 0.5 ratio or the 0 - 0.75 ratio (see Tables 5.6, page 155, for p values).

Chromosome 22q

Of twenty three patients for whom clinical follow up and LOH data was available, all had LOH at one or more loci (ratio 0 - 0.5) and all had LOH/imbalance at one or more loci (ratio 0 - 0.75). LOH 22q was not associated with survival in this group of patients (p=0.91, Log Rank Test).

The association of survival and LOH at each marker locus was assessed individually. Of twenty one patients for whom data was available, seven had LOH (0 - 0.5 ratio) and eleven had LOH/imbalance (0 - 0.75) at F8VWFP. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.37 Log Rank Test) or the 0 - 0.75 ratio (p=0.59 Log Rank Test). Of twenty one patients for whom data was available, two had LOH (0 - 0.5 ratio) and eight had LOH/imbalance (0 - 0.75) at D22S301. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.41 Log Rank Test) or the 0 - 0.75 ratio (p=0.45 Log Rank Test). Of eighteen patients for whom data was available, two had LOH (0 - 0.5 ratio) and nine had LOH/imbalance (0 - 0.75) at D22S294. Length of survival was not asociated with loss at this locus for either the 0 - 0.5 ratio (p=0.16 Log Rank Test) or the 0 - 0.75 ratio (p=0.28 Log Rank Test).

The LOH data was also analysed to assess whether there was a relationship between LOH and age ≤3 years versus age >3 years. With 2 markers there was no association with age at either the 0 - 0.5 ratio or the 0 - 0.75 ratio (see Tables 5.7, page 155 for p values), but with D22S301 there was an association between >3 years of age and a lower frequency of LOH (p=0.03 Log Rank Test).

MIN and survival

Only two out of the twenty seven tumours analysed showed MIN. Each of these tumours, IN2659 and IN2697 showed changes in electophoretic mobility, or band shifts, at two independent loci on chromosome 17p. A log rank test was performed and the presence of MIN was not associated with length of survival.

Correlation of length of survival with losses and gains determined by CGH

There was clinical follow up on thirteen of the fifteen patients whose tumours were analysed by CGH. The most frequent CNAs in this study were loss on 9q, 10q, 12q, 16q, 17p and 22 and gain on 4q, 5q, 6q and 9p. With the exception of loss on 16q, there was no correlation between presence of any of these CNAs and length of survival. However, patients whose tumours had loss of 16q survived significantly longer than those with no loss (p=0.01). This is shown in Figure 5.4, page 159, along with a summary of the microsatellite analysis 16q v survival data for comparative purposes.

Table 5.2. p values denoting the association between LOH at each chromosome 9 marker and age. With each marker there was no association with age at either the 0 - 0.5 ratio or the 0 - 0.75 ratio

Markers	**Association with LOH (0-0.5 ratio)**	**Association with LOH (0-0.75 ratio)**
D9S166	p = 0.66	p = 0.18
D9S287	p = 1.00	p = 0.65
D9S170	p = 0.60	p = 1.00

Table 5.3. p values denoting the association between LOH at each chromosome 10 marker and age. With each marker there was no association with age at either the 0 - 0.5 ratio or the 0 - 0.75 ratio. D10S580 was not assessed as the patient number was too small.

Markers	Association with LOH (0-0.5 ratio)	Association with LOH (0-0.75 ratio)
D10S581	p = 1.00	p = 0.67
D10S540	p = 0.58	p = 1.00

Table 5.4. p values denoting the association between LOH at each chromosome 12 marker and age. With each marker there was no association with age at either the 0 - 0.5 ratio or the 0 - 0.75 ratio.

Markers	**Association with LOH (0-0.5 ratio)**	**Association with LOH (0-0.75 ratio)**
D12S327	p = 0.52	p = 0.38
D12S360	p = 1.00	p = 0.65
D12S330	p = 1.00	p = 1.00
D12S342	p = 0.54	p = 0.68
D12S366	p = 1.00	p = 1.00

Table 5.5. p values denoting the association between LOH at each chromosome 16 marker and age. With each marker there was no association with age at either the 0 - 0.5 ratio or the 0 - 0.75 ratio.

Markers	**Association with LOH (0-0.5 ratio)**	**Association with LOH (0-0.75 ratio)**
D16S409	p = 1.00	p = 1.00
D16S416	p = 1.00	p = 0.35
D16S512	p = 1.00	p = 0.40
D16S516	p = 1.00	p = 1.00

Table 5.6. p values denoting the association between LOH at each chromosome 17 marker and age. With each marker there was no association with age at either the 0 - 0.5 ratio or the 0 - 0.75 ratio.

Markers	**Association with LOH (0-0.5 ratio)**	**Association with LOH (0-0.75 ratio)**
D17S720	p = 0.11	p = 1.00
D17S799	p = 0.17	p = 1.00
D17S798	p = 1.00	p = 0.66
TP53	p = 1.00	p = 0.60

Table 5.7. p values denoting the association between LOH at each chromosome 22 marker and age. With two markers there was no association with age at either the 0 - 0.5 ratio or the 0 - 0.75 ratio. However, at the D22S301 locus LOH was significantly associated with age.

Markers	**Association with LOH (0-0.5 ratio)**	**Association with LOH (0-0.75 ratio)**
F8VWFP	p = 0.12	p = 0.24
D22S301	p = 0.15	p = 0.03
D22S294	p = 0.14	p = 1.00

Table 5.8 p values denoting the association between consistent losses by CGH and age (Fisher's exact test). There was no association between any chromosomal loss and age.

CGH loss	p value
17p	1.00
10p	0.61
9p	0.14
12q	1.00
16q	0.61
22	0.64

Table 5.9 p values denoting the association between consistent gains by CGH and age (Fisher's exact test). There was no association between any chromosomal gain and age.

CGH gain	p value
4	1.00
5	0.24
6	0.33
9p	0.53

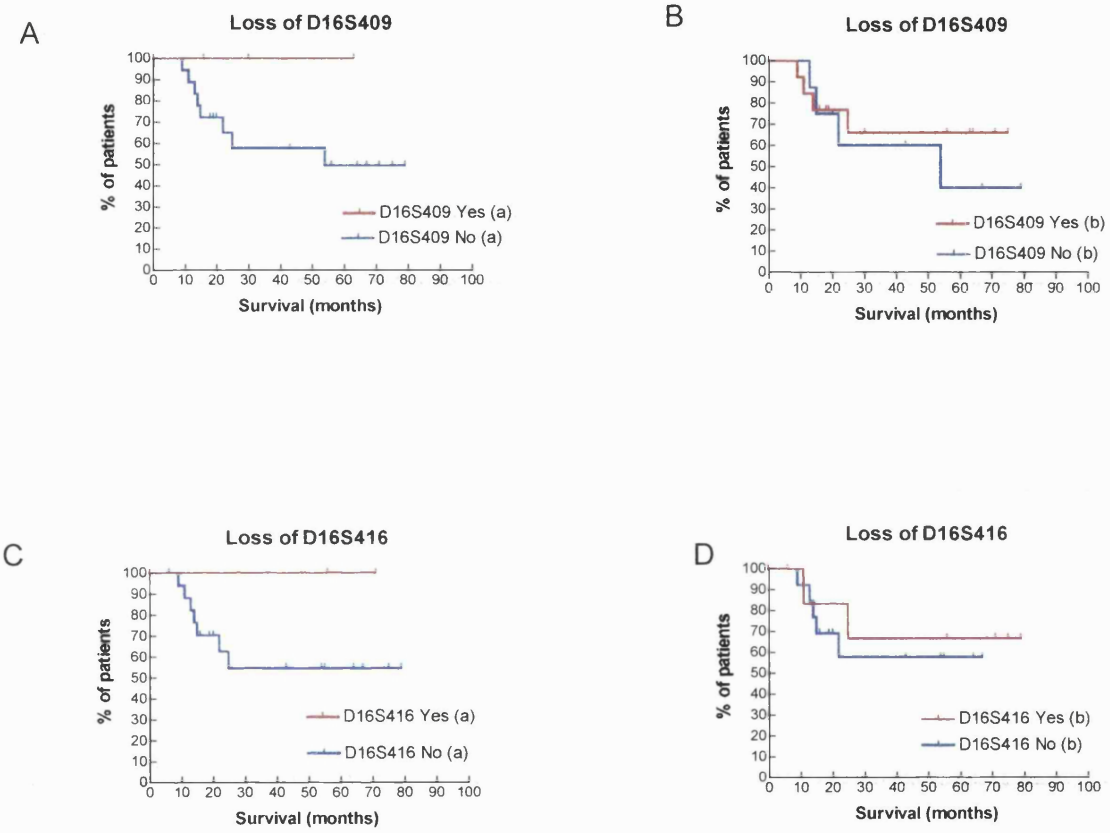


Figure 5.2 Association between LOH 16q and survival. Loss of D16S409, at a ratio of 0 - 0.5 is shown in panel A and a ratio of 0 - 0.75 is shown in panel B. Loss of D16S416, at a ratio of 0 - 0.5 is shown in panel C and a ratio of 0 - 0.75 is shown in panel D. There appeared to be no association between loss of these markers and survival at either ratio (Log-rank test, p = 0.22. 0.54. 0.29 and 0.67 respectively)

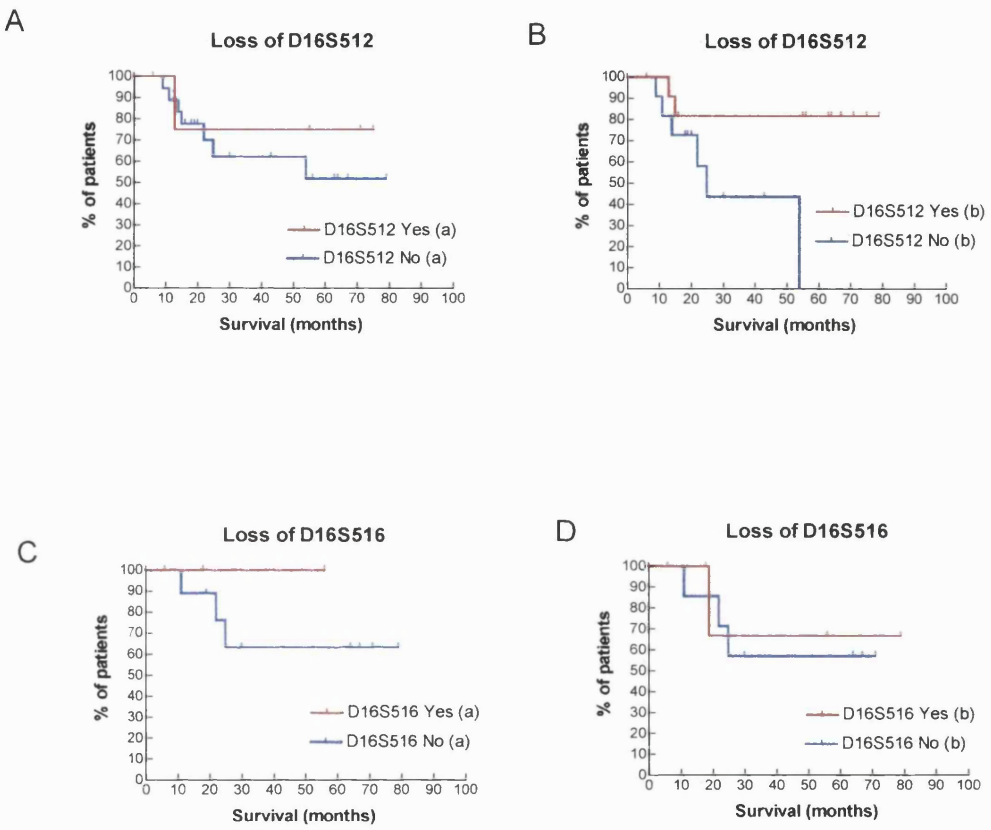
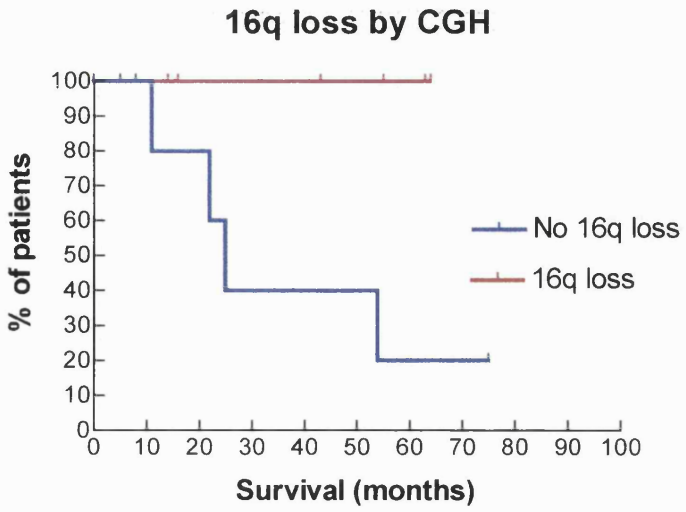
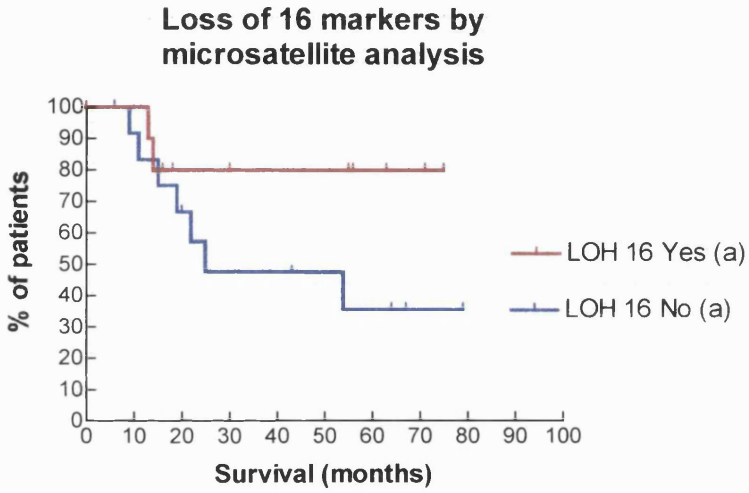


Figure 5.3 Association between LOH 16q and survival. Loss of D16S512, at a ratio of 0 - 0.5 is shown in panel A and a ratio of 0 - 0.75 is shown in panel B. Loss of D16S516, at a ratio of 0 - 0.5 is shown in panel C and a ratio of 0 - 0.75 is shown in panel D. There appeared to be no association between loss of D16S512 and survival at a ratio of 0 - 0.5 (Log-rank test, p = 0.54 although at a ratio of 0 - 0.7 there was a significant association between loss of this marker and survival (p = 0.01). There was no significant association between loss of D16S516 and survival at either ratio (p = 0.47 and 0.8).

A



B



C

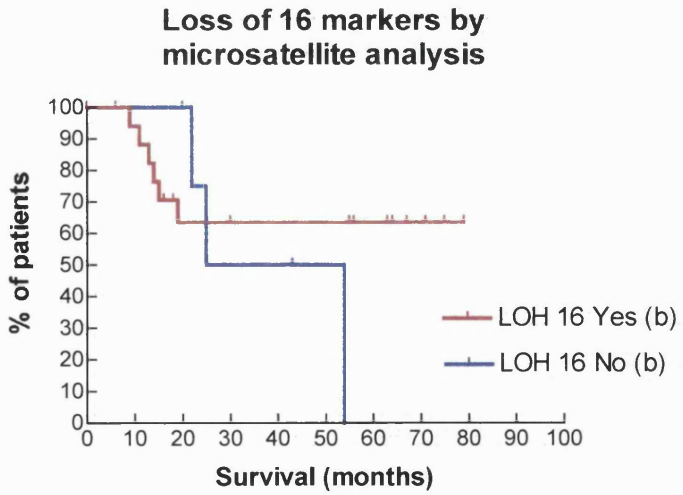


Figure 5.4 Association between LOH 16q determined by either CGH or microsatellite analysis and survival. Loss of 16q, as demonstrated by CGH, was significantly associated with survival (panel A, Log-rank test, p = 0.025), whilst loss of 16q as determined by microsatellite analysis, at either a ratio of 0 - 0.5 or 0 - 0.75, was not (panels B and C, p = 0.13 and 0.57 respectively

Correlation of patient age with CNAs determined by CGH

There was no correlation between presence of these CNAs with age (≤3 years of age and >3 years of age) (Fisher's exact test, see Tables 5.8 and 5.9, page 156 for p values).

Chapter 6

DISCUSSION

The aim of this study was to identify the genetic changes which occur in medulloblastomas. Two approaches were used to determine genetic aberrations; microsatellite analysis and CGH. Clinical correlations were assessed in order to determine which specific genetic changes could be used as prognostic indicators. This chapter will focus on giving an overall view of the study and shall discuss in depth the chromosomal regions which were found to have consistent regions of losses and gains. As the microsatellite analysis of chromosome 17p was the largest and most comprehensive undertaken in this investigation it will be discussed first. The other LOH studies, conducted as a result of what was discovered via the CGH experiments, shall be discussed later and in conjunction with CGH data for those particular chromosomal areas. Prognostic implications and how the results relate to previous investigations will also be considered. The CGH results pertaining to chromosome 17 and how they relate to the 17p LOH study will also be considered in a subsequent section.

MICROSATELLITE ANALYSIS OF CHROMOSOME 17p

Loss of p13.1 and p13.3

The results contribute to the deletion map of chromosome 17p in medulloblastoma. A clear pattern of genetic loss can be seen at several 17p13.1 loci, distal to the p53 gene. Of the twenty seven tumours analysed in this study, fourteen (51%) showed LOH at either of the adjacent marker loci D17S798 or D17S799 on 17p13.1. Two cases had LOH at both these markers and a further ten tumours showed allelic imbalance at one or both of these loci (see

table 3.1 page 85) which is highly suggestive of a putative tumour suppressor gene locus. Furthermore, 13 of 27 tumours (48%) showed LOH at D17S720, a more distal 17p13.1 locus, with a further six showing allelic imbalance at this marker. One study published 3 years ago (Scheurlen et al., 1997) used microsatellite analysis to investigate the extent of 17p deletions and LOH was found in 9 of 24 medulloblastoma samples. A high resolution deletion mapping approach was used and it could not detect several independently deleted regions on chromosome arm 17p or any tumours showing loss of chromosomal material from one particular region only. Their data suggest that a possible medulloblastoma candidate gene may be located anywhere telomeric to marker D17S953 (located at 17p11.2) and is not restricted to any particular band or sub-band. This is substantiated by an investigation in which probes in the region of 17p11.2-17p13.3 were used (Cogen et al., 1990). A common location of allelic loss for informative medulloblastoma specimens at 17p12-13 was found suggesting the presence of a tumour suppressor gene in this region.

Other recent investigations with new markers have shown that in medulloblastoma samples there is a preferential loss of DNA sequences at a site distal to p53 (McDonald et al., 1994). Indeed, some 17p LOH studies of this tumour type have defined very specific areas of loss distinct from p53 (Cogen and Daneshvar, 1990), with one investigation limiting the loss to the very distal locus D17S34 in three cases (Biegel et al., 1992), and another finding deletion within this locus in the one medulloblastoma they examined (Haataja et al., 1997) suggesting that 17p13.3 is the critical region for development of these tumours. Further evidence that a tumour suppressor locus or loci resides within the more distal region of 17p has been provided in other studies. For example, in a study comparing medulloblastoma with supratentorial PNETs, Burnett et al. (1997) found loss of 17p in 37% of the former, all of which lost the most distal marker at 17p13.3. Steichen-Gersdorf et al. (1997) observed allelic loss at 17p13.3-13.2 in 9 out of 18 tumours. Furthermore, all

tumours were informative for D17S796 and 9 out of 9 samples were deleted at this locus which is located at 17p13.3.

Thus, the 17p13.1 region may not be the only one to harbour a potential tumour suppressor gene locus on chromosome 17p. The evidence above suggests that a locus on distal 17p13.3 is involved in medulloblastoma development. It is unfortunate that the tumours used in the present study were non informative for D17S34. However LOH at the other 17p13.3 markers were seen much less frequently. For example, in the 17p13.3 region, at the marker with the most LOH seen, D17S643, loss was found in only 5 of 26 tumours (19%). However, this frequency of 20% though, makes it possible that putative tumour suppressor genes important in the pathogenesis of medulloblastoma may reside in this region. Thus there may be important loci on both 17p13.1 (distal to p53) and 17p13.3. Further evidence for this hypothesis can be seen from LOH studies of breast cancer. In a study of 168 breast tumours, Coles et al (1990) found two common areas of deletion on 17p, both distal to p53. Sato et al. (1990) reported similar results from their studies on 79 primary breast tumour patients. These findings substantiate the possibility of the existence of genes distal to p53 on 17p that may be involved in tumourigenesis. Furthermore, the extent of the deletions seen in the present study (observed across the entire region investigated) suggest that loss of more than one gene on 17p may be important.

p53 and other genes on 17p which may contribute to medulloblastoma development

Previous research has shown p53 mutations in medulloblastomas/PNETs to range from 0 out of 12 tumours and 1 out of 3 cell lines (Saylors et al., 1991), to 2 out of 11 (Ohgaki et al., 1991), 2 out of 7 (Cogen et al., 1992), 0 out of 7 (Biegel et al., 1992) and 1 out of 9 (Adesina et al., 1994). Thus, the overall incidence of mutations of p53 in these tumours is

around 8%, indicating that p53 is unlikely to play a major role in medulloblastoma development. The results of the present study provide evidence for this hypothesis in that deletions at the p53 locus were observed in only 3 of 22 tumours which constitutes 14% of the sample group.

Another mechanism of p53 inactivation is through the amplification of the *mdm2* gene, the protein product of which binds and inactivates wild type p53 (Oliner et al., 1992). Amplification of this gene has been reported in some sarcomas and astrocytomas (Oliner, et al., 1992; Ladanyi et al., 1993) thus providing another mechanism for the inactivation of p53 in tumours without p53 mutations. Adesina et al (1994) found no amplification of the *mdm2* gene in 8 of the 9 tumours they studied and, furthermore, Batra et al. (1995), observed no amplification in a set of 56 medulloblastomas that they studied. Again, these findings strengthen the hypothesis that there is another tumour suppressor gene locus, on 17p or elsewhere which may also play a role in the transformation and/or progression of medulloblastoma.

Tumour suppressor genes for various types of cancer are located on chromosome 17p. The ABR (active BCR-related gene) has been favoured as a likely candidate in medulloblastomas because it is specifically expressed in brain (McDonald et al., 1994). Interestingly, it has been shown to lie within approximately 220kb of the D17S34 locus at p13.3 (McDonald et al., 1994). The relevance of ABR is a controversial issue however. For example, although McDonald et al (1994) found this gene to be frequently deleted in medulloblastomas, they found no mutations. Furthermore, a recent study (Steichen-Gersdorf et al., 1997) which investigated LOH in eighteen medulloblastomas at fifteen different loci on chromosome 17p, found allelic loss in 17p13.3-13.2 in 50% of the tumours. The smallest region of overlap, which the authors postulate could contain a putative suppressor gene, excludes the ABR gene as well as the p53 gene.

Another candidate tumour suppressor gene is HIC -1(hypermethylated in cancer). This encodes a zinc-finger transcription factor and is expressed ubiquitously in normal tissue (Koch et al., 1996). Since it has been located to 17p13.3, the common region of overlap of deletions reported in some medulloblastomas, Koch et al (1996) investigated HIC-1 in eight medulloblastoma cell lines and came to the conclusion that this gene may contribute to the pathogenesis of this type of tumour since it is silenced by *de novo* hypermethylation in medulloblastoma, but as yet there is no more evidence.

Two other genes that fall within the smallest region of overlap of allelic loss identified in the Steichen-Gersdorf et al (1997) study have recently been characterised. These genes, identified by allelic loss mapping and positional cloning methods (Schultz et al., 1996) are OVCA1 and OVCA2. This work revealed that two distinct transcripts of approximately 2.3 and 1.1kb are ubiquitously expressed and their protein is highly conserved. Overall, the chromosomal location, plus the high degree of amino acid conservation and deregulation of mRNA expression found suggest that one or both of these genes may be involved in the pathogenesis of medulloblastomas.

The possible involvement of the 17p11.2 region in medulloblastoma development

The deletion mapping experiments of Scheurlen et al (1997) showed LOH in 9 of 24 tumours with deletion of a continuous stretch from the telomere to band 17p11.2 in all nine cases. Furthermore, it was observed that most of the chromosomal losses observed were at locations in chromosome band 17p11.2 within a region that has previously been described as the critical region for Smith-Magenis syndrome (Greenberg et al., 1991). This syndrome possesses a complex phenotype that includes developmental and growth

problems as well as facial anomalies and unusual behaviours. The molecular defect underlying the disorder is either an interstitial deletion or a duplication in chromosome band 17p11.2 (Brown et al., 1996). Although there is no increased incidence of medulloblastoma in Smith-Magenis patients, it is possible that the underlying mechanism leading to chromosomal disruption and recombination with this region might have the same molecular basis. From this, it seems clear that future LOH studies should use microsatellite markers which map to the entire chromosome 17p arm and not be restricted to the 17p13 area so that a truer picture of the involvement of 17p abnormalities in the pathogenesis of medulloblastomas can be obtained.

Associations of LOH 17p with other chromosomal losses

It is not possible to draw any conclusions between associations between LOH 17p and other chromosomal losses in the present study since LOH 17p was found in 100% of cases. What follows is a summary of what associations of loss between chromosomes have been found in previous investigations with reference to the present study. However, loss of 17p has been associated with loss of other chromosomes in previous investigations.

As deletion and mutation of the *PTC* gene may indicate a subset of medulloblastomas with poor prognosis (Scheurlen et al., 1998) and given that 17p deletions can be associated with a similar outcome (Batra et al., 1995), several investigators have looked at whether or not these two abnormalities occur together in tumours. Scheurlen et al. (1998) found LOH 9q22 in 3 of 30 (10%) of informative samples and this was the only locus on 9q which they studied. LOH 9q22 was never associated with LOH 17p which suggests alternate pathways for medulloblastoma initiation. Similarly, Albrecht et al. (1994) found LOH 17p in several tumours, none of which had LOH 9q. In the present study LOH 9q22 was only

found in 3 of 26 samples, as seen at the adjacent markers D9S287 and D9S176, whilst allelic imbalance was observed in a further 10 samples at this locus.

Scheurlen et al. (1998) found LOH 10q in 6 of 29 informative samples, and 3 of 6 (50%) showed a corresponding LOH 17p. Cogen et al. (1990) found loss of 10q in 2 of 9 samples and both of these showed a corresponding 17p loss, whilst Blaeker et al. (1996) observed LOH 10q in 4 of 6 cases, 2 of which (50%) also showed loss of 17p. In the present study, 5 of 26 (19%) showed LOH of both 10q and 17p but only 3 markers were used to study 10q. An interesting comparison can be made with a 1988 study of the molecular genetics of glioblastomas (James et al., 1988). Loss of genetic information from chromosome 10 was often accompanied by loss of genetic information from other chromosomes, most commonly 13, 17 and/or 22. In the current investigation a similar pattern of genetic alterations can be seen; in 5 of 7 cases with 10q loss, information was also lost from chromosomes 17 and 22. It appears that, unlike chromosome 9q, loss of 10q does not denote a subset of medulloblastomas with alternate molecular pathways of development, rather it is a secondary change associated with 17p and/or 22 loss.

As already stated, loss of 12q is uncommon in medulloblastomas so an examination of any such loss in conjunction with LOH 17p has not hitherto been considered. All seven samples showing 12q loss by CGH also showed loss of chromosome 22, although this is perhaps not significant when the extent of chromosome 22 deletions is considered. In 6 of 7 cases showing 12q loss, 9q loss was also seen, and in 4 of 7 tumours with deletions of 12q, deletions of 10q were found. The significance of 12q loss in association with loss of other chromosomes and the pathways involved remains to be elucidated.

Differences in desmoplastic versus classical

Scheurlen et al. (1998) reported no LOH 17p in five desmoplastic medulloblastoma samples. In the present study, 3 of 27 tumours were desmoplastic, IN2588, IN2832 and IN2917. LOH was found in all three tumours of (at 4 of 26 loci in IN2588, 3 of 22 loci in IN2832 and 2 of 22 loci in IN2917) and there was no difference in frequency to that found in the classical medulloblastomas.

CNAs DETECTED BY COMPARATIVE GENOMIC HYBRIDISATION

Summary of losses and gains of genetic material found in previous CGH studies

An i(17q) has been detected in a maximum of 50% of medulloblastomas in previous studies suggesting that additional loci may be involved in the pathogenesis of these tumours. At least one study (Biegel et al., 1989) provides evidence of tumours with single structural abnormalities other than i(17q), including deletion or monosomy of chromosome 6 and deletion of 16q. Additional findings in small subsets of tumours, either by cytogenetic analysis or LOH allelotyping, have already been cited in the introduction; trisomy 7 (Battacharjee et al., 1997), loss of 22 (Bigner et al., 1997), loss of 11p (James et al., 1990), loss of 9q (Raffel et al., 1997) and unbalanced translocations or deletions of 5q, 8, 10 and 16q (Biegel et al., 1989; Bigner et al., 1988: Raffel et al., 1990; Thomas and Raffel, 1991; Albrecht et al., 1994; James et al., 1990).

One of the advantages of the CGH technique is that it can be used to identify both losses and gains across the whole genome. Once specific regions of the genome have been implicated in medulloblastomas in this way, more detailed methods can be used to further

investigate these areas and ultimately identify candidate genes. Previous studies using CGH to examine medulloblastomas have detected consistent regions of genetic loss and gain at a much higher rate than other studies using different techniques. In one series of eighteen tumours, the most common changes were loss of 17p (22% of samples), which confirms the frequent loss of 17p and gain of 17q found by cytogenetic and FISH studies. New regions of interest were identifieed including gains on 4p, 5p, 5q, 7q, 8q, 9p and 17q in each of 3 tumours (11%) (Schütz et al., 1996). In a second study of twenty seven tumours (Reardon et al., 1997), gains were reported on 17q (48% of samples) and 7 (44%) and losses revealed on 10q (41%), 11 (41%), 16q (37%), 17p (37%) and 8p (33%). Another study of a sample group of twenty three, detected multiple chromosomal imbalances in twenty cases (Avet-Loiseau et al., 1999). Loss of 17p was seen in eight (34%) cases and gain of 17q was observed in eleven (47%) tumours; almost identical frequencies to those seen in the Reardon et al., (1997) study. Other losses found at high frequencies were those of chromosomes 11 in nine (39%) cases and 10q in eight (40%) cases. Gains were less frequent but extra copies of chromosome 7 were seen in nine (39%) cases whilst gains of 1q were detected in 6 (26%) tumours. Another study conducted last year used a very small number of samples, only six, and found gains of 7q and 17q in 4/6 (66%) cases (Nishizake et al., 1999).

Summary of major changes found in the present study

The frequency of 17p deletions (60%) found in the present study by CGH correlates with previous LOH data as well as the LOH data presented here. However, the frequency of gain of 17q (6% of cases) was lower than expected and was actually seen in only 1 of the 15 tumours studied by CGH. Although gains of 2q, 6q and 9p have been detected in medulloblastoma by cytogenetic and CGH analysis, we found a much higher incidence than in earlier studies. Similarly, losses on 9q, 12q and 22 have been identified previously

but at significantly lower frequencies. However, losses involving 10q and 16q were detected at similar frequencies to the CGH study by Reardon et al. (1997) Loss of 16p and the high copy number amplicons at 3q24-26, 9p21-23, 13q21-31 and 18q21-22 are novel findings.

Loss was detected in the highest frequencies at 22q and 9q where 14 of 15 (93%) and 13 of 15 (86%) tumours respectively had deleted regions High frequencies of loss were also seen at 17p in 9 of 15 (60%), 16q in 8 of 15 (53%), 10q in 7 of 15 (46%) and 12q in 7 of 15 (46%) cases. LOH analysis had already been carried out on chromosome 17p as discussed above, so polymorphic microsatellite markers were used to further investigate the areas of deletion on these other chromosomes.

CHROMOSOME 9

Loss of 9q

LOH of 9q was found in the present study in 54% (14 of 26) samples, whilst overall LOH and allelic imbalance was found in 25 of 26 tumours (96%). The CGH study found loss of 9q in 13 of 15 cases. The incidence of loss found contrasts markedly with previous LOH studies. For example, Albrecht et al. (1994) found LOH 9q in only 2 of 19 (15%) informative samples, although these were obtained from adult patients.

Deletions at the Gorlin Syndrome gene locus (PTC)

The gene for Gorlin Syndrome (GS) is located at 9q22.3-q31 and it is the human homologue of the Drosophila *patched (PTC)* gene (Hahn et al., 1996; Johnson et al., 1996) which has been found to be lost and/or mutated in 10-20% of sporadic

medulloblastomas as well as in GS (Raffel et al., 1997). The results from several other studies indicate that loss of 9q may involve deletion/mutation of *PTC* (Raffel, et al., 1997; Wolter et al., 1997; Vorechovsky et al., 1997; Zurawel et al., 2000) and could indicate a subset of tumours with poor prognosis (Scheurlen et al., 1998). A further investigation found that mutation/deletion of the *PTC* locus is restricted to the desmoplastic variant of this neoplasm (Cowan et al., 1997). In the present study, loss at the *PTC* locus was seen in 4 of 15 (26%) of tumours by CGH which is obviously a much higher frequency than that found previously. Moreover, the microsatellite data gleaned indicated that there may have been loss at the *PTC* locus in a similar frequency of cases; 8 of 26 (30%).

All of the tumours used in the present study were obtained from non-GS associated medulloblastoma specimens. However, the markers used mapped from 9q21 to 9q33 and LOH was seen at all of them. Also, despite the fact that 30% of tumours showed loss in the region of the *PTC* locus, of the fourteen tumours with LOH, ten (38%) showed loss at either D9S287 (mapping to 9q22.3) or D9S166 (mapping to 9q21.1). A further eleven tumours showed allelic imbalance at one or both of these loci, suggesting that there may be another putative tumour suppressor gene between 9q21.1 and 9q22.3. Furthermore, in the four cases showing loss in the region of *PTC* by CGH, this loss formed only a small area of a much bigger deletion; almost the entire chromosomal arm in some cases. Whilst these results do not exclude a role for *PTC* in the pathogenesis of sporadic medulloblastoma they make such a role less likely. Alternatively, *PTC* may be involved in a subset of tumours with a second putative tumour suppressor gene being also located on 9q.

The ABL gene

One such candidate gene is the *ABL* gene located at 9q34-qter. This gene, coding for a member of the CDC2 family of protein kinases (PITALRE), (Bullrich et al., 1995) and

which is lost in chronic myelogenous leukaemia has been mapped to this area. In the present study, 12 of 15 (80%) of tumours had loss of material on 9q34 or 9q34-qter by CGH, and in five of these cases (IN2077, IN2636, IN2823, IN2966 and IN2996) the loss was restricted to this region rather than comprising part of a wider area of deletion. These results suggest that this gene is of more interest and has more relevance than *PTC*. Loss in the region of the *ABL* locus has already been reported in paediatric glioblastoma multiforme and an astrocytoma case (Blaeker et al., 1996) arousing interest regarding the loss of this gene in other childhood brain tumours. The CGH findings described in the present study raise the possibility of a 9q34-qter tumour suppressor gene in a subset of childhood medulloblastomas.

Other possible candidate genes on 9q

ABL is not the only candidate gene with a possible involvement in the pathogenesis of sporadic medulloblastomas. Several candidate tumour suppressor genes/areas have been suggested from work on different forms of cancer. DBCCR1 (deleted in bladder cancer chromosome region candidate 1) is located at 9q32-33 (Habuchi et al., 1998), whilst in ovarian carcinoma deletions of 9q31 and 9q32-34 have been found (Schultz et al., 1995). Moreover, following studies of lung carcinoma, Suzuki et al. (1998) have suggested tuberous sclerosis complex 1 (TSC1)-associated region at 9q34 as a candidate locus for a tumour suppressor gene. It is thus clear that there are many possibilities with regard to 9q involvement in medulloblastoma development.

Prognostic implications of 9q loss

A previous study has looked at 9q loss and prognosis. Despite having only three tumours from which prognosis could be assessed in their analysis, Scheurlen et al. (1998), noted

that all patients with LOH of chromosome 9q22 had relapses after 10, 20 and 26 months respectively, despite a lack of metastatic disease and a good initial response to chemotherapy. Based on this evidence, it seems possible that the subset of medulloblastomas with deletions at this locus may have a worse prognosis than those without this abnormality. However, in the present study, although loss was seen in over 50% of tumours with both the CGH and LOH techniques, there was no significant difference in survival between those who had 9q loss and those who didn't. Furthermore, LOH at any particular microsatellite marker locus (whether defined by the 0-0.5 or the 0-0.75 ratio) did not significantly effect survival. To give examples of cases showing loss of 9q22 specifically, three tumours (IN2741, IN2832 and IN2933) showed loss of this region as seen by CGH and three tumours (IN2465, IN2741 and IN2805) as seen by LOH but the implications for prognosis were found to be statistically not significant. For example, although the patient from which IN2805 was obtained had a non CNS recurrence and died 14 months after diagnosis, the patient from which IN2465 was obtained was still alive and well almost 6 years after an initial total resection despite a recurrence 2 years subsequent to diagnosis and patient IN2832 is alive and well over 3.5 years since diagnosis. Unfortunately, follow up information is not available for patient IN2741, but, nevertheless, these results show that deletions of the 9q22 locus may not be a viable prognostic indicator.

Another prognostic variable analysed was age. Since age < 3 years has often been associated with a worse prognosis (Kopelson et al., 1983; Packer et al., 1991; Packer et al., 1997), in this study age ≤ 3 years and > 3 years versus LOH at each marker locus and versus losses seen by CGH were analysed. There was no significant correlation of 9q deletion and age. Overall, whilst there is evidence that at least two putative tumour suppressor genes are located on 9q, one at 9q21.1 to 9q22.3 and one at 9q22.3 to 9q33, the lack of evidence to suggest that loss of chromosome 9 has any association with survival

suggests that loss or mutation of these putative genes may have no effect on prognosis. Furthermore, the data presented here suggest that if the *PTC* locus is lost in a subset of medulloblastomas then, contrary to previous evidence (Scheurlen et al., 1998), this loss may not necessarily be associated with a poor prognosis.

Gain of 9p

Gain of chromosome 9p has been reported infrequently in medulloblastomas. In the current study, gain of genetic information on 9p was seen in 10 of 15 (66%) of cases and a high copy number amplification was seen at 9p21-23 in two tumours (IN2765 and IN2832). It is also possible that in this sample there is i(9p). Another case (IN2659) also shows gain of the entire 9p arm, and in all but one of the samples studied which showed gain on 9p it was accompanied by a loss of 9q.. Two previous studies have shown a gain of genetic material on 9p (Vagner-Capodano et al., 1994; Schutz et al., 1996). The former study found gain in one tumour and it was confined to the 9p24 region. The Schutz et al. (1996) investigation was a CGH analysis and 3 of 18 (16.5%) cases showed gain of 9p, encompassing the 9p24 locus in all three tumours. In concurrence, all eleven tumours with gain of 9p in the present study show gain at this locus. It is possible that a putative oncogene whose transformation leads to medulloblastoma development is locate at 9p24. However, gain at this region was usually only a small area of a much bigger area of gain in the present study.

Prognostic implications of gain of 9p

Although gain of this chromosome arm was seen in over 60% of tumours by CGH analysis there was no significant difference in survival between those who had 9p gain and those who did not. Similarly, when looking at age ($\leq$ 3 years of age and $>$ 3 years of age)

versus gains of 9q, again there was no significant difference. Overall, whilst the frequency of gain at the 9p24 locus represents a potentially exciting new area of study in the future, the lack of evidence to suggest that gain of chromosome 9p has any association with survival suggests any transformation of a putative oncogene at this locus may have no effect on prognosis.

CHROMOSOME 10q

Deletions of chromsosome 10q are one of the most frequent structural abnormalities found in medulloblastomas, but the extent to which such losses are found is variable. For example, some previous studies have demonstrated loss on chromosome 10q at only fairly low frequencies. One investigation detected allelic loss with probes from 10q in 2 of 9 (22%) of cases (Cogen et al., 1990), whilst another report relates deletions of 10q in 2 of 17 (11.5%) medulloblastomas with an abnormal karyotype (Bigner et al., 1997), and a third study showed loss in 6 of 29 (20.7%) of medulloblastomas at 10q (Scheurlen et al., 1998). However, other investigations have showed loss of this chromosome arm at a much higher frequency. In one study, 4 of 6 (66%) tumours had 10q loss (Blaeker et al., 1996), and in one CGH analysis 11 of 27 (41%) of medulloblastomas studied showed deletion of 10q (Reardon et al., 1997). Another CGH study showed deletion of 10q in 8 of 20 cases (40%) of medulloblastoma samples studied (Avet-Loiseau et al., 1999) The latter is comparable with the present study in which CGH analysis revealed loss of 10q in 7 of 15 (46%) of samples and the microsatellite analysis showed LOH on this chromosome arm in 6 of 26 (23%) of cases, and overall allelic imbalance in 12 of 26 tumours (46%). One of the tumours (IN2741) showed a corresponding loss of 10p as did one of the tumours in the Blaeker et al. (1996) study and six of the cases analysed in the Reardon et al. (1997) investigation. Also in an LOH investigation, losses on 10q included all informative markers suggesting loss of the entire chromosome arm in 4 of 6 tumours (Blaeker, et al.,

1996). The CGH results from the present work showed deletion of the whole arm of 10q in two tumours (IN2636 and IN2741) whilst the LOH studies indicate that possibly 4 of 26 (15%) of cases could show the same extent of loss. With the remaining tumours that demonstrated 10q loss by CGH, the loss was confined to the distal region of the arm, and it is possible that loss of the distal region of 10q, at 10q25-26, is important in medulloblastoma development. The LOH data revealed that of the six tumours with LOH, four (66%) showed loss at either D10S581 or D10S580. An additional seven tumours showed allelic imbalance at one or both of these loci, suggesting that a putative tumour suppressor gene is located at 10q21 to 10q23.1 which is flanked by these two markers. Thus, the results from the present study indicate that there may be an important locus in the latter region and also at the very distal aspect of chromosome 10q at 10q25-26.

Putative target genes

The loss of distal 10q is one of the most common abnormalities in high-grade gliomas (Rasheed et al., 1992). The target of loss of distal 10q may be the MXI1 (MAX-interacting protein 1) gene which maps to 10q24-25 and is deleted in 60% of high grade gliomas (Wechsler et al. 1997). Its product forms heterodimers that suppress transcription and compete with MYC for binding site occupancy, thus antagonising transcriptional activation of gene expression by MAX-MYC oncoproteins. The data presented here as well as concordance of FISH and CGH data regarding distal 10q in a previous study (Reardon et al., 1997) reinforces the potential of MXI1 as a medulloblastoma tumour suppressor gene. More recently, the gene DMBT1 (Deleted in Malignant Brain Tumours 1), located at 10q25.3-10q26.1 and a member of the scavenger-receptor cysteine-rich (SRCR) superfamily, has shown homozygous deletions or lack of expression in a medulloblastoma (Mollenhauer et al., 1999) and has thus been proposed as a candidate tumour suppressor gene. One study has determined the genomic sequence of DMBT1 to allow analyses of

mutations (Mollenhauer et al., 1999) and the data suggested that alternative splicing gives rise to isoforms of the gene with a differential utilisation of SRCR domains and SRCR interspersed domains. Further, the major part of the gene was found to harbour locus specific repeats. The authors argue that these repeats may point to the DMBT1 locus as a region susceptible to chromosomal instability. Although the MMAC/PTEN tumour suppressor gene (at 10q23) also maps within the deleted region on 10q and has been found to be lost and/or mutated in numerous human cancers, a previous study has found mutation in only 1 of 22 medulloblastomas and it is unlikely to be involved in these tumours (Rasheed et al., 1997).

Prognostic implications of 10q loss

As far as the author is aware, this is the first study to determine a correlation of 10q loss with survival in medulloblastoma. Although loss was seen in over 20% of tumours with both the CGH and LOH techniques there was no significant difference in survival between those who had 10q loss and those who didn't. Furthermore, LOH at any particular microsatellite marker locus (whether defined by the 0-0.5 or the 0-0.75 ratio) did not significantly correlate with survival. When looking at age ($\leq$ 3 years of age and $>$ 3 years of age) versus LOH at each marker locus and versus losses seen by CGH, again there was no significant difference. Overall, whilst there is evidence that a putative tumour suppressor gene is located at 10q21 to 10q23.1, the lack of evidence to suggest that loss of chromosome 10q has any relationship with survival suggests that loss or mutation of this putative gene may have no effect on prognosis.

CHROMOSOME 12

Loss of 12q

Loss of 12q was seen in 7 of 15 (46%) tumours by CGH varying from discrete deletions (eg. IN2077) to losses extending to a substantial part of the chromosome arm (eg. IN2588). A corresponding frequency of loss of 46% of samples (12 of 26) was found in the LOH studies, 61% of the thirteen tumours with LOH showed loss at either D12S330 or D12S366 suggesting that a putative tumour suppressor gene is located at 12q24.1-24.2 and is flanked by these two markers. There is also the possibility of a second locus, at 12q24.3, in a subset of cases. This high frequency of loss, and indeed identification of putative tumour suppressor gene loci, is very surprising in the light of previous studies of medulloblastoma in which loss of 12q has been observed at a very low frequency if at all (Schutz et al., 1996; Reardon et al., 1997). Furthermore, in an LOH study which analysed all chromosomes, chromosome 12 was one of only three chromosomes not to show LOH in at least one medulloblastoma in a small series of six (Blaeker et al., 1996).

In the present study, six tumours were studied using both methods of analysis (IN2266, IN2636, IN2741, IN2805, I 2823 and IN2933). No loss was found using CGH but areas of allelic imbalance and/or LOH were found at at least one marker locus using microsatellite analysis in each tumour. The example of IN2636 was particularly interesting in that informative results are available for all five markers studied and allelic imbalance is seen for the middle marker (mapping to the 12q24.1 locus) only, whilst the other markers are all preserved. This seems to particularly highlight the way in which microsatellite analysis is sensitive enough to localise small regions of loss.

Putative target genes

Little is known of putative candidate genes located on 12q. However, one study of ovarian germ cell tumours used microsatellite markers to assess LOH and found a common site of deletion at 12q22 in 53% of cases (Faulkner and Friedlander, 2000). A further microsatellite analysis identified two commonly deleted regions on 12q in pancreatic cancer (Kimura et al., 1998). Forty samples of pancreatic ductal adenocarcinoma were examined of which 67.5% showed loss at 12q21 and 60% showed loss at 12q-12q23.1. The authors argued that these frequently deleted regions in pancreatic cancer may provide new avenues for isolating novel tumour suppressor genes. In the present study, three tumours (IN2832, IN2894 and IN2912) showed loss of 12q23 by CGH analysis, whilst three other cases (IN2588, IN2659 and IN2765) showed loss at 12q23 in both the CGH and LOH investigations. Thus, as well as the two regions harbouring putative tumour suppressor genes at 12q24.1-24.2 and 12q24.3, it may be that 12q23 is also an important locus for future investigation. However, it is clear that until further studies are carried out the significance of the high frequency of deletions found in the present study will not be known.

Prognostic implications of 12q loss

Since 12q loss is so rare in medulloblastoma, this is the first study to attempt a correlation of this loss with survival to the author's knowledge. Although loss was seen in at least 50% of tumours with both the CGH and LOH techniques there was no significant difference in survival between those who had 12q loss and those who did not. Furthermore, LOH at any particular microsatellite marker locus (whether defined by the 0-0.5 or the 0-0.75 ratio) did not significantly correlate with survival. When looking at age ($\leq$ 3 years of age and > 3 years of age) versus LOH at each marker locus and versus losses

seen by CGH, again there was no significant difference. Thus, distribution of such changes is not related to age. Overall, whilst the evidence cited above suggests that multiple putative tumour suppressor genes are located on 12q, the lack of data to suggest that loss of chromosome 12q has any influence on survival suggests that loss or mutation of these putative genes may have no effect on prognosis.

Gain of 12

In 6 of 15 cases (40%) examined by CGH in the present study, gain of material on chromosome 12 was found. In five of these, the same region on 12q21 was affected. As far as the writer is aware, this is a novel finding in medulloblastomas. Additionally, in three of these cases, loss of a different region of 12q was also found. It is clear that more studies of chromosome 12 need to be conducted in order to assess whether these findings have any significance.

CHROMOSOME 16

Loss of this chromosome has been found in previous studies of medulloblastoma (Griffen et al., 1988; Bigner et al., 1988). In the present study, loss of 16q was seen in 8 of 15 (53%) cases and loss of 16p was seen in 9 of 13 (69%) by CGH. Reardon et al. (1997) found loss of 16q in 10 of 27 (37%) cases, whilst Blaeker et al. (1996) found loss of 16q in 3 of 6 (50%) cases and loss of 16p in 1 of 6 (16.5%) samples. One of their samples showed loss of every informative 16p and 16q marker, indicating the loss of the entire chromosome. Similarly, the results presented here show loss of material on both 16p and 16q in 5 of 13 (38%) cases by CGH, with one of these cases (IN2741) demonstrating loss of the entire chromosome. The microsatellite analysis reveals LOH in eleven cases (42%) and LOH or allelic imbalance of 16q in 22 of 26 (84%) samples. Three tumours (IN2266,

IN2637 and IN2715) show loss at such a range of marker loci that it is probable that the entire chromosome arm has been lost in these cases. Of the eleven tumours with LOH, seven (63%) showed loss at either the D16S514 or D16S512 loci (flanking 16q21-16q22.1), seven (63%) showed loss at either D16S512 or D16S516 (flanking 16q22.1-16q24.1) and five (45%) showed LOH at either D16S409 or D16S416 (flanking 16q12 - 16q13). These findings suggest that there may be multiple putative tumour suppressor genes located on chromosome 16q.

Candidate tumour suppressor genes on 16

Two genes on 16q which may be involved in tumourigenesis have been identified and it is possible that one or both of these may be involved in medulloblastoma development. For example, a putative candidate tumour suppressor gene for breast cancer tumourigenesis, CTCF (CCCTC-binding factor), has been localised to 16q22.1 (Filippova et al., 1998). Moreover, large deletions including mutations in *TSC2* (tuberous sclerosis 2), located at 16p13.3, are found in patients with tuberous sclerosis which indicates that this gene acts as a tumour suppressor (van Bakel et al., 1997).

Prognostic implications of 16q loss

This is the first study to attempt a correlation of this loss with survival to the author's knowledge. Loss was seen in at least 40% of tumours with both the CGH and LOH techniques and there was a significant difference in survival in that those patients who had loss of 16q by CGH had a significantly longer time of survival compared to those who did not. Similarly, when analysing the individual marker loci from the microsatellite analysis, it was found that LOH of the D16S512 locus only (mapping to 16q22.1) at the 0.0-0.75 ratio was significantly correlated with an higher survival rate. When looking at age ($\leq$ 3 years of

age and > 3 years of age) versus LOH at each marker locus and versus losses seen by CGH there was no significant correlation however. Overall, the evidence from this study suggests that multiple putative tumour suppressor genes are located on 16q and that the mutation/deletion of one of them at 16q22.1 in patients, may result in an improvement in survival regardless of age.

CHROMOSOME 22

Loss of chromosome 22 was found in 13 of 15 (86%) tumours by CGH, whilst the microsatellite analysis showed LOH in 12 of 26 cases (50%) and overall allelic imbalance was found in 23 of 26 (88%) of cases, making it the most consistent abnormality in the entire study. The fact that loss on this chromosome was seen more frequently than that observed on 17p indicates that abnormalities of this chromosome may be very important in the pathogenesis of medulloblastomas.

In addition to medulloblastoma, karyotypic analyses of various CNS tumours have shown chromosome 22 to be abnormal. One investigation (Yamada et al., 1980) demonstrated the deletion of this chromosome in gliomas and meningiomas. A later study showed chromosomal translocation t(14:22) in leukocytes of patients with familial meningioma (Bolger et al., 1985), whilst another revealed a different translocation, t(11;22) in peripheral neuroblastomas (Whang-Peng et al., 1984). More recent reports have cited loss of chromosome 22 as a major abnormality in ependymomas (James et al., 1990; Vagner-Capodano et al., 1992; Blaeker et al., 1996) and in neurofibromatosis 2 (Rouleau et al., 1987).

Previous investigations have reported loss of chromosome 22 in medulloblastoma in between 12.5 and 28.5% of samples and the importance of such deletions to the pathogenesis of medulloblastoma is a contentious issue. Deletions of were seen in 4 of 14 (28.5%) tumours investigated by Vagner-Capodano et al. (1992), whilst loss of chromosome 22 was shown in 4 of 17 (23.5%) medulloblastomas with an abnormal karyotype in a later study (Bigner et al., 1997). Indeed in 2 of 4 cases in the latter study, this loss was the sole abnormality. In the opinion of other investigators however, whilst they have found loss of chromosome 22, the frequency of loss is too insignificant for this chromosome to play a role in medulloblastoma pathogenesis. For example, a recent study by Lescop et al. (1999) found LOH of chromosome 22q in only 3 of 23 (13%) of medulloblastomas and they concluded that 22q deletions are infrequent in this type of tumour. Results reported in three other studies have found only three tumours with LOH chromosome 22 out of a total of thirty nine medulloblastoma samples analysed; 2 of 11 (18%) in a study by James et al. (1990), 0 of 20 as reported by Thomas and Raffel (1991) and 1 of 8 (12.5%) as found by Blaeker et al. (1996).

Monosomy 22 in medulloblastoma and atypical teratoid/rhabdoid tumours

Battacharjee et al. (1997) reported monosomy 22 as an abnormality in approximately 10% (3 of 20) of medulloblastomas, and all four tumours were monosomic for chromosome 22 in another study (Vagner-Capodano et al., 1994). All, incidentally, were of the desmoplastic variant. Furthermore, monosomy 22 was the single abnormality observed in these tumours. In the present study, 9 of 15 tumours (60%) showed monosomy 22 by CGH. The results contrast with other CGH analysis of large series' of medulloblastomas in which monosomy 22 has not been shown to be a frequent change (Reardon, et al., 1997; Schutz et al., 1996). Biegel et al. (1990) noted frequent monosomy 22 in rhabdoid tumours of the CNS which, according to some observers, represents a variant of

medulloblastoma when occurring in the cerebellum (Bigner et al., 1997). Rorke et al. (1995) consider these tumours to represent a unique class of CNS neoplasm. They have named them atypical teratoid/rhabdoid tumours (AT/RTs) and emphasise their poor prognosis and association with monosomy 22. It has been argued that most medulloblastoma biopsies with monosomy 22 have been obtained from patients who were diagnosed within the first 2 years of life, raising the possibility that some cases have been misdiagnosed and are in fact atypical rhabdoid tumours. One recent study states that germ-line and somatic mutations of *hSNF5/INI1* have been reported in AT/RTs of the brain, consistent with its role as a tumour suppressor gene (Biegel et al., 2000). These authors determined the frequency of deletions and mutations of *INI1* in fifty two children whose original diagnosis was medulloblastoma or PNET of the CNS. Mutations were detected in DNA isolated from four tumours, all from children less than 3 years of age at diagnosis. Two of the four were reviewed and reclassified as atypical teratoid tumour. The relatively low frequency of mutations, even in a large series of children, suggested that loss of sequences from chromosome 22 and/or mutations of *INI1* do not account for the poor prognosis of children with medulloblastoma/PNET which is generally seen in those less than 3 years of age (Biegel et al., 2000). Nevertheless, the authors assert that chromosome 22 deletion and *INI1*-mutation analysis of infants with medulloblastoma/PNET should be considered for all children who are less than 1 year of age since detection of these mutations suggests that the child has an AT/RT rather than a medulloblastoma/PNET, a finding with important prognostic value.

In the present study, five of the samples showing monosomy 22 were taken from children over the age of $\geq$ 3 years old (range 3 - 9 years) so, it seems unlikely that these are AT/RTs which has been misdiagnosed. However, the one remaining sample (IN2912) came from a child of only 3 months old at initial presentation. Whilst, it is possible that this tumour is actually a misdiagnosed AT/RT, the length of survival for the patient was 54

months so it makes it unlikely, since AT/RT patients generally have a much worse prognosis than this (Rorke et al., 1995). Therefore, it seems likely that there can be substantial loss of sequences of chromosome 22 in medulloblastoma independent of the possibility that the tumours are actually misdiagnosed AT/RTs. Moreover, loss of chromosome 22 did not signify a worse prognosis in this study using either the LOH or the CGH analyses. It seems clear that more work needs to be conducted looking at the link between loss of chromosome 22 and its implications for children >3 years of age, since the focus has so far been on those ≤ 3 years of age.

The involvement of other genes on chromosome 22

It can be argued from the report cited above (Biegel et al., 2000) that *ISI1* mutations may play a role in medulloblastoma patients <1 year of age if the diagnosis is correct. The results of the present study also suggest that a putative tumour suppressor gene plays a role in those medulloblastomas with LOH or monosomy 22. Indeed, the pattern of results from the LOH analysis of chromosome 22q undertaken here suggests that such a gene may be located at the 22q11.2-22q12.13 region as of the twelve tumours with LOH, eleven (91%) of informative samples showed LOH at the two markers flanking this region. It is clear that further studies need to be undertaken in order to elucidate the true role of chromosome 22 abnormalities in medulloblastoma development.

Prognostic implications of 22q loss

Although loss was seen in at least 50% of tumours with both the CGH and LOH techniques there was no significant difference in survival between those who had 22q loss and those who did not. Furthermore, LOH at any particular microsatellite marker locus (whether defined by the 0-0.5 or the 0-0.75 ratio) did not significantly correlate with length

of survival. When looking at age (≤ 3 years of age and > 3 years of age) versus losses seen by CGH, there was no significant association. However, when looking at age versus LOH at each marker locus, it was found that at D22S301, mapping to 22q12.13, the >3 years age group had a significantly lower frequency of LOH. Since in the study as a whole those patients >3 years of age had a significantly worse prognosis, this finding suggests that preservation of the 22q11.2 locus may be a contributory factor.

CHROMOSOME 1

Loss of 1p and gain of 1q

In the present study, deletions of distal 1p were found in 11 of 13 (85%) cases analysed using CGH. Although CGH artefact is often manifested as telomeric 1p deletions, the regions of loss found appear to be too large to be the result of artefact alone. Abnormalities of chromosome 1 are common in solid tumours but they are not considered to be associated with any particular tumour type (Berger et al., 1985). Allelic loss of this chromosome has been associated with several human malignancies including glioma (Kraus et al., 1995), melanoma (Dracopoli et al., 1989), male germ cell tumours (Mathew et al., 1994) and carcinomas of the liver (Yeh et al., 1994) and pancreas (Ding et al., 1992). There is evidence that several distinct tumour suppressor genes on chromosome 1 are involved in the pathogenesis of these tumour types. Chromosome 1 has the highest frequency of structural abnormalities found in medulloblastomas (Bigner et al., 1997; Griffin et al., 1988; Bigner et al., 1988; Biegel et al., 1989; Fujii et al., 1994). These changes are usually deletions or unbalanced translocations involving 1p. Instead of 1p being lost however, the abnormal chromosome 1 is usually represented as an extra copy so that there is a net gain of 1q. Indeed, in the present study, four tumours with loss of 1p also showed gain of 1q (IN2636, IN2765, IN2805 and IN2832). The observation that loss of 1p is uncommon in

medulloblastomas is supported by the infrequent finding of LOH for 1p in allelotyping studies (Raffel et al., 1990; James et al., 1990; Bello et al., 1995; Blaeker et al., 1996).

Similarities with neuroblastomas and P73

Medulloblastomas bear some resemblance to neuroblastomas in that members of the *MYCN* gene family are amplified in these tumours also, and some of them show i(17q) (Bigner et al., 1990) The CGH results presented here reveal another similarity in that monosomy for portions of 1p were found and this frequently occurs in neuroblastoma (Biegel et al., 1990). Loss of 1p36 is seen in neuroblastoma, indicating a possible neuroblastoma tumour suppressor gene at this locus. A previous study investigated LOH of this area in medulloblastoma and failed to find any loss (Kraus et al., 1996) whereas all 11 tumours showing 1p loss in the present study had loss at the 1p36 region. Loss of 1p36 and amplification of the *MYCN* oncogene are associated with a poor prognosis in neuroblastoma. None of the tumours in this study showed amplication at the *MYCN* locus. Nevertheless, the striking extent to which 1p36 loss was seen suggests that there may be alterations of the putative neuroblastoma tumour suppressor gene at this locus in medulloblastomas. Recently, the *P73* gene was mapped to 1p36 and its protein is known to share considerable homology with p53. It is thus possible that *P73* disregulation may contribute to the tumourigenesis of neuroblastomas (Jost et al., 1997;Kaghad et al., 1997), and, considering the results from the present study, to the development of medulloblastomas too. However, it must be said that no clear evidence supporting the importance of the loss of this gene has been published.

CHROMOSOME 7

Gain of 7q

An extra copy of chromosome 7, often seen in tumours with an i(17q) has been observed to be the second most common cytogenetic abnormality in medulloblastoma (Battacharjee et al., 1997; Biegel et al., 1989; Bigner et al., 1997; Griffen et al., 1988). In the present study, the CGH analysis shows that it is possible that one tumour (IN2741) gained a copy of chromosome 7 but there was no corresponding gain of material on 17q. Three other tumours (IN2659, IN2823 and IN2933) showed areas of gain on 7q alone. This concurs with findings of a previous CGH study in which gain of 7q was observed in 3 of 18 cases (16%) (Schütz et al., 1996). In the Reardon et al. (1997) study, gain of 7q was observed in a much higher incidence in 12 of 27 (44%) of samples. As trisomy 7 is seen in association with other structural chromosome abnormalities in medulloblastoma, it has been suggested that it is a secondary change and not an initiating event (Biegel, 1999).

Lack of amplification at EGFR (7p12)

Epidermal growth factor receptor (EGFR) amplification has previously been detected in medulloblastoma cell lines and a small number of primary tumours (Batra et al., 1995), but the biological and prognostic significance of this feature has yet to be established. In the present study, none of the tumours studied by CGH, showed amplification in the region of the EGFR locus at 7p12. None of the published studies have shown that EGFR plays a significant role in the genesis of medulloblastoma and it has been stated that it cannot serve as a marker for medulloblastoma to predict either behaviour or outcome (Goumnerova. 1996). The results of the present study add support to this argument.

CHROMOSOME 8

Changes at the *MYCC* locus

No tumour showed gain or amplification of at the site of the *MYCC* protooncogene (8q24). Two tumours, IN2741 and IN2894 demonstrated a deletion at this locus, and in fact the latter tumour displayed monosomy of chromosome 8. These findings support the assertions in the literature which has reported that such amplification of this gene is not, or only rarely, found in primary medulloblastomas (Batra et al., 1995; Biegel et al., 1995).

Involvement of *MYCC*

Amplification of the *MYCC* protooncogene may be characteristic of a subgroup of medulloblastomas with a more aggressive behaviour since two studies have reported a case of amplification of MYCC in a tumour with an usually swift and aggressive progression of the disease (Tomlinson et al., 1994; Jay et al., 1999). A study by Scheurlen et al. (1998) found *MYCC* amplification in 8 of 30 (27%) of medulloblastomas as detected by Southern blotting or FISH. All of the tumours with *MYCC* amplification displayed LOH of chromosome 17p. In the present study, amplification at the *MYCC* locus was not shown. This finding concurs with that found by Batra et al. (1995) who reported such amplification in only 6.9% of tumours studied.

In the Scheurlen et al. (1998) study, the authors report that their comparative data on LOH of chromosome 17p and amplification of the *MYCC* gene show that LOH 17p alone is not a marker of poor prognosis and that only if this abnormality was seen in conjunction with *MYCC* amplification did tumours have a significantly worse outcome. However, the results of the present study and that of Batra et al. (1995) show that as MYCC

amplification is often found in such a small subgroup of tumours, if at all, it cannot viably be used for statistical analysis of prognosis. Moreover, the Batra et al. (1995) study found that LOH 17p alone was a useful prognostic marker.

CHROMOSOME 11

Abnormalities of chromosome 11 are observed in many solid tumours (Berger et al., 1985). The CGH results from the present work show deletions in 5 of 15 (38%) of tumours, whilst, conversely, DNA gain on both 11p and 11q was observed in 1 of 15 (6%) cases. Loss of 11q was seen in 2 of 5 of the tumours with deletions, of 11p in 2 of 5 and of both arms in the remaining case.

Loss of 11p and a region of overlap

Although occasional examples of 11p and 11q deletions have been reported from cytogenetic studies of other brain tumour types (Griffin et al., 1988; Bigner et al., 1988; Chadduck et al., 1992; Vagner-Capodano et al., 1992), it is more common to find loss of the entire copy of chromosome 11, by karyotypic analysis (Karnes et al., 1992; Neumann et al., 1993). Involvement of 11p in medulloblastoma was first demonstrated by James et al. (1990) who saw loss of this chromosome arm in 3 of 11 (27%) tumours analysed. A study conducted four years ago found similar results in that loss of 11p was observed in 2 of 6 (30%), medulloblastomas. Moreover, loss of 11q was also seen in 2 of 6 (30%) and loss of both arms in 1 of 6 (16%) (Blaeker et al., 1996). In this study, for the losses on 11p, there was a region of overlap at 11p15. Similarly, in the present study, a total of 3 of 13 (23%) of tumours showed LOH at 11p, at a comparable frequency and, moreover, all three tumours showed loss at 11p15. However, one LOH study of sixteen

medulloblastoma specimens showed no loss of 11p and state particularly that the 11p15 region is unlikely to be involved in medulloblastoma development (Albrecht et al., 1994).

It is clear that further studies need to be carried out in order to elucidate whether chromosome 11 plays a role in medulloblastoma development and what that role is, particularly when one considers that, in contrast to previous reports indicating loss of this chromosome in this type of tumour, gain of a substantial part of this chromosome was seen in 1 of 13 (7.5%) cases (IN2765) in the present investigation.

CHROMOSOME 17

Previous reports cited in this study have repeatedly shown evidence that 17p loss is the most common chromosomal abnormality found in medulloblastomas. Thus, a comprehensive microsatellite study was undertaken, using twenty two markers spanning 17p13.1-17p13.3. Loss of heterozygosity was seen in 100% (27 of 27) of samples over a wide range, and allelic imbalance was also seen in 100% of cases. The CGH analysis revealed loss in 60% (9 of 15) of cases, and in most of these, it encompassed the entire 17p arm. This finding supports the conclusion that future deletion mapping experiments should focus on the whole of 17p and not just the p13.1-p13.3 area.

Lack of i(17q) and loss of 17q

As stated above, i(17q) has been shown to be a consistent anomaly in medulloblastomas, seen in 30-60% of cases (Bigner et al., 1988). However, in the current investigation, CGH analysis did not show evidence of i(17q) in a single case (0/13). Gain of genetic material on 17q was shown in only one tumour (IN2659) and was restricted to the 17q22 region. Interestingly, loss of 17q was seen in 61% of cases (8 of 13), a frequency almost

as high as 17p loss. This may not be as unusual as it at first appears as loss of 17q has been observed previously (Vagner-Capodano et al., 1992). In this study, loss of this chromosome arm was seen in 3 of 14 (21%) of cases, and these three tumours were actually monosomic for chromosome 17. In the current study, monosomy 17 was also seen - in one tumour (IN2765). These data strengthen the argument that a tumour suppressor gene is localised on the short arm of chromosome 17 and refute the hypothesis of a gene on 17q which is overexpressed in i(17q) and which is important in medulloblastoma pathogenesis.

CHROMOSOME 19

The CGH study showed loss of material from chromosome 19 in 9 of 15 cases (60%). Moreover, monosomy was seen in two thirds of these (6 of 9). Although CGH artefact is often found on chromosome 19 (Kallioniemi et al., 1994), the frequency and extent of the deletions suggest that the changes seen in this study are genuine. Whilst deletions of chromosome 19 are not common in medulloblastoma, they are seen frequently in other brain tumour types, for example in malignant gliomas (Rosenberg et al., 1996). Furthermore, in an investigation of a series of twenty five oligodendroglial tumours, Bello et al., (1995) found LOH 19q in nineteen samples (76%). The authors argued that inactivation of a tumour suppressor gene located on 19q represents an alteration occurring in the oncogenic transformation of oligodendroglial neoplasms. Whilst it is too early to say whether a putative tumour suppressor gene which plays a role in medulloblastoma pathogenesis is located on chromosome 19, the results from the present study suggest that it is an interesting region for future microsatellite investigations.

CHROMOSOME 20

Loss of material from chromosome 20 was observed in 9 of 15 cases (60%), with 4 of 9 showing monosomy. These results are surprising in the light of the fact that deletions of this chromosome has not previously been associated with medulloblastomas. However, 20q displays recurrent LOH in blast cells from children with acute lymphoblastic leukaemia (Couque et al., 1999). Indeed, a deletion of 20q is a recurring abnormality in a wide spectrum of myeloid disorders possibly through a tumour suppressor gene localised to 20q12 (Wang et al., 1998). As with chromosome 19, the frequency of deletions seen on chromosome 20 in this study are indicative of putative tumour suppressor genes which may play a role in medulloblastoma development.

GAIN OF CHROMOSOME 2q

Regions of gain on this chromosome arm were seen at a very high freqency, with 11 of 15 cases (73%) showing gain. This is surprising in that such a finding has not previously been reported in medulloblastoma. In cytogenetic analyses investigating other tumour types however, gain of 2q has been found. In a study of ten hepatoblastoma samples, Hu et al. (2000) found regions of high level gain at 2q24. In the present study, gains at this locus were observed in seven tumours. Moreover, in a group of inherited ovarian carcinomas, Tapper et al., (1998) found frequent gain at 2q24-32. As well as the seven tumours with gain at 2q24 already described in the present study, three tumours (IN2832, IN2588 and IN2765) showed gain of the entire 2q24-32 region. A specific amplified gene has yet to be identified at the 2q24 region (Hu et al., 2000) but the results presented suggest that it be used as a target for future studies.

GAIN OF CHROMOSOME 3

Gain of this chromosome was seen in 11 of 15 cases (73%), and in tumour IN2933 a high copy number amplification was found at 3q24-26. This is a novel finding in medulloblastoma, although high copy number amplification of 3q has been found in squamous cell carcinoma of the head and neck (Bergamo et al., 2000). High copy number gains have also been found in 4 of 17 (24%) adenocarcinoma samples at the 3q22-ter region in adenocarcinomas (Bjorkqvist et al., 1998). Thus, amplification of genes on 3q may be important in the tumourigenesis of some types of cancer including medulloblastoma.

Gain of chromosome 4

Only one case in the CGH study (IN2266) showed no gain of material somewhere on chromosome 4. Of the remaining fourteen cases, seven (50%) demonstrated genetic gain on both chromosome arms to varying extents and two (IN2805 and IN2894) are probably trisomic for chromosome 4. One previous CGH study (Schutz et al., 1996), detected gains on this chromosome in 3 of 18 (17%) cases, one of which was also showed trisomy. Imbalances such as these have never before been found in medulloblastoma and suggest new candidate regions for oncogenes.

GAIN OF CHROMOSOME 5q AND 6q

Gain of 5q was observed in 11 of 15 cases (73%). Moreover, all eleven cases showed gain in the 5q21 region. Only one other study of medulloblastoma has showed gain of 5q previously, in which it was found at the terminal region only (Schütz et al., 1996). Similarly, gain of chromosome 6q was seen in 10 of 15 cases (73%), all of which showed

gain of the 6q12-16 region. This is a novel finding in medulloblastoma. Furthermore, as far as the author is aware, no candidate oncogenes have been postulated for 5q or 6q.

GAIN OF CHROMOSOME 13q

Gain of 13q was found in 11 of 15 cases (73%) and high copy number amplification was seen at 13q21-31 in tumours IN2894 and IN2805. Although these changes may be due to CGH artefact, since gains of 13q have also been seen in high frequencies in paediatric astrocytomas and ependymomas by CGH analysis (T. Warr, personal communication), the fact that high copy number amplification was seen in two tumours makes this highly unlikely. Rather, it is possible that 13q harbours a putative oncogene which is transformed in a variety of paediatric brain tumours. Copy number gains of this chromosome arm have been seen in CGH studies of breast cancer (Loveday et al., 2000), Burkitt's lymphoma (Zunino et al., 2000) and prostate cancer (Sattler et al., 1999). These results add support to the hypothesis that 13q harbours a gene whose oncogenic transformation leads to a variety of human cancers including medulloblastoma.

OTHER CLINICAL CORRELATIONS (treatment v survival, age v survival)

Of the 25 cases with follow up information in which it was possible to assess survival, analysis was conducted to ascertain as to whether prognosis was influenced by the type of therapy patients received. Seven patients had surgery followed by radiotherapy only, three patients had surgery followed by chemotherapy only, whilst the remaining fifteen patients had both radiotherapy and chemotherapy following surgery. The different treatment regimens were found to have no significant effect on survival times. These findings contradict previous evidence which states that survival rates are improved with a regimen of

surgery followed by both radiotherapy and chemotherapy (Packer et al., 1999). However the numbers used in the present study are too small to draw any firm conclusions.

Patients < 3 years of age were found to have a significantly better prognosis than those >3 years of age. This is in contrast to previous studies in which an age of < 3 years is associated with a worse prognosis (Kopselson et al., 1983) since such patients may have biologically more aggressive lesions (Allen and Epstein, 1982). However, another study found that infant (those aged 0-24 months) survival was improved compared to other age groups over the first eight years of follow up (Belza et al., 1991). The follow up times in the present study are very short though and it is impossible to say whether a younger age would signify a better prognosis in a long range study.

CONSIDERATION OF EXPERIMENTAL STRATEGIES

The importance of densitometry analysis

With regard to the findings of the microsatellite analysis in the present research, it can be seen that the frequency of LOH is much higher and more widespread than that found in the past. This may be explained in part by the use of densitometry to ascertain LOH and allelic imbalance in that this analytic tool may be far more accurate than looking for evidence of LOH 'by eye'- a method which is often used (eg. Batra et al., 1995). In this study, LOH was scored if one of the two alleles in the tumour DNA was either absent or reduced in intensity by 80% as compared with normal DNA. In the present study, when the results of the densitometry analyses were compared with the raw data on the polyacrylamide gels (i.e. the allelic bands) it was revealed that on numerous occasions that even when the intensity of the tumour and normal alleles appeared to be identical looking with the naked eye, the sensitivity of the densitometry detected a significant reduction in intensity and LOH or

allelic imbalance was scored. Therefore, in other studies where LOH is scored 'by eye' there may actually be an under representation of the degree of loss.

The risk of false positive results

A major problem with the technique is that it has no standardised evaluation. Efforts to establish improved CGH data analysis procedures applying statistical concepts to the definition of diagnostic thresholds have revolved around two different methods. The first is that which was used in the present study, whereby upper and lower threshold values are established (in this case 1± 0.2) for the identification of chromosomal imbalances of the given ratio profiles, whilst the second method is based on a linear regression model with fixed and random effects (Laird et al., 1982). Simultaneous 99% confidence interval (CI) estimation of CGH ratios is included to identify relevant gains and losses which are defined by CIs that do not include a ratio of 1.0 (Reardon et al., 1997).

A recent study (Barth et al., 2000) compared the two approaches by using them to analyse a large series of B-cell lymphomas. They termed the first approach described as the fixed threshold procedure (FTP), whilst the second was referred to as the data-driven procedure (DDP). Fluorescence in situ hybridisation (FISH), with probes selected from the respective regions, was also used in the study in order to allow the independent assessment of chromosomal copy numbers. It was found that with the DDP a much higher fraction (42/218 vs. 8/218) of false positive results was obtained. Based on this, the authors recommend the use of fixed diagnostic thresholds as the alternative procedure generates an unacceptably high proportion of incorrectly scored chromosomal imbalances. Thus, even though a large number of chromosomal imbalances were found in the present study, the fact that fixed diagnostic thresholds were used means that the likelihood of any of these imbalances being false positive results is greatly diminished.

Peri-centromeric and heterochromatic repeat regions cannot be reliably evaluated by CGH as these areas are blocked to varying degrees in different chromosomes by the unlabelled Cot-1 DNA in the hybridisation, so ratio changes at or near these regions have been interpreted cautiously in the present study, and have not been included as valid if the change is confined to these regions only in a chromosome and is not part of a larger region of loss or gain.

Similarly, since the green and red fluorescence intensities gradually decrease at the chromosome telomeres and unreliable ratio changes may appear (Kallioniemi et al., 1994), again, in the present study, changes in ratio at chromosome telomeres have been interpreted cautiously and have not been included as valid if the change is confined to the telomeres only in a chromosome and is not part of a larger region of loss or gain.

Evidence has shown that the green to red fluorescence ratios at 1p32-pter, 16p, 19 and 22 may occasionally be substantially below the average ratio (Kallioniemi et al., 1994). The consequence would be a false positive interpretation of a deletion. These considerations were borne in mind when interpreting the results in the present study.

The use of two different experimental techniques

CGH is currently the optimal method to determine sites of genetic gains in genome-wide screens of medulloblastoma although the sensitivity of the technique is limited. For example deletions within a chromosomal band may not be detected. Thus, in the present study microsatellite analysis has been used to confirm genetic losses seen by CGH since this is a much more sensitive technique and can detect more discreet regions of loss (Weiss

et al., 1999). Using two methods also reduces scoring artefactual deletions asbtrue positives.

CONCLUSIONS

The microsatellite analysis has contributed to the deletion map of chromosome 17p. The results indicate that there are three putative tumour suppressor genes at independent loci on 17p13.1 distal to the p53 gene. The CGH analysis confirmed the extent of deletion found by the microsatellite analysis and located other consistent regions of loss on chromosomes 9q, 10q, 12q, 16q and 22q. Through subsequent microsatellite analysis of these areas, minimum regions of deletion were defined on each chromosome in which putative tumour suppressor genes may be located. The CGH analysis also detected consistent regions of gain on chromosomes 2q, 3, 4q, 5q, 6q and 13q, many of which are non-random novel genetic changes in medulloblastoma. Data was correlated with clinical parameters and it was found that loss of 16q, as detected by CGH, was associated with a longer survival. Furthermore, loss at the 16q marker D16S512 as found by microsatellite analysis was also associated with a more favourable prognosis. In contrast to previous reports, age ≤ 3 years was associated with a longer survival, although distribution of LOH and changes by CGH were not related to age, except with one marker, D22S301 on chromosome 22 where a lower frequency of LOH was observed in samples from patients > 3 years of age. Treatment received by the patients was not associated with survival. However, the small number of patients used in this study, the significant male:female bias of the sample group, the uncertainty as to whether this group is representative of medulloblastomas as a whole and the short follow up periods in most cases makes it impossible to draw any firm conclusions.

Suggestions for future work

The data presented here, along with those from the four previous studies conducted on medulloblastomas using CGH cited (i.e. Reardon et al. 1997; Schultz et al. 1996; Avet-Loiseau et al., 1999; Nishizake et al., 1999) provide significant evidence that the genetic pathogenesis of medulloblastoma is much more complex than originally thought, and that genes on other regions as well as those on 17p may be equally as important. Several novel consistent losses and gains have been identified which suggest candidate areas for oncogenes or tumour suppressor genes. Thus, the next stage would be to use mapping techniques in order to characterise these critical areas and identify minimum regions of gain and loss from which novel genes can be isolated.

A more extensive number of samples should be used in order to ensure validity of the results already obtained, thus the sample group should be added to and include more fresh biopsy and paired blood samples as they are received. In samples where the amount of DNA extracted is insufficient to conduct CGH, degenerative oligonucleotide primer (DOP) PCR could be used to amplify small, or even trace, amounts of DNA . DOP-PCR allows random, uniform amplification of DNA templates from any source. A low hybridisation temperature of 30^0C allows nonspecific binding of the primer to the DNA template which is amplified nonspecifically during the initial 5 PCR cycles. The PCR products carry the universal primer sequence at their termini, allowing specific amplification during the subsequent 35 cycles.

Subsequent to the CGH experiments, it seems prudent to screen the most consistent novel chromosomal areas of interest with appropriate polymorphic microsatellite markers in order to characterise the region of gain and/or deletion. Areas of loss and gain have already been localised from the CGH experiments conducted and further CGH experiments may

elucidate more such regions, but the resolution obtained with microsatellite markers is much greater and will allow the identification of each of the minimal overlapping critical regions.

An increase in DNA copy number could be determined using quantitative differential PCR (Reifenburger et al. 1993). With this technique, PCR amplification of tumour and paired normal somatic DNA with oligonucleotide primers specific for polymorphic microsatellite loci is conducted in the presence of a fluorescent-labelled nucleotide. After subsequent polyacrylamide electrophoresis, the resulting bands would be examined for copy number using FluorImager gel analysis.

Allelic loss could be determined using LOH analysis in same way as it was in the present work. Evidence may be found to implicate the genes on chromosome 17p described above (i.e. *ABR,* and *HIC-1*) as well as *PTC* (on distal 9q) and *MXI1* (on distal 10q). As described above, allelic loss and mutations of *PTC* have been reported in up to 20% of sporadic medulloblastomas (Raffel et al., 1997), but the CGH studies conducted for the present work revealed a much higher frequency of loss on 9q22-ter. Future studies could assess the mutation status of *PTC* in order to determine whether it is the target of the distal 9q deletions seen here or whether another as yet unidentified gene is involved. The status of *MXI1* could be evaluated in the same fashion to assess whether this gene is the target for the distal 10q deletions.

After assessing allelic loss by amplification of microsatellite markers from within the gene sequence, SSCP analysis could be used to screen each exon of the candidate genes for mutations. Exons which show abnormal motility after amplification of tumour and paired normal DNA and subsequent polyacrylamide electrophoresis could then be sequenced to determine base changes.

The ultimate conclusion of this work would be the isolation and characterisation of novel genes from candidate regions. After defining minimal critical areas using the techniques described above, YAC clones mapping to these regions could be isolated and the YAC inserts subcloned into cosmid vectors and subsequently used for 3'-terminal exon trapping. After amplifying exons via nested PCR, products could then be subcloned and the subclones sequenced (Krizman and Berget, 1993). This approach might be particularly interesting to use at the region of gain on 9p21-ter which was observed in 53% of tumours in this study by CGH and which showed high copy number amplification in 2 cases. Additional regions regions could then be chosen on the basis of the results of the mapping studies.

It would also be interesting to identify genetic markers which can be used as prognostic indicators in medulloblastoma. The association between both loss of 16q as found by CGH and LOH of the 16q marker D16S512 (mapping to 16q22.1) and better prognosis found in this study would provide a good starting point for the determination of the correlation between clinical parameters such as length of relapse free interval and survival, as well as the subsequent use of the other consistent genetic gains and losses detected in each tumour in the initial mapping studies. Hopefully this would ultimately lead to the identification of new molecular genetic markers which could be used in prognosis. By putting the suggestions above into practice it is hoped that future research will contribute a large piece to the puzzle of the genetic basis of medulloblastomas.

Summary of previous findings	Summary of findings of the present study and how they add to those from previous research
1. A much younger age (<3 years) is associated with a worse prognosis (Allen & Epstein, 1982)	1. The opposite was found; age ≤ 3 yrs was significantly associated with a worse prognosis.
2. Desmoplastic variant is related to a worse prognosis in children (Friedman et al., 1991)	2. No difference in prognosis found with the desmoplastic variant.
3. I(17q) most frequent cytogenetic abnormality found (eg. Bigner et al., 1988)	3. I (17q) not found with any sample.
4.Deletion of 17p13 seen in 30-50% of cases using cytogenetic studies.	4.All tumours studied showed LOH at at least one marker locus in the 17p13.1-17p13.3 region.
5.There is evidence that a putative medulloblastoma tumour suppressor gene is located somewhere between 17p11.2 & 17p13.3	5. The results concurred in that there is evidence that two tumour suppressor genes may be located on 17p13.1 and one on 17p13.3
6.MIN is uncommon in paediatric medulloblastomas (Lescop et al., 1999)	6. The results support this assertion; MIN was found at a low frequency – only 2/27 tumours.
7. The *PTC* gene may be a tumour suppressor gene and it has been mapped to 9q22.3	7.30% of tumours showed loss at the *PTC* locus by LOH but there is evidence that there may be another putative tumour suppressor gene between 9q21.1 & 9q22.3
8.p53 mutations occur in approximately 8% of medulloblastoma	8. Didn't assess p53 status.
9.Loss of 1p is uncommon as shown by LOH studies (eg Raffel et al., 1990).	9. The opposite was found by CGH; 85% of cases showed deletions of distal 1p.
10. An extra copy of chromosome 7 is the second most common cytogenetic abnormality found (eg. Bigner et al., 1997).	10.Only one tumour gained a copy of chromosome 7.
11. Loss of all/part of chromosome 6 seen as a single change in several medulloblastomas (eg. Biegel et al., 1999).	11. Not found. Conversely, gain of 6q was seen in 73% of cases via CGH.
12. Loss of chromosome 22 seen and monosomy with a desmoplastic histology.	12.Loss was seen in 86% of tumours by CGH & 50% by LOH. Five showed monosomy.
13. Loss of 11p, 11q and an entire copy of chromosome 11 has been shown.	13. This was found. 11p loss in 2 cases, 11q loss in 2 cases and loss of 11p and 11q in one case.
14.Amplification of MYCN and MYCC shown.	14. This was not found.
	Novel findings: consistent regions of gain on chromsomes 2q, 3, 4q, 5q, 6q and 13q High copy number amplification of 13q21-31 in 2 tumours. High copy number amplification at 3q24-26 Loss of 16q as found by CGH is associated with longer survival as was LOH 16q at the D16S512 locus.

Table showing a summary of what has been previously found with regard to medulloblastomas and what the results of the present study have contributed.

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APPENDIX I

SUMMARY OF MATERIALS, REAGENTS AND SUPPLIERS THEREOF

MATERIAL/REAGENT	SUPPLIER
Tissue culture	
Ham's F10 nutrient media	Life Technologies, 3 Fountain Drive, Inchinan, Renfrewshire PA9 9RF
Foetal Calf Serum (FCS)	Life Technologies
Hanks Balanced Salt Solution (HBSS)	Life Technologies
Tissue culture flasks	Life Technologies
Trypsin EDTA	Life Technologies
Dimethyl Sulphoxide (DMSO) New	Sigma Aldrich, , The Old Brickyard, Road, Gillingham, Dorset SP8 4XT
Freezing vials	Life Technologies
DNA extraction	
Blood DNA extraction kit contains: genomic tips (used with buffers C1, G2, QBT, QC, QF)	Qiagen, Boundary Court, Gatwick Road, Crawley, West Sussex RH10 2AX
Tissue DNA extraction kit contains: buffers ATL, AL, AW ready made Qiamp spin columns 2ml collection tubes	Qiagen
0.45 micron filters	Scientific Laboratory Supplies, Unit 27, Wilford Estate Nottingham NG117EP

50ml polypropylene screw cap tubes	Falcon, Marathon Lab Supplies, Unit 6 55 Park Royal Road, London NW10 7LP
Proteinase K	ICN Biochemicals, 1 Elmwood, Crockford Lane, Basingstoke, Hampshire RG24 8WG
isopropanol	Sigma Aldrich

Fluorometric quantification of DNA

DyNAQuant 200 fluorometer	Amersham Pharmacia Biotech, Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA
DQ 130 Capillary cuvette kit	Amersham Pharmacia Biotech

Microsatellite analysis

Polymorphic microsatellite markers	Amersham Pharmacia Biotech
Taq Polymerase	Amersham Pharmacia Biotech
PCR Buffer	Amersham Pharmacia Biotech
deoxyguanosine triphosphate	Amersham Pharmacia Biotech
deoxyadenosine triphosphate	Amersham Pharmacia Biotech
deoxythymidine triphosphate	Amersham Pharmacia Biotech
deoxycytidine triphosphate	Amersham Pharmacia Biotech
Thermal Cycler	Techne, Duxford, Cambridge CB24PZ
Agarose	Bio-Rad Labs Ltd, Bio-Rad House, Maylands Avenue, Hemel Hempstead, Herts HP2 7TD
ethidium bromide	Sigma Aldrich
loading dye	MBI, Helena Biosciences, Colima Avenue Sunderland, Tyne and Wear SR5

GeneRuler	MBI, Helena Biosciences
GenePhor Electrophoresis Unit	Amersham Pharmacia Biotech
GenePhor Excel 15/24 kit contains: rehydration buffer (0.001% bromophenol blue and 0.001% Orange G buffer strips polyacrylamide (T = 15%, C = 2%) gels electrode buffer	Amersham Pharmacia Biotech
Mineral oil (insulating fluid)	Sigma Aldrich
loading dye	MBI Helena Biosciences
PlusOne DNA silver staining kit contains: fixing solution staining solution developing solution stopping and preserving solution	Amersham Pharmacia Biotech

Data analysis

Scan-It gel scanning software	Silk Scientific Corporation
Flatbed Scanner	Epson

Comparative Genomic Hybridisation

Formamide	Sigma Aldrich
NP-40	Sigma Aldrich
2mM SpectrumGreen dUTP	Vysis, Rosedale House, Rosedale Road, Richmond, Surrey TW9 2SW
0.3mM dTTP	Vysis
0.3mM dATP	Vysis
0.3mM dGTP	Vysis
0.3mM dCTP	Vysis
Nick translation kit contains: nuclease free water 10X nick translation buffer nick translation enzyme	Vysis
DNA size marker: lamda DNA/Eco 911	MBI, Helena Biosciences

normal metaphase CGH target slides	Vysis
SpectrumRed total genomic reference DNA	Vysis
Human COT-1 DNA	Vysis
DAPI II	Vysis
QUIPS CGH Analysis system	Vysis

DETAILS OF PREPARATION OF BUFFERS AND SOLUTIONS

PREPARATION OF BUFFERS FOR BLOOD DNA ISOLATION AND CULTURED CELLS (Qiagen)

C1 109.54 g saccharose (sucrose), 1.02g $MgCl_2x6H_2O$ and 1.211 g Tris base were dissolved in 800 ml dH_2O and 100 ml 10% Triton X-100 was added subsequently. The pH was adjusted to 7.5 with HCl.

G2 76.42 g GuHCl, 11.17g NA_2-EDTA$x2H_2O$, and 3.633 g Tris base were dissolved in 600 ml dH_2O followed by the addition of 250ml 20% Tween-20 and 50 ml 10% Triton X-100. The pH was adjusted to 8.0 with HCl.

QBT 43.83 g NaCl and 10.46 g MOPS (free acid) were dissolved in 800 ml dH_2O and the pH adjusted to 7.0 with NaOH. Subsequently, 150ml pure ethanol and 15 ml 10% Triton X- 100 solution were added.

QC 58.44 g NaCl and 10.46 g MOPS (free acid) were dissolved in 800 ml dH_2O and the pH adjusted to 7.0 with NaOH before 150 ml of pure ethanol was added.

QF 73.05 g NaCl and 6.055 g Tris base was dissolved in 800 ml dH_2O before the pH was adjusted to 8.5 with HCl and 150 ml of pure ethanol added.

TE (buffer in which to keep the extracted DNA):

10 mM Tris Cl (pH 7.4) was added to 1 mM EDTA (pH 8.0) and dissolved in 800 ml dH2O before the pH was adjusted to 7.4 with HCl.

For extraction from cultured cells only:

Phosphate-Buffered Saline (PBS): 8 g NaCl, 0.2 g KCL, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 was dissolved in 800 ml dH_20 before the pH was adjusted to 7.4 with HCl.

All solutions were allowed to cool to room temperature before the final adjustment of the pH, and were made up to a final volume of 1 litre with dH_20. Buffers were stored at room temperature with the exception of C1 which was stored at 4^0C. All buffers were filter sterilised through 0.45 micron filters (Scientific Laboratory Supplies) before use.

BUFFERS USED IN THE PROCEDURE OF ISOLATION OF GENOMIC DNA FROM TISSUE

Buffers ATL, AL, and AW were supplied ready made as part of a Qiagen tissue kit.

BUFFERS AND SOLUTIONS USED IN THE FLUOROMETRIC QUANTIFICATION OF DNA

Hoechst 33258 stock dye solution (1mg/ml) 10 mg of Hoechst 33258 was added to 10 ml distilled filtered water

10X TNE buffer stock solution (1000 ml) 12.11 g Tris base [Tris (hydroxymethyl) aminomethane] (MW = 121.14), 3.72g EDTA, disodium salt, dihydrate (MW= 372.20) and 116.89 g Sodium chloride (MW= 58.44) were dissolved into 800 ml of distilled water.

The pH was adjusted to 7.4 with concentrated HCl and distilled water was added to make a final volume of 1000 ml. The buffer was filter sterilised through 0.45 micron filters (Scientific Laboratory Supplies) before use.

Calf thymus DNA (1:10 dilution of standard stock, 200 µg/ml, for low range assay) 200 µl calf thymus DNA standard, 1 mg/ml and 100 µl 10X TNE was added to 800 µl distilled filtered water.

2X Capillary assay solution (CAS)

(a) Low range (up to 10 ng/ml final DNA concentration)

2 µg/ml H 33258 in 1X TNE (0.2 M NaCl, 10 mM Tris-Cl, 1 mM EDTA, pH 7.4)

2 µl H 33258 stock solution and 100µl 10X TNE buffer was added to 898 µl distilled filtered water

(b) High range (10 to 100 ng/µl final DNA concentration)

20 µg/ml H 33258 in 1X TNE (0.2 mM NaCl, 10 mM Tris-Cl, 1 mM EDTA, pH 7.4)

20 µl H 33258 stock solution and 100 µl 10X TNE buffer were added to 880 µl distilled filtered water.

For the sample:

All solutions were diluted 1:1 with 2X CAS.

Blank 1X TNE buffer was added to 2X CAS.

Standard: 200 ng/ml DNA was added to 2X CAS (for final concentration of 100 ng/µl).

Sample: 2 µl sample was added to 2X CAS.

PREPARATION OF TRIS-ACETATE (TAE) BUFFER FOR USE IN AGAROSE GELS

1 X 0.04 M Tris-acetate and 0.0001 M EDTA were dissolved in 800 mls dH_2O and the pH adjusted to 8.0 with HCl.

DETAILS OF SOLUTIONS USED FROM THE PlusOne SILVER STAINING KIT (Amersham Pharmacia Biotech)

Fixing solution: 25 ml 5X fixing solution (benzene sulphonic acid: 3.0%w/v in 24% v/v ethanol) was added to 100 ml 24% ethanol.

Staining solution: 25 ml 5X staining solution (silver nitrate: 1.0%w/v; benzene sulphonic acid: 0.35%w/v) was added to 100 ml water.

Developing solution: 25 ml 5X sodium carbonate (sodium carbonate: 12.5%w/v), 125 µl formaldehyde (formaldehyde: 37%w/v iwater), 125 µl sodium thiosulphate (sodium thiosulphate: 2%w/v in water) was added to 100 ml water.

Stopping and Preserving solution: 25 ml 5X stopping and preserving solution (acetic acid:5%w/v, sodium acetate: 25%w/v and glycerol: 50%v/v) was added to 100 ml water.

PREPARATION OF REAGENTS USED IN COMPARATIVE GENOMIC HYBRIDISATION

20X SSC, pH 5.3: 66 g 20X SSC was mixed in 200 ml purified dH_20. The pH was adjusted to 5.3 using concentrated HCl, adjusted to a final volume of 250 ml and filtered.

Denaturation solution: 49 mL formamide, 7 ml 20X SSC (pH 5.3) and 14 ml purified dH_2O was mixed together and added to a coplin jar.

Ethanol wash solutions (70%, 85% and 100%): 100% ethanol was diluted with purified dH_2O to prepare the wash solutions.

0.4X SSC/0/3% NP-40 wash solution: 20 ml 20X SSC was mixed with 950 ml purified H_2O and 3 ml NP-40 was added. The pH was adjusted to between 7.0 and 7.5 with NaOH. Purified H_2O was added to bring the final volume to 1 litre.

2X SSC/0.1% NP-40 wash solution:
100 ml 20X SSC was added to 850 mL purified H_2O and 1 ml NP-40 added. The pH was adjusted to between 7.0 and 7.5 with NaOH. Purified H20 was added to bring the final volume to 1 litre.

Nick translation components: 10 µl of 1 mM SpectrumGreen dUTP 50 nmol (Vysis) was added to 40 µl nuclease-free water whilst 10 µl of 0.3 mM dTTP (Vysis) was added to 20 µl nuclease-free water and 10µl each of 0.3 mM dATP, 0.3 mM dCTP and 0.3 mM dGTP (Vysis) were mixed together. A solution of between 0.6 µg/µl and 1 µg/µl solution of extracted genomic tumour DNA in Tris-EDTA (10 mM Tris, 1 mM EDTA,pH 8.5) buffer was prepared.

APPENDIX II: DENSITOMETRY DATA FROM MICROSATELLITE ANALYSIS

		D9S166		D9S287		D9S176	
IN	Normal Tumour	Peak Area	Tumour/Normal Ratio	Peak Area	Tumour/Normal Ratio	Peak Area	Tumour/Normal Ratio
1241	N1	442.783		28720.3			
	N2	773.59		27685.9			
	T1	510.401		24048.1			
	T2	321.985	0.36	25385.2	0.91		
1482	N1	1010.42		4364.85		201.835	
	N2	1634.1		7092.58		430.844	
	T1	1275.63		3446.66		149.039	
	T2	1440.9	0.70	5333.97	0.95	275.899	0.86
1545	N1	787.188		14149		621.752	
	N2	1655.51		23694.9		751.932	
	T1	658.275		2695.31		599.179	
	T2	1796.16	0.77	5892.96	0.77	816.813	0.89
2077	N1	363.47		7820.69			
	N2	1392.41		8114.97			
	T1	1403.79		2745.55			
	T2	5994.75	0.90	4872.86	0.58		
2266	N1	3642.29		72540.7			
	N2	7546.16		116232			
	T1	950.49		-20398			
	T2	5441.78	0.36	39179	-0.83		
2289	N1	866.723		22945		347.882	
	N2	1050.95		22231.5		635.818	
	T1	1067.78		10599.4		606.82	
	T2	540.914	0.42	16937.6	0.61	1594.79	0.70
2465	N1	4533.34		3372.19		496.322	
	N2	294.52		5922.02		434.731	
	T1	5972.93		2113.96		388.641	
	T2	8833.94	0.04	4576.91	0.81	1739.83	0.20
2529	N1	2750.4		10601.7			
	N2	2913.27		15939.6			
	T1	2622		6863.25			
	T2	1494.08	0.53	16531.4	0.62		
2588	N1	5524.55		71073.9		331.739	
	N2	13168.6		68306.2		1545.09	
	T1	6073.82		44987.7		694.651	
	T2	13961.6	0.96	55416.6	0.78	-11.426	0.00
2590	N1	1847.38		3344.47			
	N2	1689.7		10273.7			
	T1	737.625		2145.97			
	T2	685.081	0.98	6231.19	0.95		
2636	N1	6377.49		53410.8			
	N2	20383.1		92415.5			
	T1	3109.73		55999.1			
	T2	11777	0.84	91299.7	0.94		
2637	N1	340.348		2956.65			
	N2	549.546		3871.8			
	T1	445.254		2253.87			
	T2	1813.4	0.40	4673.13	0.63		
2659	N1	735.39		4371.07			
	N2	951.9		3545.4			
	T1	667.35		4676.01			
	T2	1295.34	0.67	5422.27	0.70		
2697	N1	4760.92		7562.98			
	N2	11076.9		9638.39			
	T1	2680.26		5507.4			
	T2	4074.42	0.65	5265.42	0.75		
2715	N1	4431.84		47373.4			
	N2	11141.9		79313.6			
	T1	6543.05		25454.5			
	T2	8163.57	0.50	62598.5	0.68		
2741	N1	463.52		57943.9		-368.401	
	N2	7683.94		104468		2766.57	
	T1	1569.16		55546		-54.364	
	T2	5309.02	0.20	97871.7	0.98	2592.83	0.16
2765	N1	540.17		8842.63			
	N2	506.31		9193.5			
	T1	1376.19		13884.1			
	T2	2047.15	0.63	16855.8	0.86		
2805	N1	3244.94		40359.1		-389.446	
	N2	4617.06		106860		301.869	
	T1	3127.98		4460.74		-232.672	
	T2	7160.05	0.62	58766.3	0.20	5316.97	0.03
2823	N1	7671.43		36048.8		1832.46	
	N2	10038.8		44023.7		1425.8	
	T1	6697.27		43622		570.568	
	T2	14847.3	0.59	57727	0.92	549.818	0.81
2832	N1	1195.3		15541.4			
	N2	2542.03		20756.9			
	T1	4442.03		10790.2			
	T2	16020.8	0.59	18891	0.76		
2894	N1	634.7		11099.1			
	N2	897.52		13234.3			
	T1	118.74		21455.1			
	T2	640.63	0.26	24630.8	0.96		
2901	N1	1322.86		19973.1			
	N2	1683.72		42390.2			
	T1	1006.29		30437.7			
	T2	1773.34	0.72	69462.6	0.93		
2912	N1	1453.23		24867.4			
	N2	4045.43		25228.1			
	T1	1828.61		28035.7			
	T2	3046.72	0.60	23416.1	0.82		
2917	N1			2371.17			
	N2			6407.32			
	T1			1228.53			
	T2			2998.38	0.90		
2920	N1	561.487		9652.79			
	N2	954.143		15928.5			
	T1	698.983		8716.8			
	T2	1080.51	0.91	20168.9	0.71		
2933	N1	8975.3		31337		2046.48	
	N2	48930.1		39497.3		1903.53	
	T1	1839.03		44794.4		1516.78	
	T2	3428.32	0.34	84009.7	0.67	1834.83	0.77

		D9S170		D10S581		D10S580	
IN	Normal Tumour	Peak Area	Tumour/normal Ratio	Peak Area	Tumour/Normal Ratio	Peak Area	Tumour/Normal ratio
1241	N1	11139.9		1087.28			
	N2	14736.4		1000.51			
	T1	6220.45		861.412			
	T2	10044.1	0.82	770.328	0.97		
1482	N1	9461.98		1178.93		226.649	
	N2	11278.7		1075.92		685.479	
	T1	13247		1033.71		170.826	
	T2	9304.08	0.59	2542.02	0.37	491.795	0.95
1545	N1	9265.95		1693.04			
	N2	1800.5		16657			
	T1	5194.17		1754.23			
	T2	6417.47	0.97	1119.5	0.65		
2077	N1	24136.8		746.69			
	N2	32873.8		6360.66			
	T1	30777.3		1349.17			
	T2	82379.7	0.51	2930.08	0.52		
2266	N1	23462.4		6326.97			
	N2	25551.3		27990.1			
	T1	6407.01		6115.52			
	T2	11923.5	0.59	9230.25	0.34		
2289	N1	13664.1		1278.38		1011.93	
	N2	13057.4		1121.19		2659.61	
	T1	12877		1440.5		377.179	
	T2	16198.6	0.76	1872.65	0.67	2048.71	0.48
2465	N1	43201.8		2782.17			
	N2	48576.1		2219.66			
	T1	33446.8		1786.61			
	T2	39171.7	0.96	2436.27	0.59		
2529	N1	5082.58		1811.41			
	2	7381.41		2650.9			
	T1	7512.16		1063.2			
	T2	11490.4	0.95	1024.05	0.66		
2588	N1	72.161		33877.1			
	N2	439.886		47297.7			
	T1	1404.63		35780.1			
	T2	1175.2	0.14	49996.6	1.00		
2590	N1			1656.57			
	N2			2310.79			
	T1			1667.64			
	T2			1847.37	0.79		
2636	N1	40856.4		16700.2			
	N2	38185.4		21208.4			
	T1	23052		16119			
	T2	33825.5	0.64	28460.7	0.72		
2637	N1	5125.32		295.245			
	N2	11532.6		1961.45			
	T1	1806.7		827.966			
	T2	4202.13	0.97	5249.38	0.95		
2659	N1	2615.16		1383.75			
	N2	2023.84		936.148			
	T1	1359.31		402.276			
	T2	3114.2	0.34	413.145	0.66		
2697	N1	4730.61		393.449			
	N2	7518.9		730.241			
	T1	2544.65		660.016			
	T2	1734.36	0.43	1376.77	0.89		
2715	N1	47850.8					
	N2	54974.8					
	T1	12048.7					
	T2	21168.7	0.65				
2741	N1	42859.9		9533.78			
	N2	34738.7		14656.2			
	T1	48926.2		23506.8			
	T2	43445.4	0.91	41483.8	0.87		
2765	N1	-108.522		11173		-15917	
	N2	9.931		21235.3		18558.7	
	T1	1780.93		12008.4		148.19	
	T2	1927.31	-0.08	22403.4	0.98	1338.08	-0.13
2805	N1	31723		22456.4			
	N2	39285.5		26829.8			
	T1	34687.1		25041			
	T2	32919.9	0.77	25206	0.84		
2823	N1	4396.78		6029.32			
	N2	4674.93		14865.8			
	T1	6810.16		10444.5			
	T2	7080.84	0.98	19391	0.75		
2832	N1	6132.2		6138.22			
	N2	10791.7		34839.8			
	T1	3637.46		5901.39			
	T2	4458.33	0.70	28294.1	0.84		
2894	N1	3244.99		3211.94			
	N2	2879.59		4161.46			
	T1	8788.21		1682.82			
	T2	11549	0.68	2236.54	0.97		
2901	N1	3052.24		30442.8			
	N2	1394.03		34940.7			
	T1	-1443.27		41829.6			
	T2	1566.89	-0.42	49413.4	0.97		
2912	N1	3594.36		2566.42			
	N2	1285.88		3241.41			
	T1	1308.76		5226.31			
	T2	1939.98	0.24	5791.77	0.88		
2917	N1	5335.43		599.03			
	N2	3274.33		1341.51			
	T1	4602.47		686.74			
	T2	3984.18	0.71	1563.72	0.98		
2920	N1	4932.2					
	N2	6902.43					
	T1	2979.69					
	T2	5050.51	0.83				
2933	N1	-962.53		42534.8			
	N2	-111.427		37435.2			
	T1	326.33		13027.6			
	T2	1364.37	0.03	79607.7	0.14		

		D10S540		D12S327		D12S360	
IN	Normal Tumour	Peak Area	Tumour/normal Ratio	Peak Area	Tumour/Normal Ratio	Peak Area	Tumour/Normal ratio
1241	N1	8095.1		2053.69		1367.6	
	N2	9026.14		4467.84		5334.65	
	T1	7920.02		2078.44		2709.8	
	T2	8756.45	0.99	5816.02	0.78	7096.06	0.67
1482	N1	8072.22		4442.14		7142.3	
	N2	9507.68		7483.8		8562.04	
	T1	5280.71		2485.88		6940.66	
	T2	6009.69	0.96	5834.24	0.72	13991.1	0.59
1545	N1	13140.1		2443.79		7616.6	
	N2	15885.2		6752.16		5961.46	
	T1	10165.3		2459.68		11849.6	
	T2	12383.9	0.99	6712.75	0.99	8132.61	0.88
2077	N1	8580.11		9996.74		5860.15	
	N2	10637.6		10868.1		11609.6	
	T1	2681.46		6018.07		6110.18	
	T2	5067.54	0.66	6579.63	0.99	16587.6	0.73
2266	N1	87837.6				2445.59	
	N2	116847				5593.54	
	T1	8874.73				881.89	
	T2	17083.2	0.69			3852.69	0.52
2289	N1	9189.98				4486.53	
	N2	12621.3				7920.6	
	T1	7686.19				5152.18	
	T2	21142.9	0.50			7036.41	0.77
2465	N1	18419.1		8960.91		4736.08	
	N2	24764.8		10008.6		8379.4	
	T1	13102.3		10983.1		8501.38	
	T2	13817.3	0.78	87765.1	0.14	16591.1	0.91
2529	N1					5882.69	
	N2					11312.1	
	T1					1454.8	
	T2					5817.89	0.48
2588	N1	2836.94		10869.8			
	N2	8252.46		26031.2			
	T1	3758.67		4346.37			
	T2	8902.4	0.81	11735.7	0.89		
2590	N1	12556.8		6317.78		1109.13	
	N2	21316.5		9881.05		2840.92	
	T1	12485.4		3897		1578.28	
	T2	23898.1	0.89	6637.3	0.92	6057.16	0.67
2636	N1	102975		17179.4		2421.01	
	N2	90782.2		52329.4		8819.29	
	T1	46977		9788.43		2178.77	
	T2	57190.2	0.72	34578.9	0.86	6190.33	0.78
2637	N1	1342.1		1310.27			
	N2	16658.1		12185.1			
	T1	11286.4		2300.57			
	T2	16019.3	0.11	9444.62	0.44		
2659	N1	21387.4		2939.63		610.107	
	N2	23538.7		15083		2661.31	
	T1	17303		3822.42		2057.47	
	T2	19567.4	0.97	10313.1	0.53	5418.82	0.60
2697	N1	5889.08		11244.2		4063.67	
	N2	9487.65		29798		6342.66	
	T1	8650.38		6552.95		3471.48	
	T2	16333.8	0.85	13822.5	0.80	4505.69	0.83
2715	N1					4836.94	
	N2					7991.52	
	T1					2214.39	
	T2					4765.99	0.77
2741	N1	20011.1		6900.9		2528.89	
	N2	20295.7		20053.4		4084.87	
	T1	20881.3		4050.95		1205.91	
	T2	24499.8	0.86	6605.14	0.56	2750.92	0.71
2765	N1	37021.2		2944.51		1233.6	
	N2	42060.3		7340.43		2197.23	
	T1	12645.9		6672.22		1599.06	
	T2	13181.3	0.91	17367.9	0.96	2866.02	0.99
2805	N1	33241.3		4543.26		507.19	
	N2	35684.7		18402.8		622.552	
	T1	47133.8		3738.65		770.782	
	T2	49737.8	0.98	11447.9	0.755	1277.79	0.74
2823	N1	4050.27		4940.09		7399.54	
	N2	31632.9		17560		15431.4	
	T1	31067		6189.51		1911.44	
	T2	49065.4	0.20	20169.8	0.92	5130.17	0.78
2832	N1	80712.4		16981.6			
	N2	89998.1		41814.9			
	T1	70604.5		10971.3			
	T2	91421	0.86	36216.2	0.75		
2894	N1	13738.7		1996.93		1780.97	
	N2	16913.9		9944.14		1631.04	
	T1	16298.1		15590.5		6723.99	
	T2	18939.9	0.94	29746.5	0.38	11184.9	0.55
2901	N1	78768.4		4962.45		1259.92	
	N2	85469.5		22581.9		2449.57	
	T1	57779.2		2377.33		1075.99	
	T2	74703.7	0.84	19645.5	0.55	2513.97	0.83
2912	N1	8279.18		8730.47		4266.95	
	N2	18525.7		18741.5		7661.59	
	T1	8116.18		11112.3		3429.97	
	T2	23161.5	0.78	17700.2	0.74	6094.53	0.99
2917	N1	6362.72		6206.2		482.32	
	N2	16597.3		19215.3		273.78	
	T1	4466.87		21795.6		877.551	
	T2	13101.6	0.89	34078.4	0.51	500.495	1.00
2920	N1	1937.36		5602.5		3977.19	
	N2	4343.7		13256.2		5752.12	
	T1	1447.98		3294.89		3636.72	
	T2	3829.41	0.85	9964.42	0.78	5232.97	0.99
2933	N1	104105		20960.2		2002.53	
	N2	107454		47776.6		5674.6	
	T1	103506		25613		869.046	
	T2	112920	0.95	37990.5	0.65	3967.18	0.62

		D12S330		D12S366		D12S342	
IN	Normal Tumour	Peak Area	Tumour/normal Ratio	Peak Area	Tumour/Normal Ratio	Peak Area	Tumour/Normal ratio
1241	N1	9237.16				4704.29	
	N2	21565.5				6274.49	
	T1	1589.6				6951.98	
	T2	8353.56	0.44			9192.77	0.99
1482	N1	7125.41				4026.38	
	N2	18537.2				5285.33	
	T1	4750.45				2095.35	
	T2	15093.9	0.82			3583.46	0.77
1545	N1	12685		3606.81		8154.16	
	N2	17145.5		3716.72		7284.22	
	T1	13001.2		1243.06		7041.01	
	T2	19494.7	0.90	347.071	0.27	6327.7	0.99
2077	N1			562.191		10033.2	
	N2			486.336		15440.2	
	T1			1600.35		5034.62	
	T2			1053.89	0.76	7776.45	1.00
2266	N1	1657.24		1241.45		10445	
	N2	10607		463.605		12216.9	
	T1	224.9		773.058		4464.86	
	T2	4029.96	0.36	296.467	0.97	6905.37	0.76
2289	N1	2764.65		1111.63		17064.1	
	N2	15609.6		707.956		19725.3	
	T1	2972.06		3543.73		23446.3	
	T2	14717	0.88	4885.26	0.46	20674.1	0.76
2465	N1			4795.48		11895.3	
	N2			2023.52		11179.6	
	T1			12963.5		1265.25	
	T2			19413.9	0.28	2745.35	0.43
2529	N1	9015.72				11087.2	
	N2	20900.4				7948.75	
	T1	1696.85				2622.42	
	T2	8113.84	0.48			3418.36	0.55
2588	N1					1910.37	
	N2					2358.53	
	T1					2847.74	
	T2					3127.75	0.89
2590	N1					2744.47	
	N2					6101.89	
	T1					2994.88	
	T2					3081.27	0.46
2636	N1	6811.98		47240.3		22659.9	
	N2	17893.9		39920.2		24137.2	
	T1	4823.59		4812.29		23237.1	
	T2	17753	0.71	3056.13	0.75	26100.5	0.95
2637	N1	1455.89				846.479	
	N2	14146				1691.76	
	T1	1655.69				3593.96	
	T2	8812.45	0.55			4254.75	0.59
2659	N1					5020.72	
	N2					5145.33	
	T1					11458.5	
	T2					13753.9	0.85
2697	N1			3746.53		1147.89	
	N2			5513.57		3373.73	
	T1			9527.81		6629.1	
	T2			13385.3	0.95	6998.72	0.36
2715	N1			10875.7		6541.87	
	N2			11936.4		6771.67	
	T1			6918.61		8670.86	
	T2			9094.55	0.83	13419.4	0.67
2741	N1	1130.89		1078.75		11438.6	
	N2	9009.3		1762.54		8870.86	
	T1	2636.65		1075.14		3328.59	
	T2	18555.2	0.88	1882.53	0.93	5257.21	0.49
2765	N1	2235.09		28832.27		5475.17	
	N2	14823.6		29469.7		8677.65	
	T1	2536.73		17293.9		3374.34	
	T2	16368.2	0.97	32213.1	0.55	8149.53	0.66
2805	N1			2559.35		5371.25	
	N2			1171.65		8933.94	
	T1			4632.42		4303.3	
	T2			1479.1	0.70	7648.68	0.94
2823	N1			5634.11		5033.55	
	N2			3393.29		8590.16	
	T1			2334.84		5486.76	
	T2			5401.13	0.26	8038.68	0.85
2832	N1	2713.46		1142.79		2535.95	
	N2	14529		609.08		5153.91	
	T1	729.35		2113.36		1535.43	
	T2	10672.4	0.37	5535.76	0.20	3521.01	0.89
2894	N1			3549.97		624.52	
	N2			6866.05		1854.43	
	T1			1580.37		3735.52	
	T2			5656.84	0.54	7227.94	0.65
2901	N1			13662.2		7633.87	
	N2			70931.6		10525.4	
	T1			7476.17		7636.04	
	T2			58791.6	0.66	17985.6	0.59
2912	N1			8385.04		2606.49	
	N2			10284.6		2711.85	
	T1			3328.44		2459.64	
	T2			3810.12	0.93	4326.49	0.59
2917	N1			7506.39		4067.67	
	N2			5130.69		4704.9	
	T1			3889.29		649.57	
	T2			2189.13	0.82	591.72	0.79
2920	N1			2800.29		3108.43	
	N2			6203.11		4574.71	
	T1			2980.59		4169.36	
	T2			5674.83	0.85	4605.55	0.75
2933	N1	4755.22		46701.8		3835.68	
	N2	11403.1		64065.4		8699	
	T1	4281.42		6282.48		753.81	
	T2	11739.9	0.87	14117.7	0.61	2608.41	0.66

		D16S409		D16S416		D16S514	
IN	Normal Tumour	Peak Area	Tumour/normal Ratio	Peak Area	Tumour/Normal Ratio	Peak Area	Tumour/Normal ratio
1241	N1	1317.11		3323.81			
	N2	1497.16		9511.27			
	T1	684.289		1029.15			
	T2	825.354	0.94	2518.76	0.85		
1482	N1	1366.5		4262.03			
	N2	1676.63		13047.6			
	T1	871.602		5667.73			
	T2	1400.91	0.76	16911.9	0.97		
1545	N1	980.932		2888.72			
	N2	1208.75		8958.03			
	T1	1562.95		6940.87			
	T2	2989.96	0.64	16376.4	0.76		
2077	N1	833.39		3053.62			
	N2	1466.22		6749.52			
	T1	467.865		5967.92			
	T2	1175.34	0.70	6850.22	0.51		
2266	N1	2004.99		4365.44			
	N2	2130.48		7633.88			
	T1	764		483.392			
	T2	1483.3	0.55	1295.49	0.65		
2289	N1	925.109		9157.65			
	N2	1154.48		13395.4			
	T1	1150.64		3540.66			
	T2	1620.17	0.89	3412.23	0.66		
2465	N1	313.888		730.277			
	N2	371.28		721.774			
	T1	731.57		1840.37			
	T2	1310.22	0.66	5432.26	0.33		
2529	N1	204.365		5623.85		817.67	
	N2	482.649		12648.4		2465.95	
	T1	772.937		2494.58		436.5	
	T2	1171.9	0.64	5191.88	0.93	692.05	0.52
2588	N1	734.39					
	N2	2452.41					
	T1	493.84					
	T2	715.83	0.43				
2590	N1	648.944		2710.99			
	N2	841.869		8859.97			
	T1	950.646		2775.14			
	T2	1474.36	0.84	8526.88	0.94		
2636	N1	449.988		3552.5			
	N2	504.068		9489.83			
	T1	2898.53		1976.43			
	T2	4601.09	0.71	6661.54	0.79		
2637	N1			721.648			
	N2			1123.34			
	T1			404.202			
	T2			894.77	0.70		
2659	N1			879			
	N2			1757.6			
	T1			4321.6			
	T2			8113.31	0.94		
2697	N1	732.057					
	N2	1868.75					
	T1	689.094					
	T2	1295.05	0.73				
2715	N1	486.586		2225.31			
	N2	821.121		4634.71			
	T1	364.044		223.287			
	T2	1038.63	0.59	2062.14	0.23		
2741	N1	245.736		4048.5		4298.05	
	N2	664.77		6171.47		15466.2	
	T1	1842.98		5155.86		7341.38	
	T2	1955.06	0.39	7203.53	0.92	9351.2	0.35
2765	N1	845.462		3817.02			
	N2	1008.56		3347.84			
	T1	872.212		2176.04			
	T2	1190.41	0.87	2220.94	0.86		
2805	N1	420.464		3856.25		1829.25	
	N2	985.038		3785		15160.6	
	T1	1090.55		1681.02		5263.05	
	T2	1682.34	0.66	2068.71	0.80	6080.71	0.14
2823	N1	308.118		752.282			
	N2	216.793		1716			
	T1	938.39		1910.93			
	T2	1170.14	0.56	3901.89	0.89		
2832	N1	1813.42		6476		9662.01	
	N2	3631.86		16832.2		10866.1	
	T1	3945.46		10170.4		5201.21	
	T2	8260.99	0.96	22308.3	0.84	5821.39	1.00
2894	N1	463.302		345.491			
	N2	1176.49		674.13			
	T1	537.554		248.884			
	T2	981.413	0.72	256.157	0.53		
2901	N1	1974.45					
	N2	1308.53					
	T1	968.718					
	T2	2264.09	0.28				
2912	N1	1020.9		9270.65			
	N2	1977.61		17986.4			
	T1	716.821		6016.37			
	T2	1620.55	0.86	13676.9	0.85		
2917	N1	3628.47		5939.48			
	N2	5425.56		14120.8			
	T1	4888.97		4996.6			
	T2	7734.89	0.95	13047.4	0.91		
2920	N1	1192.72		3552.68			
	N2	1365.08		9737.27			
	T1	2423.59		2589.85			
	T2	4490.06	0.62	6960.09	0.98		
2933	N1	2792.86		12273.7			
	N2	3558.57		27244.4			
	T1	1266.33		6844.65			
	T2	2734.34	0.59	22161.9	0.69		

		D16S512		D16S516		F8VWFP	
IN	Normal Tumour	Peak Area	Tumour/normal Ratio	Peak Area	Tumour/Normal Ratio	Peak Area	Tumour/Normal ratio
1241	N1	305.936				331.63	
	N2	1509.79				13420.7	
	T1	105.354				1596.37	
	T2	245.662				3278.94	0.05
1482	N1	231.621					
	N2	170.817					
	T1	326.425					
	T2	349.156	0.69				
1545	N1	1035.621					
	N2	1420.43					
	T1	626.81					
	T2	750.634	0.87				
2077	N1	441.964		3269.67		9799.93	
	N2	480.516		6772.79		140.95	
	T1	522.183		3663.44		71178.8	
	T2	730.81	0.78	6374.63	0.84	568.96	0.56
2266	N1	231.081				1131.53	
	N2	455.003				2399.73	
	T1	239.717				8847.61	
	T2	1094.15	0.43			15711.8	0.84
2289	N1	460.356		2897.89		235.61	
	N2	1155.63		4110.12		1572.91	
	T1	238.976		799.624		323.504	
	T2	823.785	0.73	1545.56	0.73	1625.41	0.75
2465	N1	216.322		1037.54		4072.26	
	N2	409.42		3136.24		16251.3	
	T1	47.755		403.721		16056.3	
	T2	288.815	0.31	1582.83	0.77	29678.5	0.46
2529	N1	1543.34		902.317			
	N2	3436.1		1225.62			
	T1	894.454		605.742			
	T2	2529.55	0.79	613.766	0.75		
2588	N1	581.029					
	N2	2681.62					
	T1	299.655					
	T2	770.246	0.56				
2590	N1	677.811		1929.77			
	N2	1894.95		5796.19			
	T1	603.364		721.87			
	T2	955.674	0.57	1915.52	0.88		
2636	N1	953.377		9445.95			
	N2	1251.06		7986.73			
	T1	127.118		2149.37			
	T2	97.72	0.59	1708.86	0.94		
2637	N1	101.417		275.69		27.607	
	N2	96.943		596.17		1450.94	
	T1	215.458		1882.57		3556.02	
	T2	490.498	0.42	1649.09	0.41	6746.68	0.04
2659	N1	210.498				4917.64	
	N2	211.084				21104.1	
	T1	209.016				1030.92	
	T2	651.458	0.32			5981.47	0.74
2697	N1	3720.04		629.495		274.212	
	N2	8586.34		1514.74		25829.7	
	T1	2782.19		652		14025.1	
				.972			
	T2	8203.44	0.78	1639.3	0.96	15014.1	0.01
2715	N1	3007.29		1002.18			
	N2	7747.69		1309.05			
	T1	221.705		228.317			
	T2	400.356	0.70	88.634	0.30		
2741	N1	2221.65				69821.5	
	N2	4255.48				42655.1	
	T1	363.995				61458.1	
	T2	1034.03	0.67			80277.4	0.47
2765	N1	594.427		6728.03		4746.95	
	N2	703.758		7119.46		9885.02	
	T1	1098.56		3252.72		2913.65	
	T2	1381.29	0.94	3207.07	0.81	14868.9	0.41
2805	N1	2718.84				54168.8	
	N2	6618.71				11528.4	
	T1	3535.92				45626.3	
	T2	7478.06	0.87			111675	0.87
2823	N1	1030.98		2061.47		19608.3	
	N2	2169.01		2324.45		80472.5	
	T1	3305.19		7438.52		52175.1	
	T2	6127.76	0.88	10364.3	0.81	110308	0.51
2832	N1	6117.17				1101.75	
	N2	13366.1				2359.92	
	T1	5303.43				6037.74	
	T2	12466.2	0.93			2359.92	0.18
2894	N1	375.36		9435.32		15895.8	
	N2	937.72		12112.3		63044.6	
	T1	6145.11		4970.69		35908.5	
	T2	11671.1	0.76	6921.05		129869	0.91
2901	N1	3110.25		954.274			
	N2	14970.6		1992.99			
	T1	1113.67		1167.73			
	T2	5397.61	0.99	2847.99	0.86		
2912	N1	1043.76				3403.18	
	N2	3345.72				2182.8	
	T1	3271.27				20591.8	
	T2	9904	0.94			6876.21	0.52
2917	N1	779.979				122.372	
	N2	1386.68				16587.5	
	T1	861.77				3228.48	
	T2	1461.96	0.95			3931.4	0.01
2920	N1	518.284		502.41		2588.9	
	N2	1210.58		3938.84		3329.52	
	T1	689.096		2347.68		3386.68	
	T2	1338.4	0.88	3248.86	0.18	4809.57	0.91
2933	N1	534.027				19588.7	
	N2	1481.92				34096.8	
	T1	1628.74				27505.6	
	T2	3966.6	0.88			42217.4	0.88

		D22S301		D22S294		D17S34	
IN	Normal Tumour	Peak Area	Tumour/normal Rati	Peak Area	Tumour/Normal Ratio	Peak Area	Tumour/Normal ratio
1241	N1	977.061					
	N2	1123.96					
	T1	1481.82					
	T2	1797.26	0.95				
1482	N1			3141.6			
	N2			9522.49			
	T1			2130.65			
	T2			6703.42	0.96		
1545	N1	2607.48		5013.05			
	N2	4252.51		12299.1			
	T1	2819.26		1619.31			
	T2	4119.01	0.90	2811.67	0.70		
2077	N1	8957.23		21087.6			
	N2	10027.3		47792.9			
	T1	10075		5588.3			
	T2	7823.49	0.69	13209.4	0.96		
2266	N1	9288.03					
	N2	18409.8					
	T1	281.48					
	T2	1714.65	0.33				
2289	N1	3546.15		1074.			
				22			
	N2	4039.04		4179.81			
	T1	2727.55		1020.77			
	T2	3776.44	0.82	3299.78	0.83		
2465	N1	4550.85		2615.16			
	N2	4289.62		2023.84			
	T1	11717.8		1359.31			
	T2	13554.7	0.81	3114.2	0.34		
2529	N1	861.23		3937.76			
	N2	1404.16		10038			
	T1	973.584		1639.68			
	T2	1693.53	0.94	2738.89	0.66		
2588	N1	1226.72		4730.61			
	N2	10253.4		7518.9			
	T1	2288.5		2544.65			
	T2	10664	0.56	1734.36	0.43		
2590	N1	1579.79		1937.69			
	N2	2651.98		2474.06			
	T1	2188.25		2389.79			
	T2	3081.68	0.84	2664.99	0.87		
2636	N1	11128		11890			
	N2	22527.1		72965.7			
	T1	7934.05		15717.8			
	T2	9740.5	0.61	72614.8	0.75		
2637	N1	1024.36					
	N2	3471.61					
	T1	791.31					
	T2	2384.48	0.89				
2659	N1	6258.89		4396.78			
	N2	14370.2		4674.93			
	T1	8086.07		6810.6			
	T2	17063.4	0.92	7080.84	0.98		
2697	N1	3487.73		4058.51			
	N2	2481.02		5754.79			
	T1	8286.72		3008.23			
	T2	7404.56	0.80	4220.26	0.99		
2715	N1	2346.69		7047.56			
	N2	2988.69		26794.2			
	T1	5211.87		12539			
	T2	3455	0.52	39613.8	0.83		
2741	N1	22291.3		6132.2			
	N2	40987.6		10791.7			
	T1	25291.4		3637.46			
	T2	43280.3	0.93	4458.33	0.69		
2765	N1	8795.24		3244.99			
	N2	7062.99		2879.59			
	T1	3068.45		8788.21			
	T2	2226.21	0.90	11549	0.68		
2805	N1	7001.32		2776.96			
	N2	17560.8		13220.9			
	T1	6408.32		34580.1			
	T2	21542.7	0.75	101411	0.62		
2823	N1	11398.6		6499.93			
	N2	24273.4		56004.7			
	T1	15315.1		20626			
	T2	12947.2	0.40	87982.1	0.50		
2832	N1	8299.44		17745.5			
	N2	11667.1		39503.8			
	T1	7309.11		13889.9			
	T2	8490.99	0.83	48788.9	0.63		
2894	N1	7084.71		30376.4			
	N2	6876.52		56298.1			
	T1	4109.63		22027.2			
	T2	8103.71	0.49	39944.4	0.98		
2901	N1	3611.92					
	N2	5193.66					
	T1	2220.81					
	T2	3331.72	0.96				
2912	N1	2253.64		4083.5			
	N2	2919.12		4239.89			
	T1	-2.173		3309.29			
	T2	1197.56	0.00	3680.65	0.93		
2917	N1	4136.96		518.86			
	N2	5172.04		1687.96			
	T1	838.565		632.84			
	T2	1016.96	0.97	1890.87	0.92		
2920	N1	5117.78					
	N2	8790.92					
	T1	912.43					
	T2	2197.56	0.71				
2933	N1	9969.77		22016.4			
	N2	15073.8		102157			
	T1	12685.3		7785.85			
	T2	12712.9	0.66	53395.4	0.68		

		D17S849		D17S926		D17S643	
IN	Normal Tumour	Peak Area	Tumour/normal Ratio	Peak Area	Tumour/ Normal Ratio	Peak Area	Tumour/ Normal ratio
1212	N1	86279				2305.44	
	N2	112113				5228.5	
	T1	7345.01				2586.32	
	T2	5329.26	0.56			5013.96	0.85
1241	N1	11572.6		69080.9		977.92	
	N2	9004.76		49393.5		2443.44	
	T1	9423.69		44333.5		1312.15	
	T2	11298.3	0.51	30372.5	0.96	4256.1	0.77
1482	N1	29796.8		12554.9		1349.82	
	N2	38697.1		12880.8		5849.56	
	T1	150676		2532.49		1202.44	
	T2	200046	0.98	2367.96	0.91	4794.11	0.92
1545	N1	6262.44		2233.55		5455.86	
	N2	6255.47		5713.07		7105.1	
	T1	950.028		943.1		3501.25	
	T2	978.707	0.97	3919.22	0.62	7371.75	0.62
2077	N1			12954.3		2079	
	N2			23873		5095.11	
	T1			5860.22		2195.37	
	T2			19521.6	0.55	3844.77	0.71
2266	N1	10104.3				1254.61	
	N2	16573				3436.29	
	T1	7948.76				1686.59	
	T2	14029.1	0.93			2858.77	0.62
2289	N1	7856.04				256.269	
	N2	10196.8				605.49	
	T1	829.998				89.811	
	T2	1004.01	0.93			125.313	0.59
2465	N1	37350.4		52207.6		3566.12	
	N2	51129.1		45181.6		6662.74	
	T1	10781		10997.2		3052.38	
	T2	24812.8	0.59	13645.4	0.70	4076.87	0.71
2529	N1	15912.5		1032.18		3707.76	
	N2	17042.9		2575.58		5480.62	
	T1	1248.73		12492.8		1331.51	
	T2	1149.96	0.86	20040.7	0.64	2500.64	0.79
2588	N1	26973.4		5768.07		569.718	
	N2	125421		4809.48		1000.74	
	T1	17396.7		20011.5		1046.64	
	T2	81220.4	1.00	14893.9	0.89	175.92	0.10
2590	N1	36471		18881.9		1560.96	
	N2	54938.2		24555.8		2763.45	
	T1	41655.3		5438.52		203.97	
	T2	65903.5	0.95	10007.5	0.71	362.83	1.00
2636	N1			61493.7		7108.55	
	TN2			83160.6		8538.75	
	T1			32487.2		3250.08	
	T2			45709.9	0.96	12281.2	0.32
2637	N1	492.212		7450.29		1474.32	
	N2	246.373		15454.6		4926.48	
	T1	460.902		5109.95		1208.95	
	T2	18.073	0.08	4495.36	0.42	4741.34	0.85
2659	N1	616.32		7347.11		1397.58	
	N2	1450.98		12378		6366.54	
	T1	2283.07		15222		5877.37	
	T2	9018.49	0.60	27320.6	0.94	10638.6	0.40
2697	N1	1674.3		7601.13		21417.3	
	N2	9650.71		8503.04		25451.1	
	T1	3041.01		6685.06		13730.7	
	T2	10467.5	0.60	9217.49	0.81	14320	0.88
2715	N1	92387.9		3195.61		4490.8	
	N2	202739		4954.74		10421.7	
	T1	64174.2		1401.79		918.99	
	T2	179094	0.79	4051.53	0.54	7406.48	0.29
2741	N1	66506		37287.9		4865.84	
	N2	102021		39943.6		6947.21	
	T1	7262.99		656.62		4255.36	
	T2	18416.1	0.6	4033.91	0.17	5701.24	0.94
2765	N1	11281.6		22504.6		862.64	
	N2	19564.4		22235.4		5098.32	
	T1	4413.18		19678.3		2404.09	
	T2	14771.9	0.52	19903.1	0.98	7403.57	0.52
2805	N1	3859.33		18533.1		4553.63	
	N2	3832.42		18768.3		16140.6	
	T1	17186.8		7337.94		1198.55	
	T2	17782.5	0.96	8715.44	0.85	7377.87	0.58
2823	N1	8828.93		997.34		2258.64	
	N2	20120.3		1666.08		5791.25	
	T1	20309		2593.12		823.54	
	T2	34317	0.74	2575.65	0.59	3853.48	0.55
2832	N1	7933.81		7735.17		2258.64	
	N2	15589		12281.8		5791.25	
	T1	5383.16		6438.15		823.54	
	T2	12796.6	0.83	9295.07	0.91	3853.48	0.55
2894	N1	151.582		440.063		661.185	
	N2	134.02		768.972		1069.63	
	T1	5760.17		672.442		256.679	
	T2	7712.45	0.66	770.956	0.66	805.185	0.52
2901	N1	8527.47		3668.02		1592.09	
	N2	21257.3		6690.19		4581.25	
	T1	6910.97		7219.62		769.52	
	T2	11321.3	0.66	14165.2	0.93	1712.01	0.77
2912	N1	3422.69		2721.53			
	N2	7154.88		3011.05			
	T1	796.55		4895.86			
	T2	1493.89	0.90	1741.33	0.32		
2917	N1	6647.95		2688.6			
	N2	10981.1		4065.12			
	T1	5200.83		7113.65			
	T2	9180.57	0.94	7179.54	0.67		
2920	N1	7580.66		1196.7			
	N2	10180.7		1878.01			
	T1	5300.65		1411.43			
	T2	9113.27	0.78	5342.24	0.41		
2933	N1	2115.23		850.16		2794.01	
	N2	7967.51		1845.49		1558.72	
	T1	3130.68		1696.45		1677.91	
	T2	8716.77	0.74	2476.89	0.67	1043.14	0.90

		D17S695		D17S654		D17S675	
IN	Normal Tumour	Peak Area	Tumour/normal Ratio	Peak Area	Tumour/ Normal Ratio	Peak Area	Tumour/ Normal ratio
1212	N1	2403.27				11995.5	
	N2	5960.94				20061.3	
	T1	4675.85				7536.36	
	T2	5722.95	0.49			14630.5	0.86
1241	N1	9157.45		2455.45		17390.1	
	N2	4196.94		3294.01		30758.4	
	T1	4859.97		3287.64		10750.8	
	T2	3153.32	0.71	4679.19	0.94	23648.4	0.80
1482	N1	7595.58		4834.09		1420.64	
	N2	6230.55		6578.68		30589.4	
	T1	4482.4		2450.37		910.99	
	T2	6470.05	0.57	4255.78	0.78	16277.1	0.83
1545	N1	3192.15		3141.96		5087.89	
	N2	2712.55		4672.74		3570.72	
	T1	770.22		1432.95		5235.51	
	T2	516.02	0.79	2105.95	0.99	5418.3	0.68
2077	N1	2807.99		1572.52		2169.21	
	N2	4350.23		2340.93		18603.6	
	T1	2802.59		1474.93		2365	
	T2	7510.72	0.58	3879.53	0.57	27547.9	0.74
2266	N1	1654.46		3585.24		12052.7	
	N2	6468.68		4980.77		23210.7	
	T1	2646.7		3840.86		9795.8	
	T2	8596.95	0.83	5892.36	0.91	24017	0.79
2289	N1			5938.8		6524.65	
	N2			8257.15		27204.9	
	T1			4302.23		1354.67	
	T2			7394.01	0.81	8566.55	0.66
2465	N1	6903.47				383.37	
	N2	1385.65				21325.2	
	T1	6068.74				-4033.77	
	T2	6018.96	0.10			20499.4	-0.09
2529	N1			1111.55		17700.6	
	N2			2165.78		32233.1	
	T1			9730.77		13724.3	
	T2			11479.1	0.61	261.34	0.96
2588	N1	2203.22		2014.61		12768.5	
	N2	2927.6		4238.99		23153.4	
	T1	5496.91		4838.16		9721.9	
	T2	5222.55	0.72	7962.04	0.78	19527.9	0.90
2590	N1	6852.13		4727.86		508.34	
	N2	8184		7113.31		13206.1	
	T1	2302.66		4164.48		1036.26	
	T2	2009.13	0.73	5338.72	0.85	19453.7	0.72
2636	N1			1866.7		9362.2	
	TN2			3397.56		17318.6	
	T1			1566.77		13118.4	
	T2			3867.35	0.74	22678	0.93
2637	N1			796.842		2078.15	
	N2			2189.42		1370.44	
	T1			2413.39		1493.62	
	T2			2653.68	0.40	1376.94	0.72
2659	N1			4052.04		1637.06	
	N2			9874.72		2321.87	
	T1			1059.13		1367.88	
	T2			4776.86	0.54	1888.07	0.97
2697	N1	8110.03		9765.43		3254.51	
	N2	8873		9923.18		12455.5	
	T1	14920.2		5329.8		3879.87	
	T2	11481.4	0.70	7487.27	0.72	18116.5	0.82
2715	N1	9155.55		284.082			
	N2	9894.01		577.347			
	T1	2188.46		394.746			
	T2	4714.79	0.50	753.999	0.94		
2741	N1			3566.48		1055.27	
	N2			6537.21		1634.41	
	T1			5335.63		2122.68	
	T2			8405.78	0.86	4165.16	0.79
2765	N1			11105.9		2562.86	
	N2			12076.1		11378.5	
	T1			9233.83		1876.28	
	T2			12080.6	0.83	14428.2	0.58
2805	N1			3914.14			
	N2			3799.18			
	T1			1032.41			
	T2			1736.95	0.58		
2823	N1			8752.75			
	N2			13755.3			
	T1			4721.19			
	T2			9367.86	0.79		
2832	N1	13730.8		7400.62		414.66	
	N2	9288.2		12490.2		23196	
	T1	9871.54		10291.6		1191.04	
	T2	6982.02	0.96	16462.5	0.95	29321.9	0.44
2894	N1					4989.05	
	N2					5629.92	
	T1					1225	
	T2					1802.74	0.77
2901	N1			579.49		397.2	
	N2			3072.3		898.59	
	T1			405.82		698.33	
	T2			1160.93	0.54	1092.13	0.70
2912	N1			971.9			
	N2			2781.38			
	T1			1800.29			
	T2			5099.47	0.99		
2917	N1			3507.3		4485.89	
	N2			6462.37		5359.2	
	T1			1870.53		2441.14	
	T2			7662.59	0.45	2655.28	0.91
2920	N1	3778.27		6841.27			
	N2	1972.75		10326.1			
	T1	58.272		5682.65			
	T2	4648.78	0.01	10304.2	0.83		
2933	N1			1012.57			
	N2			1479.3			
	T1			39.82			
	T2			245.8	0.24		

		D17S619		D17S513		D17S678	
IN	Normal Tumour	Peak Area	Tumour/normal Ratio	Peak Area	Tumour/Normal Ratio	Peak Area	Tumour/Normal ratio
1212	N1	610.03					
	N2	1538.23					
	T1	688.52					
	T2	1831.79	0.95				
1241	N1	1469.52		7284.68			
	N2	1059.95		8711.15			
	T1	2173.13		3414.63			
	T2	2404.68	0.65	3824.17	0.94		
1482	N1	638.59		8550.91			
	N2	5079.91		12620.3			
	T1	1330.54		3325.35			
	T2	3007.03	0.28	4285.51	0.87		
1545	N1	885.29					
	N2	2651.37					
	T1	1407.16					
	T2	2743.83	0.65				
2077	N1	1031.81					
	N2	1321.13					
	T1	1245.43					
	T2	1838.25	0.87				
2266	N1	498.84					
	N2	1523.08					
	T1	872.8					
	T2	2288.1	0.86				
2289	N1						
	N2						
	T1						
	T2						
2465	N1	1595.94		12490.7			
	N2	3105.56		20719.6			
	T1	1006.63		4104.48			
	T2	815.49	0.42	12280.4	0.55		
2529	N1	1577.84					
	N2	5026.28					
	T1	1537.45					
	T2	3775.28	0.77				
2588	N1	1529.2					
	N2	1818.7					
	T1	2311.43					
	T2	2232.51	0.81				
2590	N1	792.66					
	N2	803.126					
	T1	595.999					
	T2	1178.44	0.51				
2636	N1	3264.04					
	N2	2718.03					
	T1	2279.18					
	T2	2107.86	0.90				
2637	N1	669.097					
	N2	757.304					
	T1	168.946					
	T2	474.06	0.40				
2659	N1	1217.66					
	N2	3292.21					
	T1	2130.81					
	T2	2782.69	0.48				
2697	N1	7582.46					
	N2	4999.58					
	T1	6629.56					
	T2	5112.89	0.85				
2715	N1						
	N2						
	T1						
	T2						
2741	N1	2290.31		2758.04			
	N2	4579.11		4129.17			
	T1	1565.44		9407.63			
	T2	3136.21	1.00	7282	0.52		
2765	N1	1239.61		9588.46			
	N2	1050.1		18481.8			
	T1	3984.83		5028.46			
	T2	3983.03	0.85	6097.12	0.63		
2805	N1	2115.26					
	N2	3395.77					
	T1	3325.39					
	T2	3912.94	0.73				
2823	N1	3729.96		8956.74			
	N2	3233.34		7426.86			
	T1	6289.48		6979.6			
	T2	4648.13	0.85	12085.8	0.48		
2832	N1	5986.1					
	N2	3398.63					
	T1	6482.22					
	T2	4719.96	0.78				
2894	N1	5082.71				1761.94	
	N2	4012.53				4473.18	
	T1	4998.28				849.73	
	T2	4457.42	0.89			4439.07	0.49
2901	N1	6328.6		15678.7			
	N2	3634.14		33788.8			
	T1	4395.58		9997.07			
	T2	3447.02	0.73	22341.3	0.96		
2912	N1	579.011					
	N2	789.992					
	T1	226.321					
	T2	414.898	0.74				
2917	N1	3367.23					
	N2	2452.7					
	T1	3632.84					
	T2	4008.41	0.66				
2920	N1	4528.23				2319.74	
	N2	2796.75				4740.75	
	T1	3320.57				723.34	
	T2	3547.86	0.58			5428.83	0.27
2933	N1						
	N2						
	T1						
	T2						

		D17S1149		D17S796		AKG2-1	
IN	Normal Tumour	Peak Area	Tumour/normal Ratio	Peak Area	Tumour/Normal Ratio	Peak Area	Tumour/Normal ratio
1212	N1	6995.32		4114.93		5386.91	
	N2	6219.01		3758.68		5658.82	
	T1	6744.23		4644.43		9241.68	
	T2	6764.36	0.89	4446.29	0.95	9012.25	0.93
1241	N1	7850.79		6434.64		8973.44	
	N2	11034.2		13560.4		11350	
	T1	8419.81		3808.4		18301	
	T2	10769.1	0.91	8117.01	0.99	17696.7	0.76
1482	N1			3454.93		10894.3	
	N2			2884.21		13249.1	
	T1			1975.92		7441.87	
	T2			1057.77	0.64	13844.5	0.65
1545	N1			3840.89			
	N2			2938.02			
	T1			150.862			
	T2			419.334	0.28		
2077	N1			14932.2		23531.3	
	N2			18577.6		71735.9	
	T1			22368.8		15644.8	
	T2			26386.6	0.95	53871.4	0.89
2266	N1						
	N2						
	T1						
	T2						
2289	N1			3784.63			
	N2			2836.69			
	T1			7291.34			
	T2			11257.5	0.49		
2465	N1			371.98		43002.3	
	N2			1601.42		57203.1	
	T1			58.358		51839.3	
	T2			657.219	0.38	48685.2	0.71
2529	N1	2041.32		514.943			
	N2	1601.24		773.444			
	T1	7675.01		83.123			
	T2	5471.89	0.91	224.694	0.56		
2588	N1			1527.23		4330.95	
	N2			3197.86		5511.41	
	T1			13517.7		5296.94	
	T2			11950.6	0.42	5088.07	0.75
2590	N1			8859.83		5403.58	
	N2			10933.2		11569.2	
	T1			17042.2		12319.4	
	T2			20385.4	0.97	37622.9	0.70
2636	N1			46199.7		5578.83	
	TN2			47649.6		33092.9	
	T1			42237.4		1904.77	
	T2			43257.1	0.99	18713.6	0.60
2637	N1			10019.5			
	N2			9827.97			
	T1			14203.6			
	T2			35090.9	0.40		
2659	N1			7404.72		587.313	
	N2			2945.48		1347.33	
	T1			16327		475.24	
	T2			17128.6	0.38	855.414	0.78
2697	N1			664.379		1793.92	
	N2			1540.06		10418.2	
	T1			1397.71		510.66	
	T2			1924.79	0.59	7869.54	0.38
2715	N1			1414.39			
	N2			3565.3			
	T1			1226.93			
	T2			2761.2	0.89		
2741	N1			2449.11			
	N2			5983.76			
	T1			4965.22			
	T2			8276.3	0.68		
2765	N1			39151.5		3960.32	
	N2			30603.2		24311.1	
	T1			12922.8		620.08	
	T2			9065.02	0.90	7869.94	0.48
2805	N1	7234.66		17081.5		174.893	
	N2	8215.97		14083.2		317.507	
	T1	10953.1		9807.96		1075.43	
	T2	9501.98	0.76	11714.4	0.69	3271.28	0.60
2823	N1	1644.51		2993.85		9498.54	
	N2	1701.37		4797.5		8432.13	
	T1	2042.95		53094.8		39157.3	
	T2	1845.59	0.87	114663	0.74	31844.8	0.92
2832	N1			1134.2		3128.13	
	N2			10098.6		10308.8	
	T1			1353.49		3035.21	
	T2			11349.2	0.94	12377.2	0.81
2894	N1			1689.06		972.667	
	N2			1099.75		2774.73	
	T1			8245.51		373.985	
	T2			8434.24	0.64	1155.95	0.92
2901	N1			12385.6		3620.01	
	N2			17698.8		6594.68	
	T1			9908.64		3779.4	
	T2			16915.5	0.84	7790.75	0.88
2912	N1			1242.73		516.32	
	N2			2105.67		4919.62	
	T1			968.15		3918.36	
	T2			2185.71	0.75	12798.2	0.34
2917	N1			4002.29		7278.42	
	N2			9219.29		16686.9	
	T1			1744.44		1899.44	
	T2			5615.3	0.72	4877.38	0.89
2920	N1			2949		3319.94	
	N2			6742.69		6140.91	
	T1			5221.09		3227.04	
	T2			8801.3	0.74	7044.4	0.85
2933	N1			3081.52		6591.22	
	N2			4813.17		7800.49	
	T1			1814.46		4736.92	
	T2			3541.25	0.80	11093.5	0.51

		D17S720		D17S952		D17S786	
IN	Normal Tumour	Peak Area	Tumour/Normal Ratio	Peak Area	Tumour/Normal Ratio	Peak Area	Tumour/Normal ratio
1212	N1			3155.81			
	N2			9493.72			
	T1			2346.02			
	T2			16575.2	0.43		
1241	N1	10941.1		1885.62		9812.2	
	N2	28566.9		13390.5		10171.5	
	T1	2226.51		2558.1		2363.74	
	T2	2724.98	0.47	18805.8	0.97	2697.01	0.91
1482	N1	1780.79		2962.04		2139.79	
	N2	881.5		25188.1		2212.47	
	T1	1897.32		2554.58		7331.43	
	T2	6194.03	0.15	13020	0.60	7980.59	0.95
1545	N1	27704.4		1559.66		8138.87	
	N2	3598.87		6270.15		9906.1	
	T1	13045.9		811.37		6079.46	
	T2	1563.17	0.92	5796.75	0.56	8108.65	0.91
2077	N1	2168.21		18372.6		27668.4	
	N2	4531.8		41683		21287.3	
	T1	5486.39		9410.46		43378.3	
	T2	18760	0.61	18913.8	0.89	39264.4	0.85
2266	N1	10351.9		4283.77			
	N2	26202.6		14112.9			
	T1	5889.6		1643.34			
	T2	12586.4	0.84	6782.92	0.80		
2289	N1	1770.48		2745.03		24298.3	
	N2	3705.61		13819.1		34967.6	
	T1	1575.3		1736.84		17697.7	
	T2	11508	0.29	2640.39	0.30	20226.1	0.79
2465	N1	5771.66				1650.43	
	N2	3292.86				3631.2	
	T1	3966.5				4854.02	
	T2	1219.99	0.54			4903.11	0.46
2529	N1	56.6		2643.81		1796.56	
	N2	313.2		6655.45		4221.85	
	T1	380.97		878.471		4099.89	
	T2	259.88	0.12	2047.37	0.93	8461.79	0.88
2588	N1	2566.02		2996.38		4996.66	
	N2	1939.72		18576.6		8640.14	
	T1	11442.2		3359.83		6001.88	
	T2	22700.7	0.38	27440.1	0.84	12572.7	0.83
2590	N1	652.62		6009.42		25871.9	
	N2	340.28		29853.1		26834.8	
	T1	4005.32		4397.45		10542.4	
	T2	4466.2	0.47	34661.6	0.63	14001.7	0.78
2636	N1	4217.24		6037.74		35297.2	
	N2	3913.57		25457.3		59779.4	
	T1	3405.99		1101.75		44093.1	
	T2	11979.5	0.26	2359.92	0.51	50869	0.68
2637	N1	2961.54		4746.95		27850.2	
	N2	3894.2		9885.02		67402	
	T1	395.149		2913.64		18420.9	
	T2	771.086	0.67	14768.9	0.41	48595.8	0.92
2659	N1	7118.13		861.05		1155.72	
	N2	6975.59		10408.2		1556.96	
	T1	5944.84		5187.8		125.366	
	T2	5939.12	0.98	37208.6	0.59	705.108	0.24
2697	N1	1576.69		1205.76		3152.48	
	N2	6659.25		6627.11		4577.06	
	T1	1605.24		1309.08		2681.26	
	T2	4376.82	0.65	6948.17	0.97	3659.7	0.94
2715	N1	605.119		5634.36		359.568	
	N2	2127.31		13434.6		381.507	
	T1	500.382		16004.1		632.31	
	T2	956.45	0.54	31322.1	0.82	82.102	0.12
2741	N1	3032.06				4812.83	
	N2	15566.1				6798.33	
	T1	8937.6				3084.06	
	T2	8064.98	0.18			3200.26	0.73
2765	N1	1238.21		3917.54			
	N2	656.15		11103.1			
	T1	4209.75		2031.92			
	T2	9480.98	0.24	6981.48	0.82		
2805	N1	724.86		5163.29		37898.8	
	N2	3078.25		33725		44352.7	
	T1	2632.11		8589.79		1924.8	
	T2	5601.71	0.50	52375.1	0.93	50507.9	0.04
2823	N1	3228.58		9004.22			
	N2	7387.99		11979.9			
	T1	2554.77		9985.6			
	T2	12073.1	0.48	25636.5	0.52		
2832	N1	314.496		2590.23		3555.84	
	N2	1266.29		6099.08		7924.45	
	T1	1323.46		1673.49		3407.3	
	T2	3353.55	0.81	5659.5	0.70	9783.77	0.78
2894	N1	184.66		4893.03			
	N2	336.57		32603.5			
	T1	677.92		4237.26			
	T2	497.88	0.40	29036.3	0.97		
2901	N1	468.33		7026.46		5614.45	
	N2	453.75		8120.23		8099.04	
	T1	1354.47		9233.36		7113.2	
	T2	1704.42	0.77	15847.7	0.67	11883.1	0.86
2912	N1	1359.82		916.11		5357.56	
	N2	1818.13		7322.95		7969.85	
	T1	101.61		1069.78		2054.56	
	T2	370.061	0.37	8059.92	0.94	2996.5	0.98
2917	N1	360		8678.92		2052.93	
	N2	525.38		12374.3		2427.68	
	T1	475.39		3274.45		1115.48	
	T2	509.57		7544.38	0.62	1075.88	0.82
2920	N1	1591.76		2289.43		1807.48	
	N2	1829.27		7305.6		1832.63	
	T1	3968.63		4530.04		1119.69	
	T2	4446.08	0.97	9794.83	0.68	1582.45	0.72
2933	N1			843.46		1125.04	
	N2			8495.88		978.7	
	T1			1081.57		1522.34	
	T2			7906.51	0.73	1488.86	0.89

		D17S804		D17S945		D17S954	
IN	Normal Tumour	Peak Area	Tumour/Normal Ratio	Peak Area	Tumour/Normal Ratio	Peak Area	Tumour/Normal Ratio
1212	N1	15743.7		4096.85		12141.7	
	N2	20692.4		8566.47		36485.7	
	T1	18967.1		9148.04		27952.2	
	T2	25112.1	0.99	10618.3	0.56	63256.9	0.75
1241	N1	39531.2		14807.7		36376.5	
	N2	42012.9		26007.7		85206.7	
	T1	34772.6		4228.42		562.96	
	T2	31573.7	0.85	11782.6	0.63	42751	0.03
1482	N1	59711.9		16423.5		55152.3	
	N2	45060.9		21323.7		72434.9	
	T1	34635.1		19246.4		3793.05	
	T2	40567.8	0.64	27966.5	0.89	12612.9	0.39
1545	N1	3253.9					
	N2	3909.12					
	T1	4877.08					
	T2	8155.02	0.72				
2077	N1	79122.2				26960.3	
	N2	171115				102518	
	T1	80710.1				39396.3	
	T2	204833	0.85			103169	0.69
2266	N1					32879.4	
	N2					99084.3	
	T1					28563.3	
	T2					79816.7	0.93
2289	N1	55735.3		11601.5		9832.98	
	N2	44197.4		9482.87		15664.1	
	T1	42362.5		11707.4		1055.31	
	T2	34203.8	0.98	13629.8	0.70	5560.37	0.30
2465	N1	17876.6		35434		15958.5	
	N2	20283		34843.5		33476.6	
	T1	9443.94		9987.01		6368.63	
	T2	12461.1	0.86	28783	0.34	22843.2	0.58
2529	N1	12794.2		3115.25		6644.63	
	N2	16624.5		4936.9		21880.8	
	T1	11991.9		992.73		1585.61	
	T2	11341.2	0.73	1974.29	0.80	7512.35	0.70
2588	N1	4936.53		12438.2		9027.57	
	N2	6749.89		27282.6		38304.2	
	T1	59954.5		18569.3		2424.41	
	T2	59748.6	0.73	30385.1	0.75	22953	0.45
2590	N1	39821.5		3667.87		15477.5	
	N2	39821.5		3779.73		53823	
	T1	15772.9		4790.63		9782.3	
	T2	23164.5	0.68	7798.24	0.88	37367.9	0.91
2636	N1	62807.6				5036.91	
	N2	66770.9				21129.1	
	T1	133063				3492.99	
	T2	109216	0.77			21046.4	0.70
2637	N1	14931.1		5997.72		3561.76	
	N2	64870.1		9954.96		19542.8	
	T1	54012.5		10437.4		805.23	
	T2	85528	0.36	14858.8	0.86	5287.56	0.84
2659	N1	5478.01		5943.98		3865.65	
	N2	4256.65		28501.3		24434.8	
	T1	9695.11		31391.1		35161.5	
	T2	12695.9	0.59	36838.9	0.24	57697.7	0.26
2697	N1	1571.27		12556.4		15116.4	
	N2	4032.54		12845.6		23083.8	
	T1	14119.6		10268.4		17045.9	
	T2	21641	0.60	9195.37	0.88	21428.3	0.82
2715	N1			40210.1		46382.6	
	N2			39895		106778	
	T1			27072.4		28747.8	
	T2			32524.4	0.83	85180.7	0.78
2741	N1	11374.2		26025.1		9258.2	
	N2	17483.1		25136.5		29260.3	
	T1	10931.9		10957		13512.7	
	T2	13007.8		24154.7	0.44	40646.4	0.95
2765	N1	41413.4		37180.7		5091.89	
	N2	50978.7		43611.6		14048.1	
	T1	5621.7		31971.4		6346.44	
	T2	9096.42	0.76	44294.2	0.85	15243.9	0.87
2805	N1	64947.8		54747.1		22936.8	
	N2	39997.6		50274.9		38980.7	
	T1	27810.6		35249.8		19563.9	
	T2	34999.2	0.49	50909.4	0.64	31400.6	0.94
2823	N1	1097.31		45485.5		11327.4	
	N2	4101.66		44302.6		18019.4	
	T1	3916.11		21896.8		5067.25	
	T2	3466.18	0.24	23820.4	0.90	12739.8	0.63
2832	N1	13784.8		9364.75		1930.01	
	N2	23027.9		7245.23		10663.5	
	T1	6764.14		3120.5		6421.23	
	T2	14398.9	0.78	1744.76	0.72	25155.5	0.71
2894	N1			28957.6		48623.3	
	N2			23500.6		96695.3	
	T1			39288.4		33196.6	
	T2			35332	0.90	51104.7	0.77
2901	N1	18677		20592.6		6371.56	
	N2	33159		27767.7		21597	
	T1	30911.3		8244.51		2933.08	
	T2	47641.7	0.87	17486.8	0.64	12012.3	0.83
2912	N1	10298.9		2744.87		13908	
	N2	14612.7		3447.5		21998.3	
	T1	4905.66		3314.73		15967.4	
	T2	6755.36	0.97	3449.42	0.83	21868.3	0.87
2917	N1	70182.2		820.17		37466.3	
	N2	71288.6		1686.04		80619.3	
	T1	45080.3		965.49		17173.6	
	T2	64690.8	0.71	2790.8	0.71	54604.6	0.68
2920	N1	105974		11895.7		57353.3	
	N2	86439		10907.2		64119.6	
	T1	44781.9		2824.63		22113	
	T2	55636.8	0.66	3907.69	0.66	32859.7	0.75
2933	N1	9132.32		6426.58		2453.93	
	N2	12014.7		9263.72		7596.04	
	T1	10848		1191.18		3797.75	
	T2	12963.6	0.91	1134.32	0.66	9939.58	0.85

		D17S799		D17S798		TP53	
IN	Normal Tumour	Peak Area	Tumour/Normal Ratio	Peak Area	Tumour/Normal Ratio	Peak Area	Tumour/Normal Ratio
1212	N1	34942.5		10692.7		1070.12	
	N2	35999.4		30101.8		1739.6	
	T1	18934.2		17655.9		871.81	
	T2	20874.5	0.93	33813.9	0.68	2426.04	0.58
1241	N1	22538.6		3393.48		27758.9	
	N2	25400.7		8201.16		22569.6	
	T1	1878.68		4250.81		13786.2	
	T2	412.35	0.19	7533.55	0.73	14327.5	0.78
1482	N1	186.25		7539.47		1345.89	
	N2	337.59		7065.69		2061.01	
	T1	21382.1		5411.2		7245.04	
	T2	27110	0.70	9955.1	0.51	10244.8	0.92
1545	N1	20769.4		6167.67			
	N2	29026.4		6617.94			
	T1	22816.9		4740.28			
	T2	30976.7	0.97	6793.05	0.75		
2077	N1	36201.4		27908.4			
	N2	41708.7		54309.6			
	T1	15612		29366			
	T2	27981.1	0.64	64947.1	0.88		
2266	N1			7257.06			
	N2			7197.01			
	T1			7609.86			
	T2			8023.71	0.94		
2289	N1	3271.39		9341.86		4238.05	
	N2	16317.8		12406.4		7197.2	
	T1	1952.08		7159.83		5174.18	
	T2	14514.9	0.67	3052.21	0.32	4177.67	0.48
2465	N1	17876.6		376.89		12729.7	
	N2	20283		26552.6		31787.2	
	T1	9443.94		9306.88		14987.2	
	T2	12461.1	0.86	15501.9	0.02	33936.5	0.91
2529	N1	1139.66		1358.85			
	N2	11966.7		2752.1			
	T1	137.38		1350.96			
	T2	1452.6	0.99	3555.21	0.77		
2588	N1	710.78		2951.04		45430.5	
	N2	1186.64		3558.3		34616.3	
	T1	14357.2		13186.1		55461.4	
	T2	15336.5	0.64	13836	0.87	39675.7	0.94
2590	N1	5836.62		24783.4		4405.92	
	N2	10210.2		34502.4		4920.62	
	T1	17629.7		39521.4		20037.9	
	T2	25591.7	0.83	41753.5	0.76	18430.4	0.82
2636	N1	5993.96		8993.22		2124.41	
	N2	13424.8		31976.6		2272.79	
	T1	429.93		4038.14		3858.05	
	T2	4062.33	0.24	21443	0.67	4028.98	0.98
2637	N1	22734.2		2577.82		7778.64	
	N2	22845.7		13038.1		4941.04	
	T1	41381		9272.75		3584.87	
	T2	16364.7	0.39	50141.3	0.94	1529.63	0.67
2659	N1	275.7		6254.31		241.21	
	N2	1753		13419.7		2147.96	
	T1	11019.1		15509.2		3617.81	
	T2	14808.1	0.21	24135.3	0.73	4198.7	0.13
2697	N1	2372.98		19996.1		2931.1	
	N2	13384.9		17857.7		3355.87	
	T1	4147.92		8203.54		1779.28	
	T2	15817	0.68	12189	0.60	2373	0.86
2715	N1	254.31		99.56		17512.7	
	N2	663.47		1637.33		16079.3	
	T1	6664.48		9914.77		22010.9	
	T2	7246.24	0.42	14696.7	0.09	21037.8	0.96
2741	N1	2190.66		1185.76		8118.86	
	N2	2238.76		17563.8		4691.46	
	T1	2264.66		5774.31		36725.8	
	T2	4634.68	0.50	13627.4	0.16	24477.4	0.87
2765	N1	3243.37		9551.36		6291.64	
	N2	10881.3		22303.6		8128.88	
	T1	3368.71		281.82		7688.23	
	T2	5928.33	0.52	4251.44	0.15	7012.36	0.71
2805	N1	20081.4		22860.9		15447.4	
	N2	21980.6		36390.6		9994.99	
	T1	2519.65		30555.5		23878.1	
	T2	4759.35	0.58	58877.2	0.83	16726.5	0.92
2823	N1	1369.52		9939.35		47877.1	
	N2	1999.56		25085.2		30954.7	
	T1	900.591		865.08		39044.4	
	T2	4606.35	0.29	4680.08	0.47	22344.7	0.89
2832	N1					3394.42	
	N2					2989.47	
	T1					9556	
	T2					8299.26	0.99
2894	N1	2438.61		5378.09			
	N2	378.34		9887.35			
	T1	26745.9		349.92			
	T2	2438.61	0.59	7292.64	0.09		
2901	N1	5835.03		8398.08		19375.5	
	N2	12392.2		18735.1		27110.6	
	T1	4301.86		5400.14		11811.5	
	T2	8096.59	0.89	4069.51	0.34	14509.8	0.88
2912	N1			8098.86		1763.54	
	N2			19690.2		2088.44	
	T1			10661.4		6970.96	
	T2			20110.2	0.78	7037.06	0.85
2917	N1	3098.1		1023.31			
	N2	6236.64		4878.7			
	T1	1180.12		626.85			
	T2	6327.92	0.38	1805.23	0.60		
2920	N1	5863.41		28361.2			
	N2	15214.7		31821.1			
	T1	1846.05		11478.6			
	T2	6618.87	0.72	23093.1	0.56		
2933	N1	1420.43		11261.5		2631.64	
	N2	2123.18		12850.7		2348.97	
	T1	594.062		8896.5		4169.29	
	T2	1545.71	0.57	11195.2	0.91	4147.21	0.90

APPENDIX III

Summary of clinical protocols used in the treatment of children included in this study

1. Baby Brain Protocol (UKCCSG CNS 9204)

For patients less than 3 yeaers of age with malignant brain tumours.

After surgery, patients received vincristine carboplatin, methotrexate, cyclophosphamide and cisplatin over a 43 day cycle with a maximum of 7 cycles, a total treatment time of 379 days.

2. CHP455 Protocol

For patients 3 years of older with metastatic medulloblastoma or intracranial PNET.

Following surgery, patients received radiotherapy with concurrent chemotherapy of 6 cycles of vincristine. Chemotherapy comprising a combination of vincristine, CCNU and cisplatin was started 6 weeks after the end of radiotherapy. A total of 8 cycles was given.

3. Infant PNET

Following surgery, all patients were given induction chemotherapy of cyclophosphamide, carboplatin, vincristine and G-CSF (lenograstin) on day 1 followed by white blood harvest on day 14, a further cycle of combination chemotherapy on day 15 and white blood infusion on day 17. Those patients with posterior fossa tumours which responded to

therapy subsequently received local radiotherapy and oral etoposide whilst those patients with supratentorial primaries or posterior fossa tumours which failed to respond received craniospinal therapy, oral etoposide or entered a phase II study.

4. PNET III Protocol

For patients with non-metastatic disease aged 3 years or older.

Following surgery, patients were randomised to receive either radiotherapy or chemotherapy followed by radiotherapy. The chemotherapy comprosed of 4 cycles of vincrisine, etoposide and either carboplatin or cyclophosphamide in alternate cycles.

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