THE UNIVERSITY OF HULL

The Role of Chromosome 22 in the Progression of Head & Neck Squamous Cell Carcinoma

Being a thesis submitted for the degree of Doctor of Medicine

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By

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This thesis is dedicated to my wife Claire and my sons George and Joseph.

Abstract

Head and neck squamous cell carcinoma (HNSCC) is one of the ten most frequent cancers in the world. Despite advances in surgery, radiotherapy and chemotherapy over the last 30 years, there has been little improvement in the mortality rate from this disease. HNSCC remains a leading cause of cancer death, particularly once it has metastasised to the regional lymph nodes. The presence of metastatic cervical lymphadenopathy at the time of diagnosis reduces the likelihood of 5-year survival by 50%.

Preliminary comparative genomic hybridisation (CGH) work in our laboratory, aimed at identifying genetic markers of metastasis, suggested that amplification of chromosome arm 22q may be important in enabling malignant cells to metastasise. Overamplification of 22q was observed in 5/17 (29%) lymph node metastases (LNM) compared with none of their matched primary tumours. In a further group of 12 non-metastasising primary tumours there was no evidence of either gross deletion or amplification of 22q, supporting its unique association with metastasis. However, a literature search revealed two other studies that had identified significant aberration of 22q in primary head and neck cancers, as opposed to metastases.

Thus two contrasting bodies of evidence exist for the role of a putative oncogene on 22q in HNSCC. Two studies support a role during initial tumorigenesis, whilst another supports a role in the process of lymph node metastasis.

The aim of this thesis was to determine which of these two roles for 22q is more likely in HNSCC, and to narrow the search for the putative oncogene in question. For patients with HNSCC, microsatellite markers were used to investigate allele imbalance (AI) along 22q and in doing so to construct a high resolution AI map of this chromosome arm.

The 9 microsatellite markers selected covered approximately 25Mb (72%) of 22q, spanning 22q11.23 to 22q13.31. A series of 28 patients with nodal metastases from laryngeal or hypopharyngeal squamous cell carcinoma were selected. DNA was extracted from normal tissue and primary tumour for each patient, and microsatellite marker analysis performed. The microsatellite loci examined, and rates of AI were as follows: D22S420 (11%), D22S539 (25%), D22S117 (4%), D22S315 (0%), D22S1163 (15%), D22S280 (20%), D22S277 (15%), D22S283 (11%) and D22S1169 (0%). Overall there was no significant AI (mean rate AI = 8.2%), thus confirming our null hypothesis based on our CGH results that there would be no significant AI between the normal and tumour DNA samples. This finding would tend to refute a role for 22q in initial tumorigenesis, and future work will examine the corresponding lymph node metastases for AI. Microsatellite instability was a very infrequent event (1% cases), suggesting that HNSCC does not primarily arise through defects in the mismatch repair system.

The role of 22q at the protein level was also examined using immunohistochemistry. Gene databases were searched for potential candidate oncogenes, which may be responsible for the metastatic competence of head and neck tumours with 22q amplification. The matrix metalloproteinase (MMP)

Stromelysin III (MMP-11) is encoded by a gene that resides on 22q11.2. Evidence from studies of other malignancies supports a role for MMP-11 in the initial invasive stages of the metastatic cascade. The second part of the thesis tested the hypothesis that there would be no difference in MMP-11 expression between normal tissue and primary tumour, but that there would be increased MMP-11 expression in lymph node metastases. This was found to be the case, with a significantly higher level of MMP-11 detected in the lymph node metastases ($p=0.01$). MMP-11 may therefore be a putative proto-oncogene responsible for allowing head and neck squamous cell carcinoma cells to metastasise.

The sequencing results do not support a specific role for aberrations of chromosome 22q in initial head and neck tumorigenesis. They also exclude defects in the mismatch repair system as a significant mechanism of tumorigenesis in head and neck cancer. The immunostaining results do support a role for MMP-11 overexpression in the progression from primary tumour to metastasis. It remains to be seen whether the observed overexpression of MMP-11 in metastatic carcinoma cells is due to amplification of chromosome 22q. This work will be continued, with microsatellite analysis of the matched lymph node metastases. These findings will increase our understanding of the molecular mechanisms of metastasis, and hopefully provide novel therapeutic targets.

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Abbreviations

AI	allele imbalance
AJCC	American Joint Committee on Cancer
bp	base pairs
BV	blood vessel
CCD	charged coupled device
cDNA	complementary DNA
CEPH	Centre d'Etudes Polymorphisme Humain
CGH	comparative genomic hybridisation
cM	centimorgan
CT	connective tissue
DAB	3, 3'-diamino-benzidine tetrahydrochloride
DCC	deleted in colorectal carcinoma
EBV	Epstein-Barr virus
ECM	extra cellular matrix
EGFR	epidermal growth factor receptor
FHIT	fragile histidine triad gene
FISH	Fluorescence *in situ* hybridization
GST	glutathione s-transferases
H&E	haemotoxylin and Eosin
HNSCC	head and neck squamous cell carcinoma
HPV	human papilloma virus
HRP-StrepABC	horseradish peroxidase-streptavidin-biotin complex
IHC	immunohistochemistry
KP	keratin pearls
LCM	laser capture microdissection
LOH	loss of heterozygosity
LNM	lymph node metastases
MI	microsatellite instability
MMP	matrix metalloproteinase
MMP-11	Stromelysin
mRNA	messenger RNA

MT-MMP	membrane-type matrix metalloproteinase
MVD	microvessel density
NK	natural killer
NPC	nasopharyngeal carcinoma
NS	not significant
PCR	polymerase chain reaction
PRB	retinoblastoma protein
PRKM 1	protein kinase, mitogen activated 1
Rb	retinoblastoma gene
SC	squamous cell
SCC	squamous cell carcinoma
TBS	tris buffered saline
TIMP	tissue inhibitors of MMP
TNM	tumour, nodal metastases, distant metastases
TSG	tumour suppressor gene
UADT	upper aero digestive tract
UICC	L'Union Internationale Contre le Cancer
VEGF	vascular endothelial cell growth factor
WT	wild-type

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Declaration

The planning, experimental design and execution of the studies and technical work involved in this thesis were all performed by the candidate unless otherwise stated.

No part of this thesis has been submitted in support of an application for any degree or qualification in any other institute of learning.

Publications and presentations arising during the course of this thesis

Publications

Overexpression of Bcl-2 in Carcinoma of the Larynx – a marker of Radioresistance.
L.T. Condon, J.N.E. Ashman, S.R. Ell, N.D. Stafford, J. Greenman and L. Cawkwell
International Journal of Cancer 2002; 100: 472-475.

Prognostic Value of Genomic Alterations in Head and Neck Squamous Cell Carcinoma Detected By Comparative Genomic Hybridisation.
JNE Ashman, HS Patmore, **LT Condon,** L Cawkwell, ND Stafford and J Greenman.
British Journal of Cancer 2003; 89: 864-869.

Published abstracts

Overexpression of Bcl-2 in Carcinoma of the Larynx – a marker of Radioresistance.
L.T. Condon, J.N.E. Ashman, L. Cawkwell, S.R. Ell, J. Greenman, N.D. Stafford.
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JNE Ashman, HS Patmore, **LT Condon,** L Cawkwell, ND Stafford and J Greenman.
Journal of the American College of Surgeons (In press).

Presentations

The Identification of Genes with Prognostic value in HNSCC.

L.T. Condon

"Advancing the Frontiers of Surgical Research" to the President and benefactors of The Royal College of Surgeons of England. London, April 2001.

Overexpression of Bcl-2 in Carcinoma of the Larynx – a marker of Radioresistance.

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Inhibition Of Apoptosis Is Associated With Radioresistance In Laryngeal Squamous Cell Carcinoima.

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Prognostic Value of Genomic Alterations in Head and Neck Squamous Cell Carcinoma Detected By Comparative Genomic Hybridisation.

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Meeting of the American College of Surgeons, Chicago, October 2003.

Manuscripts in preparation

A study of allele imbalance and microsatellite instability at 22q in head and neck squamous cell carcinoma.

LT Condon, HS Patmore, ND Stafford, RP Baker, J Greenman and L Cawkwell.

Overexpression of MMP-11 in the lymph node metastases of head and neck squamous cell carcinoma.

LT Condon, PA Nix, ND Stafford, J Greenman and L Cawkwell.

CHAPTER 1

INTRODUCTION

Introduction

1.1 The nature of head & neck cancer

The term "head and neck" cancer refers to a primary malignancy arising within the head and neck region (Figure 1.1). This definition encompasses a broad range of tumours, but excludes central nervous system and ocular tumours.

Squamous cell carcinoma is the commonest histological subtype, accounting for over 95% of head and neck cancers (Benninger, 1992), and gives rise to the collective term "head and neck squamous cell carcinoma" (HNSCC). This entity mainly encompasses squamous cell carcinoma (SCC) of the upper aerodigestive tract (UADT). In practice, this comprises the lips, oral cavity, oropharynx, nasopharynx, nose and paranasal sinuses, hypopharynx, cervical oesophagus and the larynx. Far less commonly involved head and neck subsites include the outer and middle ear, salivary glands and trachea.

The remaining histological subtypes of head and neck malignancy include adenocarcinomas, usually of the nasal cavity and paranasal sinuses, mucoepidermoid and adenoid cystic carcinomas of the salivary glands and papillary and follicular carcinomas of the thyroid gland. Primary lymphomas, lymphoepitheliomas and sarcomas can also arise in the head and neck region.

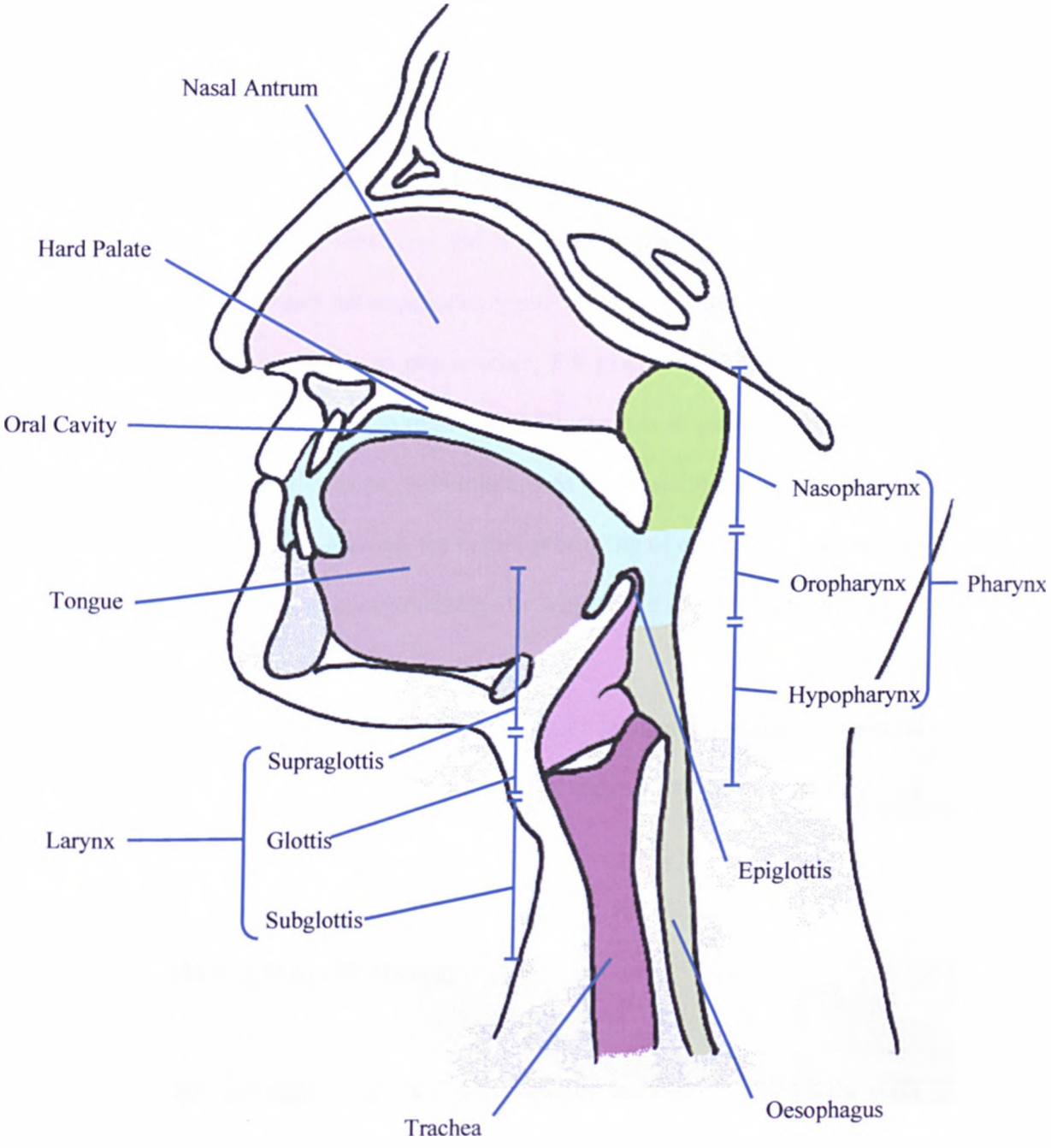


Figure 1.1 Anatomy of the Head and Neck region. Head and neck squamous cell carcinoma encompasses a diverse group of tumours arising in the epithelium lining the structures depicted above. (Adapted, with kind permission of J. Ashman)

1.1.1 Tumour heterogeneity

Most molecular research in HNSCC considers all squamous cell carcinomas of the head and neck region together, on the assumption that they share a common aetiology and pattern of biological behaviour, regardless of subsite. This may be an over-simplification, however, and it may be more appropriate to consider them as a diverse and heterogeneous group of tumours, that share little more than anatomical proximity to one another. For example, whilst smoking is an aetiological factor common to most HNSCCs, the role of genetic predisposition and the Epstein-Barr virus are well established in the aetiology of most cases of nasopharyngeal SCC. Likewise, the earlier propensity of oral SCC to metastasise to regional lymph nodes contrasts markedly with that of glottic laryngeal SCC.

This thesis is concerned only with the study of laryngeal and hypopharyngeal SCC, in an attempt to minimise the impact of tumour subsite as a confounding factor.

1.2 Incidence and epidemiology

Over 500,000 new cases of HNSCC are diagnosed each year, making it the sixth most common cancer and accounting for 5% of all cancers worldwide (Parkin *et al.*, 1993; Vokes *et al.*, 1993).

The marked global variations in incidence reflect the variable prevalence of aetiological factors (both genetic and environmental) in different parts of the world. In the Asian subcontinent the incidence is far greater, with oral cancer reported to be the third most common malignancy after cancer of the cervix and stomach (Parkin *et al.*, 1988). In Bombay cancer of the oral cavity is the most common malignancy accounting for 50% of all cancers. This relates to the local practices of chewing betel quid and habitual reverse smoking of homemade cigars (Squier *et al.*, 1984).

Around 7,000 new cases of head and neck cancer are diagnosed each year in the U.K., giving an overall incidence of 11.8 per 100,000 and accounting for 2.8% of all cancers (Yorkshire Cancer Organisation, 1995). The relative site distribution of head and neck cancers in Yorkshire is shown in Figure 1.2, and is fairly typical for the whole of the U.K. However, there is considerable geographical variation in incidence, with almost twice the incidence in Wales (15.3 per 100,000) compared to South-East Thames (7.7 per 100,000) (Effective Head and Neck Cancer Management, Third Consensus Document, 2002).

HNSCC is more common in males than females with an average overall predominance of 2:1 (Yorkshire Cancer Organisation, 1995). The only exception to this is post cricoid carcinoma, which is commoner in females due to its association with iron deficiency anaemia as part of the Patterson-Brown-Kelly syndrome (Morton *et al.*, 1997). The sex ratio of laryngeal carcinoma has been approximately 10:1 male to female, but an increase in the incidence in women over the last ten years has seen this ratio fall to 7:1. Laryngeal tumours are also

twice as common in heavily industrialised areas. Incidence increases with age, the condition typically being diagnosed in the seventh or eighth decade of life.

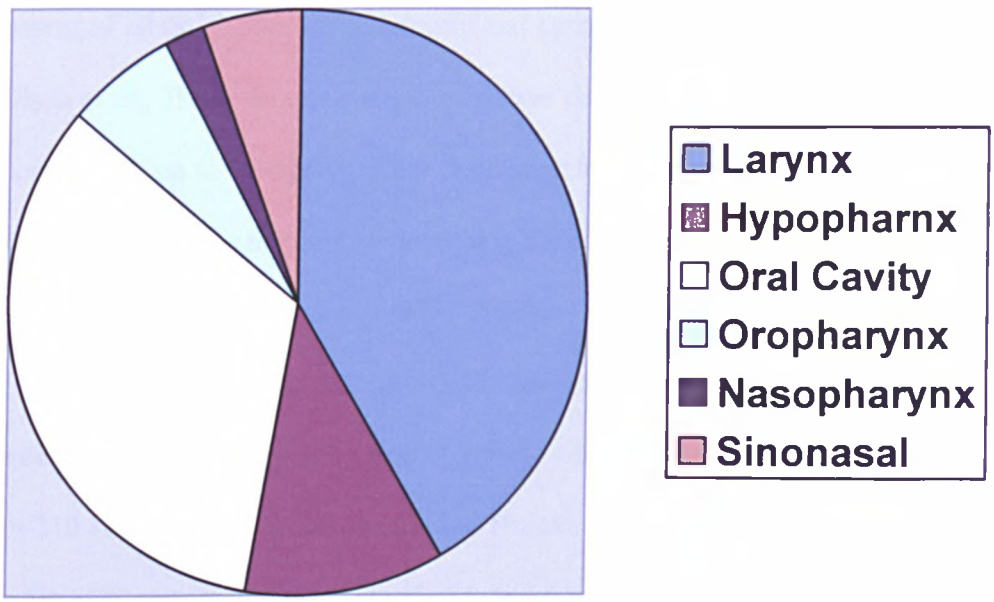


Figure 1.2 Site distribution of HNSCC in Yorkshire, 1985-1989 (Yorkshire Cancer Organisation).

1.3 Aetiology

HNSCC is a largely preventable disease in that exposure to certain environmental factors, particularly tobacco and alcohol, appears to play a far greater role in its aetiology than inherited genetic predisposition. These and other carcinogens have mainly been identified through epidemiological studies, in which a correlation has been observed between prevalence of exposure and incidence of HNSCC.

1.3.1 Tobacco and alcohol

Tobacco and alcohol use are the two strongest aetiological factors for the development of HNSCC both independently and synergistically (Merletti *et al.*, 1989; Olsen *et al.*, 1985). In one study the relative risk of laryngeal cancer was 4.4 for smokers of up to 10 cigarettes per day, rising to 34.4 for smokers of over 40 per day, demonstrating the dose-dependent relationship between smoking and incidence of HNSCC (Wynder *et al.*, 1976). Tobacco smoke consists of a potent mix of over 4000 chemicals, 43 of which have so far been identified as carcinogens and include polycyclic hydrocarbons, nitrosamines and radioactive poloniun-210 (US Department of Health and Human Services, 1989). The link between HNSCC subsite and the method of tobacco use (e.g. smoking, chewing and reverse smoking) was discussed earlier in Section 1.2.

The precise role of alcohol consumption in the development of HNSCC has been more difficult to ascertain. Blot *et al.* (1988) have postulated that as a solvent, ethanol enhances the contact of tobacco carcinogens with the upper aerodigestive tract mucosa. Other groups have implicated nitrosamines (also present in tobacco), which are present in high levels in beer, lower levels in distilled spirits and negligible levels in wine (Berger, 1998). The picture has been further complicated by the recent discovery of resveratrol in grapes and wine, and the subsequent demonstration of its cancer chemopreventive activity (Jang *et al.*, 1997). It has therefore emerged that the type of alcoholic beverage is relevant in considering risk, and not simply the total quantity of ethanol imbibed. The most recently published study to examine the association between different

alcoholic drinks and cancer of the upper aerodigestive tract was a Danish population based study of 28,180 (Gronbaek *et al.*, 1998). Compared with non-drinkers, subjects who drank 7-21 units/week of beer/spirits but no wine had a relative risk of 3.0 of developing oropharyngeal or oesophageal cancer. However, subjects who drank a similar amount but who also drank wine as >30% of their total alcohol intake had a relative risk of 0.5. For subjects who consumed >21 units/week the relative risks, including and excluding wine, were 5.2 and 1.7 respectively. Thus, while there is a strong association between beer and spirit consumption and upper aerodigestive tract cancer, those who drink a similar quantity of wine are at a lower risk, whilst moderate wine drinkers may be afforded an overall degree of protection, compared to their tee-total counterparts.

1.3.2 Viruses

Human papilloma virus (HPV) is believed to play a central role in the aetiology of squamous cell carcinoma of the cervix (Giannoudis and Herrington, 2001) and has recently been implicated in HNSCC (Snijders *et al.*, 1996). HPV genotypes have been classified into three risk categories for the onset of malignant phenotypes: high (types 16, 18), medium (types 31, 33) and low risk (types 6, 11) (de Villiers, 1994). Adult onset recurrent respiratory papillomatosis, caused by infection with low risk HPV types 6 and 11, is associated with a small chance (3%) of malignant transformation to veruccous SCC (Klozar *et al.*, 1997). Using polymerase chain reaction (PCR) based analysis, DNA of the high risk HPV types 16 and 18 has been detected in

HNSCC at a frequency of 35-60% with the highest frequency in tumours of the oral cavity (Anwar *et al.*, 1993; Clayman *et al.*, 1994; McKaig *et al.*, 1998). Another study demonstrated that the presence of HPV 16/18 DNA was associated with an increased risk of head and neck cancer formation, with an odds ratio of 4.32 (Nishioka *et al.*, 1999). Two protein products of HPV 16/18 DNA encoded by exons 6 and 7 (E6 and E7) have been shown to inactivate the tumour suppressor gene products p53 and pRb (retinoblastoma protein), thereby potentiating cell immortalization (Flaitz *et al.*, 1998). Rather than acting as a major independent risk factor for HNSCC, a recent review of the literature concluded that high risk HPV infection is more likely to play a synergistic role with behavioural risk factors, such as cigarette smoking and alcohol consumption (Almadori *et al.*, 2002).

The most compelling evidence for a viral aetiology exists between Epstein-Barr virus (EBV) and nasopharyngeal carcinoma (NPC). In South-East Asia, where this malignancy is endemic, the EBV genome is consistently detected in nasopharyngeal carcinoma cells (Vasef *et al.*, 1997). The EBV genome not only encodes oncogenic proteins itself (e.g. EBNA-LP) but also induces host expression of proto-oncogenes (e.g. *bcl-2*, *bcl-10* and *jun/fos*) (Wattre and Hober, 1996). Incidentally, the consumption of nitrosamines in salted fish, which is a dietary standard in China, has also been identified as an independent risk factor for nasopharyngeal carcinoma (Fedder and Gonzalez, 1985). It should be emphasised that NPC is quite different to other HNSCC subsites in terms of tumour aetiology and behaviour.

1.3.3 Familial predisposition to HNSCC

Inherited genetic factors are often overlooked in what are perceived to be environmentally induced cancers such as HNSCC. In a large retrospective case-controlled study the adjusted relative risk for HNSCC development was 3.79 for first- degree relatives of patients with HNSCC, rising to 7.89 in first-degree relatives of patients with multiple HNSCC (Foulkes *et al.*, 1996). A possible explanation for this variability in susceptibility may lie in the genetic polymorphisms of several families of enzymes involved in the metabolism of carcinogens. Glutathione s-transferases (GSTs) are one such family, which catalyse the conjugation of carcinogens to glutathione (Tsuchida and Sato, 1996). GSTP1, an important member of this family, is polymorphic in humans, with allelic variants differing in their catalytic activities towards a range of carcinogens (Park *et al.*, 1997). The GSTP1 AA genotype is thought to confer relative resistance to mutagens, and has been found at a significantly lower frequency in individuals with oropharyngeal and laryngeal cancers (Jahnke *et al.*, 1999). Conversely, less active allelic variants of cytochrome-p450 (Wu *et al.*, 1997) and N-acetyltransferase (Katoh *et al.*, 1998) have been associated with an increased risk of upper aerodigestive tract and oral squamous cell carcinoma, respectively.

1.3.4 Occupational and dietary factors

Nickel and chromate refining workers have an increased incidence of laryngeal cancer, whilst hard-wood dust has been identified as an aetiological factor in the development of adenocarcinoma of the paranasal sinuses (Acheson *et al.*, 1968). There is an association between Paterson-Brown-Kelly syndrome (iron deficiency anaemia, glossitis, koilynchia and upper oesophageal webbing) and postcricoid carcinoma. The underlying iron deficiency in this condition may explain the relative preponderance of postcricoid carcinoma in young females (Morton *et al.*, 1997). Paterson-Brown-Kelly syndrome, which can be reversed with iron replacement and vitamin B therapy, is becoming less common with dietary improvements, and so less important as a risk factor for postcricoid carcinoma (Maran *et al.*, 1993). The association between a salted fish diet, particularly in childhood, and the later development of nasopharyngeal carcinoma in Chinese individuals with EBV was discussed in section 1.3.2 (Yu *et al.*, 1986). Epidemiological data suggest a protective role of dietary carotenoids and an inverse association between the consumption of fruits and vegetables and the incidence of head and neck cancer (Vokes *et al.*, 1993).

1.4 The Natural history of HNSCC

1.4.1 Histology of HNSCC

The diagnosis of SCC of the larynx and hypopharynx is a histological one, which depends on the microscopic examination of an adequate biopsy of the

tumour, usually obtained at endoscopy in the anaesthetised patient. SCC arises from squamous epithelium via a series of well-defined histopathological steps (see Figure 1.3).

The majority of the upper aerodigestive tract is lined with non-keratinising stratified (stacked) squamous (flat) epithelial cells (Wheater *et al.*, 1987; see Figure 1.3). Epithelium is defined by its adherence to an underlying basement membrane, which is composed of Type IV collagen and adhesive glycoproteins (e.g. laminin) embedded in a proteoglycan ground substance (e.g. heparan sulphate) (Kumar *et al.*, 1992a).

The first histological manifestation of progression towards a malignant phenotype is an increase in the rate of cell proliferation. The cells retain the appearance of normal an increase in the rate of cell proliferation. The cells retain the appearance of normal epithelial cells, but exhibit an increased rate of proliferation. This appearance is termed hyperplasia, and is potentially reversible upon removal of the causative agent. Histologically the epithelium is thickened with increased mitotic activity in the basal layer.

Progression to dysplasia entails a change in the microscopic appearance of the cells, as well as increased proliferation over the basal rate. The cells are less well differentiated than normal squamous cells, and demonstrate nuclear hyperchromia (dark staining nuclei), hyperplasia (enlarged nuclei) and pleomorphism (altered nuclear shape), prominent nucleoli and an increased cellular density and cellular pleomorphism (Speight *et al.*, 1996).

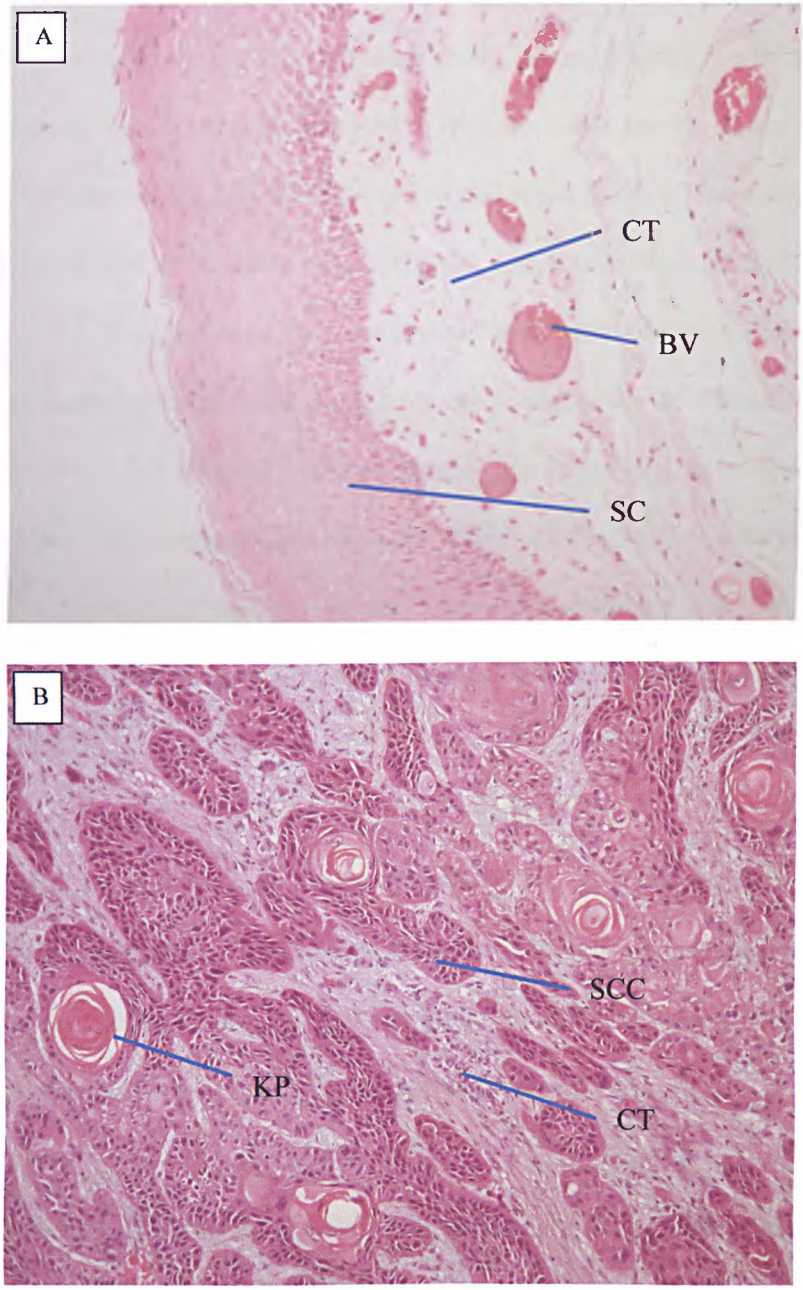


Figure 1.3 Histology of Head and Neck Squamous Cell Carcinoma. Haemotoxylin and Eosin (H&E) stained tissue sections (Magnification x100). Figure 1.3(A) depicts the appearance of normal, stratified squamous epithelium of a human uvula removed from a chronic snorer. A regular layer of stacked squamous cells (SC) can be seen with underlying connective tissue (CT). Blood vessels (BV) are also visible in the connective tissue. Figure 1.3(B) depicts an H&E stained section of a well differentiated SCC. 'Nests' of malignant squamous cell carcinoma (SCC) are seen invading the surrounding connective tissue. Characteristic keratin pearls (KP), whorls of accumulated keratin, are also seen.

Dysplasia is graded according to how much of the epithelium between the basal and the uppermost layer is affected by the above changes. Mild, moderate and severe degrees signify the presence of dysplastic cells in the lower third, extension into the middle and involvement of the upper thirds of the epithelium respectively. Whilst it is likely that epithelial dysplasia always precedes the appearance of cancer, dysplasia does not invariably progress to cancer (Kumar *et al.*, 1992b). Indeed mild to moderate degrees of dysplasia may be reversible and, with removal of the putative inciting agents, the epithelium may revert to normal. Hence, the treatment for these two grades of dysplasia is cessation of exposure to aetiological factors (primary cancer prevention), and "watchful waiting" to ensure that, if it occurs, malignant transformation is detected as early as possible.

Severe dysplasia, however, in which dysplastic changes involve the entire thickness of epithelium, is considered a pre-invasive form of cancer, and is referred to as carcinoma-in-situ. This is deemed irreversible, and therefore requires treatment to prevent progression to invasive carcinoma.

Histologically, invasion of the basement membrane is the seminal event that separates carcinoma-in-situ and carcinoma. Breech of the basement membrane and local invasion of tissues by malignant cells represents the malignant phenotype.

HNSCC is graded by the histopathologist into well, moderately or poorly differentiated, according to the degree of keratinisation and cell stratification. A

well differentiated tumour closely resembles normal squamous epithelium (> 75% keratinisation and uniform stratification; see Figure 1.3B), whilst a poorly differentiated tumour has lost many of the features of normal squamous epithelium and bears little resemblance to it (<25% keratinisation and disordered or absent stratification). In a retrospective study of 1,266 patients treated for HNSCC, Fortin *et al.* (2001) demonstrated an association between advanced histological grade and the likelihood of distant metastases.

1.4.2 Field cancerization

Field cancerization is the theory that areas of UADT mucosa exposed to the same carcinogenic insults might respond in a similar fashion to each other (Slaughter *et al.*, 1953). Slaughter *et al.* originally proposed the theory to account for the presence of non-contiguous, multicentric oral cancers, separated by areas of histologically normal or dysplastic mucosa. The concept has also been extended to account for the observation that 1-2% of patients presenting with a HNSCC have a synchronous second primary tumour, and that around 15% of patients with HNSCC will go on to develop a metachronous primary UADT during their lifetime (Jones *et al.*, 1995; Haughey *et al.*, 1992). Second primary tumours are usually squamous cell carcinomas of the aerodigestive tract, most often arising in the lung. Both histopathological and genetic evidence exists to support the theory of field cancerization. Widespread histopathological changes have been observed throughout the respiratory tract epithelium of smokers both with and without lung cancer (Auerbach *et al.*, 1961). More recently, loss of heterozygosity (LOH) studies have identified loss of genetic

material from chromosome 9p in histologically normal UADT mucosa of patients with HNSCC (see Section 1.8.2; Lydiatt *et al.*, 1998).

However, an alternative theory exists, that multiple primary tumours share a clonal origin. It is postulated that cells from the original primary tumour migrate within the mucosa to give rise to distant primaries, or re-implant at distant mucosal sites after being shed from the primary (Sidransky *et al.*, 1992). Support for this theory would require the identification of a marker of clonality. One study examined the pattern of X chromosome inactivation in a small population of female patients with HNSCC, and found the same X chromosome to be inactivated in all informative cases (n=4) (Bedi *et al.*, 1996). This supports a clonal origin, but the study is very small, and it may be that X chromosome inactivation occurs at such an early stage of embryogenesis that all cells in the UADT share a clonal embryological origin.

Several authors have studied patterns of acquired mutations in non-contiguous tumours, working on the theory that tumours of a clonal origin will share identical genetic aberrations. Such work has given conflicting results, with Gasperotto *et al.* (1995) demonstrating identical p53 mutations (see Section 1.8.2) in up to half of non-contiguous tumour pairs (n=9) and Chung *et al.* (1993) identifying different p53 mutations in all non-contiguous tumour pairs (n=21). The discordance of p53 mutations between non-contiguous tumour pairs in these studies would appear to support an independent origin for multiple primary tumours (i.e. field cancerization). However, the clonal theory of origin cannot be rejected, as further independent mutations may accumulate in cells of

the same clonal origin after they have micrometastasised to different sites, leading to genetic discordance between non-contiguous tumours. The lack of a reliable marker of clonality so far, means that the debate continues, although the two theories of field cancerization and clonal origin may not necessarily be mutually exclusive.

1.4.3 Cell biology of HNSCC (The principles of invasion)

This section deals with the changes that are necessary at a cellular level to achieve the malignant phenotype, whilst Section 1.7 will consider the genetic alterations under-pinning these changes. The hallmark of malignancy is uncontrolled proliferation of abnormal cells, leading to tumour formation. The net accumulation of tumour cells may be due to an increased rate of proliferation, or a decreased rate of cell death (or "apoptosis"), or both. Apoptosis, often known as "programmed cell death", is a distinct mode of cell loss responsible for the physiological deletion of cells in normal tissues (for review see Gastman, 2001). Unlike necrosis, it is an active process characterised by cell shrinkage and condensation and cleavage of nuclear chromatin. The cell cycle controls cell fate, and determines whether a cell undergoes mitosis, apoptosis, or remains senescent. Regulation of the cell cycle is under the control of the protein products of over forty genes, including both proto-oncogenes and tumour suppressor genes. The genetic aberrations responsible for the uncontrolled cell proliferation seen in malignancy are discussed in Section 1.8.

This escape from normal growth restraints represents neoplasia. However the malignant phenotype is further characterised by the invasion of surrounding tissues. Invasion is the active translocation of malignant cells across tissue boundaries and through host cellular and extracellular matrix (ECM) barriers. In the case of an epithelial malignancy such as HNSCC, this equates to invasion of the basement membrane in the first instance, and then deeper tissues such as fat, connective tissue, muscle, cartilage and even bone. To successfully achieve invasion a cell must breech the adjacent tissue and then migrate through it. The mechanisms are likely to be the same as those used by normal cells for physiological invasion (e.g. during embryogenesis and angiogenesis), and again it is deregulation of these processes that is responsible for invasion by malignant cells (Kendal, 1993). Each class of proteinase enzyme (e.g. matrix metalloproteinases) is paired with its own class of natural inhibitor (e.g. tissue inhibitor of metalloproteinases). Proteolysis of normal host tissue by malignant cell depends on the local balance between proteinase and inhibitor being tipped in the favour of proteolysis. Migration of malignant cells requires coordinated attachment and de-attachment to components of the extracellular matrix (via cell-matrix adhesion molecules e.g. E-cadherin). Forward locomotion of a malignant cell through a three dimensional matrix requires spatial and temporal organization of these processes. Thus, local proteolysis is followed by attachment of the advancing pseudopodium to matrix components in the zone of lysis. At the rear of the cell, this must be coordinated with dissociation from adjacent cells (via cell-cell adhesion molecules e.g. integrins) and detachment from previous attachment sites to release the cell, before motility factors stimulate contraction of the cytoskeleton and so propel the cell forward

(Aznavoorian and Liotta, 1992). The affinity of the integrin receptor for its ligand can be modulated by intracellular signals, and so the same receptor can be used at one time point for adhesion, and subsequently to release the adhesion (Thomas, 1997).

Growth of the primary tumour is naturally dependent on the recruitment of new blood vessels, both for its metabolic needs and for access to the circulation for metastasis (Folkman, 1971). Host endothelial cell proliferation is stimulated by a number of tumour produced growth factors, the most studied of which is VEGF (vascular endothelial cell growth factor). VEGF has been shown to be over-expressed in HNSCC (reviewed by Homer *et al.*, 2000), and elevated levels of VEGF in the circulation, as detected by enzyme-linked immunosorbent assay, have been significantly correlated with increased recurrence rates and reduced disease free intervals in HNSCC (Eisma *et al.*, 1997). VEGF inhibitors are currently undergoing clinical trials, and are showing promise in the treatment of a number of solid tumours (reviewed by Ferrara *et al.*, 2003).

The common theme in this discussion has been the concept that malignancy is a defect in the regulation of the preexisting cellular program for growth and invasion. Cell proliferation, apoptosis, proteolysis, cell motility and angiogenesis are all normal physiological processes, which escape normal physiological control and become deregulated in malignancy.

1.5 Metastasis

1.5.1 The importance of metastasis

Cancer describes a group of diseases, having in common the uncontrolled growth of aberrant cells, invasion of normal organs through direct extension, and metastasis. Metastasis is defined as the spread of disease from one organ or part to another not directly connected with it through the blood, lymph, or serosal surfaces (Alberts *et al.*, 1994). The study of the metastatic process is of paramount importance, as most cancer deaths can be directly attributed to the effects of metastatic disease (Fidler, 1991; Frost and Levin, 1992). This was appreciated by Lord Moynihan, who in 1904 remarked that "*the surgery for malignant disease is not the removal of the tumour but the anatomy of the lymphatics*". The presence of regional metastatic disease at diagnosis in HNSCC is the single most important clinical predictor of survival. Overall the presence of cervical metastases in HNSCC reduces the 5-year survival of the individual patient by 50% (British Association of Otolaryngologists Head & Neck Surgeons, 2002).

1.5.2 Metastasis as an active process

Whilst local tumour invasion must precede metastasis, the two processes can be considered separately. The process of cancer metastasis is a dynamic multi-step process (Ponta *et al.*, 1995) and not just a consequence of increasing tumour burden at the primary site. Experiments on rodents with mammary cancer reveal

that despite a $1cm^3$ tumour liberating millions of tumour cells into the circulation each day, very few metastases are produced (Weiss, 1986). Radiolabelling studies in a murine system have revealed that less than 0.1% of all circulating tumour cells survive to form secondary tumours (Fidler, 1970). Placement of peritoneal-venous shunts in women with ascites due to ovarian cancer does not result in lung metastases, despite the presence of billions of tumour cells in the in the venous circulation of these patients (Tarin *et al.*, 1984a; Tarin *et al.*, 1984b), suggesting that these malignant cells have not progressed to the metastatic phenotype. Also the occasional clinical finding of metastatic squamous cervical lymphadenopathy in the absence of a clinically identifiable primary tumour (the "occult primary") supports this notion. Breast carcinoma also has a preponderance to micrometastatic spread in the presence of minimal primary disease.

1.5.3 Metastasis as a specific process ("seed & soil" theory)

Lymphatic and venous drainage are not the only reasons for the location of metastases in specific organs (Frost, 1992). Over a century ago Paget (1889) proposed the "seed and soil" theory of metastasis, based on his analysis of the autopsies of a large number of patients with breast cancer. He observed that the pattern of metastases was non-random, and that breast cancer cells had bypassed the lung in order to establish bone metastases. He postulated that this was due to some tumour cells ("seeds") having an affinity for growth in the environment provided by certain organs ("soil"). This supports the notion that the

development of secondary tumours is dependent on interplay between host factors and intrinsic characteristics of the metastatic cells (Fidler, 1991).

1.5.4 The metastatic cascade

In order for a malignant tumour cell to leave its primary tumour of origin and establish itself as a metastasis, it must successfully complete a well-defined series of steps, known collectively as the metastatic cascade. Malignant transformation must be followed by progressive growth of neoplastic cells. Simple diffusion initially supplies the expanding tumour mass with nutrients, but neovascularisation must occur if it to exceed 1-2 mm in diameter. Initiation of the metastatic cascade is defined by loss of adhesion to neighbouring cells and penetration of the epithelial basement membrane. Increased cell motility and degradative enzymes also contribute to the subsequent invasion of the non-malignant host stroma. Successful vascular or lymphatic intravasation will then provide access to distant sites. Indeed, according to Liotta *et al.* (1991) proliferation, migration and matrix degradation are the key cellular processes responsible for tumour invasion. Circulating tumour cells must then arrest in a distant organ capillary bed or lymph node, by adhering to subendothelial basement membrane. Subsequent extravasation and proliferation in the organ parenchyma or lymph node then completes the metastatic cascade. Growth of the metastatic colony beyond 1 cm^3 again requires neovascularisation (Folkman, 1987). At any stage of tumorigenesis, tumour cells must overcome host immune cell killing (Schirrmacher, 1985). It has recently been proposed that the immediate microenvironment of disseminated tumour cells may be important in

this respect. Intravascular tumour cells may be "camouflaged" by fibrin or extracellular matrix proteins, and so be less susceptible to killing by circulating T-cells and natural killer (NK) cells (Ruiter *et al.*, 2001).

1.5.5 Regional metastasis in HNSCC

Around 35-40% of all patients with HNSCC will have lymphatic metastases at presentation (Jones *et al*, 1998). The incidence varies considerably with subsite within the head and neck region, reflecting the interplay of tumour factors and host factors. Metastatic potential depends both on the intrinsic propensity of the malignant tumour to metastasise, and on the lymphatic drainage of the site of origin of the tumour. This is illustrated by the low incidence of lymphatic metastases arising from carcinomas of the glottic larynx (poor lymphatic drainage) compared with the high incidence from carcinomas of the supraglottic larynx (rich lymphatic drainage). In a series of 1394 patients with glottic tumours, 4.8% had regional cervical metastases, compared with 38.8% of a series of 598 patients with supraglottic tumours (Robin *et al*, 1997). These two subsites are separated by millimetres, and are exposed to the same environmental aetiological factors. This suggests that there may be instances where glottic tumour cells acquire the metastatic phenotype, but fail to metastasise because of poor access to the regional cervical lymph nodes. Conversely, certain primary sites (e.g. oropharynx and hypopharynx) and subsites (e.g. supraglottic and subglottic larynx) are associated with a greater tendency to metastasise, most probably because of their rich lymphatic drainage (Magnano, 1997).

A higher T-stage is associated with a greater chance of nodal metastasis in laryngeal cancer (Bataini *et al.*, 1985). Within a single T-stage category, tumour volume can vary considerably (Parmeijer *et al.*, 1997) and has been shown to be an independent factor in predicting regional metastasis (Johnson et al., 1995). In a study of 94 patients with laryngeal cancer, a significant correlation between the depth of invasion of the primary and the presence of lymph node metastasis was found (Yilmaz *et al.*, 1998).

Neovascularisation is known to be essential in the growth and progression of both primary and metastatic tumours. The relationship between angiogenesis, measured by tumour microvessel density (MVD), and nodal metastasis has been established in many solid tumour types, such as breast, ovarian, endometrial, non-small cell lung, prostate, colorectal and oesophageal carcinomas (Petruzzelli, 2001). The evidence in HNSCC, however, remains conflicting. Gasparini *et al.* (1993) evaluated MVD using the CD-31 endothelial monoclonal antibody in biopsy specimens of 70 patients with advanced head and neck cancer. Patients with a MVD > 25 / 200x field had a significantly higher ($p<0.0046$) incidence of local or distant metastases. Likewise, tumour angiogenesis has been shown to positively correlate with nodal metastasis in carcinoma of the tongue, floor of mouth and nasopharynx (Klijanienko *et al.*, 1995; Roychowdhury *et al.*, 1996; Zatterstrom *et al.*, 1995). Conversely, other studies looking independently at tongue, supraglottic larynx and tonsil have reported no significant difference in vascularity between metastasising and non-metastasising tumours (Kinsella *et al.*, 1994; Leedy *et al.*, 1994; Williams *et al.*,

1994). The role of genetics in the establishment of metastases will be discussed in Section 1.8.

1.5.6 Distant metastasis in HNSCC (& second primary tumours)

In contrast to regional cervical metastases, distant metastases are rarely detected at presentation in HNSCC (Hibbert, 1997). The true incidence is difficult to estimate, because of the high incidence of second primary tumours in HNSCC. Distant metastases may be difficult to distinguish from second primaries, especially as both are most commonly found in the lung (Calhoun *et al.*, 1994). Distant metastases have been demonstrated at post mortem in as many as 37% of patients who die from HNSCC (Nishijima *et al.*, 1993).

The strongest single predictor of distant metastases is the presence of extracapsular extension in the cervical lymph nodes (Petruzzelli, 2001). A review of 130 stage III and IV patients treated with primary surgery, revealed that 30 went on to develop distant metastases, of whom 88% had extracapsular extension in the cervical lymph nodes. The presence of three or more histologically positive nodes was also associated with a higher incidence of distant metastases (Alvi and Johnson, 1997). It may be that extracapsular spread is a prerequisite for successful haematogenous access to distant sites.

1.5.7 The clinical problem of metastasis in HNSCC

Assessment of cervical lymph node status is critical in planning treatment and predicting prognosis. The gold standard for assessment of cervical lymph node status is histological scrutiny of a neck dissection specimen (pathological nodal status). Clearly this is only possible after surgical treatment has been implemented and its only therapeutic use is in deciding whether to administer post-operative radiotherapy.

Prior to treatment the neck is therefore assessed clinically by palpation and radiologically by computerised tomography (CT), magnetic resonance (MR) or ultrasound (US) scanning, to give a clinical nodal status. This clinical staging is used to plan subsequent treatment. Unfortunately clinical nodal status has been shown to have both a poor sensitivity and specificity. Up to 40% of clinically N0 necks will be falsely negative, and up to 50% of clinically positive necks will be falsely positive and have no pathological disease (Ali *et al.*, 1985; Madison *et al.*, 1994). The former group of patients may therefore be under-treated and placed at increased risk of regional and distant relapse. The latter group of patients may be subjected unnecessarily to the morbidity of a neck dissection, which has both functional and cosmetic implications. Clearly, an understanding of the molecular biology of metastases may open new avenues in diagnosis, treatment and even prevention of lymph node metastases. For example, a molecular marker of the "occult" lymph node metastasis, that failed to be detected by conventional clinical assessment in up to 40% of patients presenting

with a primary HNSCC, may prevent under-treatment and the increased rate of regional relapse seen in this group of patients.

Interestingly, a false-positive clinical N1 status may be preferable to a true-negative clinical N0 status (Gallo *et al.*, 1995). It may be that reactive lymphadenopathy indicates a better host immune response to the tumour.

1.6 Principles of staging and treatment

1.6.1 Staging of HNSCC

Cancer staging attempts to classify tumours according to their local extent and degree of regional and distant spread. Staging thus assists in the planning of treatment and acts as a guide to prognosis. Furthermore, it aids research by facilitating the exchange of information between different centres, for example when evaluating the results of different treatment strategies. The staging of cancer is based on the TNM (tumour, nodal metastases, distant metastases) system. The internationally accepted TNM system is that devised by the joint committees of the American Joint Committee on Cancer (AJCC) and L'Union Internationale Contre le Cancer (UICC), and was last revised in 1997 (UICC, 1997). A score is given for each of the three parameters described. The staging may be based on the results of clinical and radiological investigations (a clinical staging) or on the basis of information arising from resected specimens (pathological staging).

The primary tumour (T) is scored from 0 to 4, lymph node metastases (N) from 0 to 3 and distant metastases (M) either 0 or 1. The definitions of the TNM system for laryngeal and hypopharyngeal tumours are given in Appendix 1. Several such systems exist for the different subsites within the head and neck, and for simplification, stage grouping may be done. This combines the T, N and M stages from different subsites to give an overall measure of tumour advancement. This is expressed as one of four categories (I to IV), and is particularly useful when studying large heterogeneous groups of tumours (Appendix 2).

1.6.2 An overview of the treatment of HNSCC

The curative treatment of HNSCC is by surgery, radiotherapy or a combination of the two. The general consensus in the U.K. is that chemotherapy has no routine role in the curative treatment of HNSCC (Jones, 1997).

Generally speaking, large aggressive tumours require dual modality treatment, usually in the form of surgery followed by adjunctive radiotherapy. A balance must be struck between maximizing the chances of cure, and minimizing the morbidity associated with dual modality treatment. Typically in the U.K. dual modality treatment is given to patients with stage III and IV disease, as determined either clinically before treatment is begun, or pathologically, as a result of histological scrutiny of the resected specimen. The latter may show, for example, pathological involvement of lymph nodes in a neck that was clinically assessed as N0. It is widely thought that the prognosis in HNSCC has not altered

in the last few decades. However there is some evidence to suggest that the increased use of post-operative radiotherapy has improved survival rates (Jones, 1998).

Smaller tumours (e.g. T1 or T2, N0) can usually be treated with single modality treatment. The choice of radiotherapy or surgery for such tumours depends on several factors, such as potential surgical access to the tumour, an assessment of the potential morbidity of surgery and radiotherapy and, increasingly, patient preference. Until recently in the U.K., radiotherapy has been the usual treatment of most T1/T2 tumours, based on the general consensus that radiotherapy confers less morbidity than surgery. However this view is changing, as organ-preserving surgical techniques (including endoscopic laser resection) become more popular.

Metastases in the neck are usually treated with surgery, with or without subsequent radiotherapy depending on the histological findings. Treatment of the neck where there is no clinical evidence of disease (the "N0 neck") is more controversial. The current trend is to perform a selective (less radical) neck dissection or give radiotherapy to the neck, with the exception of patients with early glottic cancers and certain small oral cavity lesions with little chance of lymph node metastases (British Association of Otolaryngologists Head & Neck Surgeons, 2002). The argument for this approach is based on the observation that around 20% or more of tumours (excluding those referred to above) will have metastasised to the neck even though the disease is not clinically detectable. Improved loco-regional control and survival have been demonstrated with this

approach in some retrospective studies (August *et al.*, 1996; Haddadin *et al.*, 1999).

The treatment of loco-regional disease recurrence is limited by whether the patient has already received radiotherapy. Generally speaking, radical radiotherapy cannot be given to the same site more than once. Salvage surgery in a previously irradiated site is more difficult, and is prone to more complications than primary surgery. Disease recurrence may signify an extremely poor prognosis and further mutilating treatment may not be justified. Another issue pertinent to disease recurrence is its detection. It can be difficult, both clinically and pathologically, to confirm the persistence or recurrence of disease in HNSCC, especially after radiotherapy. This is because the typical post treatment appearances of oedema (early after treatment) and fibrosis (later) can mimic those of malignant disease. However, if a treatment policy of radiotherapy with salvage surgery as necessary is adopted, the early detection of disease recurrence or persistence is crucial.

Two caveats to this overview are the assumption that the patient is generally fit enough to endure radical surgery and / or radiotherapy, and that he or she has neither distant metastatic disease nor a synchronous primary tumour. The presence of distant metastases or a primary carcinoma of the bronchus or oesophagus is associated with a very poor prognosis. The pre-treatment assessment aims to identify such patients and so prevent them from inappropriately undergoing radical treatment.

1.7 The genetics of HNSCC

1.7.1 Cancer as a genetic disease

Features common to all cancers are unregulated proliferation of cells, invasion of surrounding tissues and a variable potential for metastatic spread to other parts of the body. The regulation of the growth and proliferation of the 30 trillion cells in the human body is tightly controlled by the 40 or so known proteins that regulate the cell cycle. These proteins are the products of proto-oncogenes and tumour suppressor genes (TSGs). Under normal conditions, the co-ordinated expression of these genes leads to an equilibrium between growth promoting and growth restraining signal transduction, and natural cell loss, such that cell turnover is appropriate to the tissue and physiological circumstance. Mutations may produce an imbalance of these factors, through proto-oncogene activation and tumour suppressor gene inactivation, which leads to an unbalanced mitogenic signal and consequent aberrant cell proliferation. A variety of mechanisms produce mutations (i.e. a change in the sequence of genomic DNA) including point mutations, deletions and amplifications of segments of DNA, and chromosomal rearrangements, where entire chromosomes in part or in whole are lost, translocated or re-duplicated. The accumulation of these genetic alterations underlies the progression from a normal cell to a cancer cell, and is referred to as multi-step carcinogenesis (Califano *et al.*, 1996). It has been estimated that between 6 and 11 specific mutations are required for the development of HNSCC (Renan, 1993). Thus, acquisition of the malignant phenotype may require as few as 6 critical genetic alterations throughout the

entire genome, to allow a cell to overcome the mechanisms that tightly control its proliferation and motility.

Theodor Boveri recognised that this growth advantage could be passed on to the daughter cells following mitosis. In 1914, this led him to propose the "somatic mutation theory" of cancer, which postulates that the mode of transmission of the malignant genotype is via mitotic replication of permanent mutations in the genome (Boveri, 1914). Extension of this concept gives rise to the clonal evolution theory of tumours, whereby continued proliferation of the genetically identical daughter cells leads to tumour formation (Nowell, 1976). This theory will be discussed further in Section 1.7.2.

1.7.2 Intra-tumour heterogeneity

A primary carcinoma of the head and neck region arises from a single cell that has acquired the malignant phenotype. This occurs once the collective effect of its accumulated genetic aberrations enables it to proliferate in an uncontrolled manner, invade surrounding tissues and potentially metastasize to distant sites in the body. The subsequent clonal expansion of this malignant cell then gives rise to the tumour. This sequence of events is the basis of the "clonal evolution model of tumour cell populations", and may lead one to assume that all cells in a tumour are genetically identical (Nowell, 1976).

However tumours are actually genetically heterogeneous, and consist of multiple subclones all competing for dominance. The reason for this is that the progeny of

the original malignant cell are genetically unstable and may continue to be exposed to carcinogens. They therefore acquire their own further mutations which may in turn enhance or diminish their ability to proliferate, invade or metastasize. Thus, whilst all the cells in a tumour originate from the original malignant cell, and so share a clonal origin, each is susceptible to the acquisition of new mutations, and so they tend to give rise to subclones. Naturally these subclones will compete with each other for the limited supply of nutrients, and so the subclones evolve, with more aggressive subclones flourishing and weaker ones perishing altogether (Kerbel, 1990).

This tumour heterogeneity has methodological implications when considering tumour sampling as a means of studying the genetics of primary tumours that have metastasised. Fidler and Hart (1982) proposed that metastasis-competent cells constitute a subpopulation within a heterogeneous primary tumour. They have provided substantial evidence that tumours are genotypically heterogeneous, due to the selective pressure predicted by Paget's "seed and soil theory" (Fidler, 1995). Clearly, the size of such subclones is of critical importance, as they could be missed altogether when sampling the primary if they constitute only a small proportion of the tumour. In studies using fluorescence *in situ* hybridization (FISH), metastatic cells were rarely detected in the primary tumour, supporting the notion that the metastatic clone constitutes a small proportion of the primary tumour (Kuukasjarvi *et al.,* 1997). Indeed, it has been estimated that fewer than one in ten million cells in a primary tumour acquires metastatic capacity (Poste & Fidler, 1980). Conversely, Kerbal (1990) proposed that the metastasis-competent subclone will eventually rise to

dominance within a tumour, and subsequently demonstrated that the metastatic subclone dominates the tumour mass early in its growth. In their metastasis review, Liotta *et al*, (1991) concluded that "measurement of the average level of a molecular marker in a primary tumour is likely to reflect the general metastatic propensity of the entire tumour". The fact that this thesis is concerned with the genetic discordance between primary tumours and their matched lymph node metastases would tend to refute the claim of Kerbal that the metastasis competent subclone rises to dominance early in tumour progression. However, another possible explanation for this discordance, which does not necessarily refute Kerbel's proposal, is the continued clonal evolution of cells after they have seeded in the lymph node. Figure 1.4 illustrates these possibilities, and the fact that discordance between the two tumour cell populations from the same patient is suggestive of either subclone heterogeneity within the primary tumour at the time of establishment of the metastasis or further clonal evolution of both tumours after metastasis (Sun *et al.*, 1995).

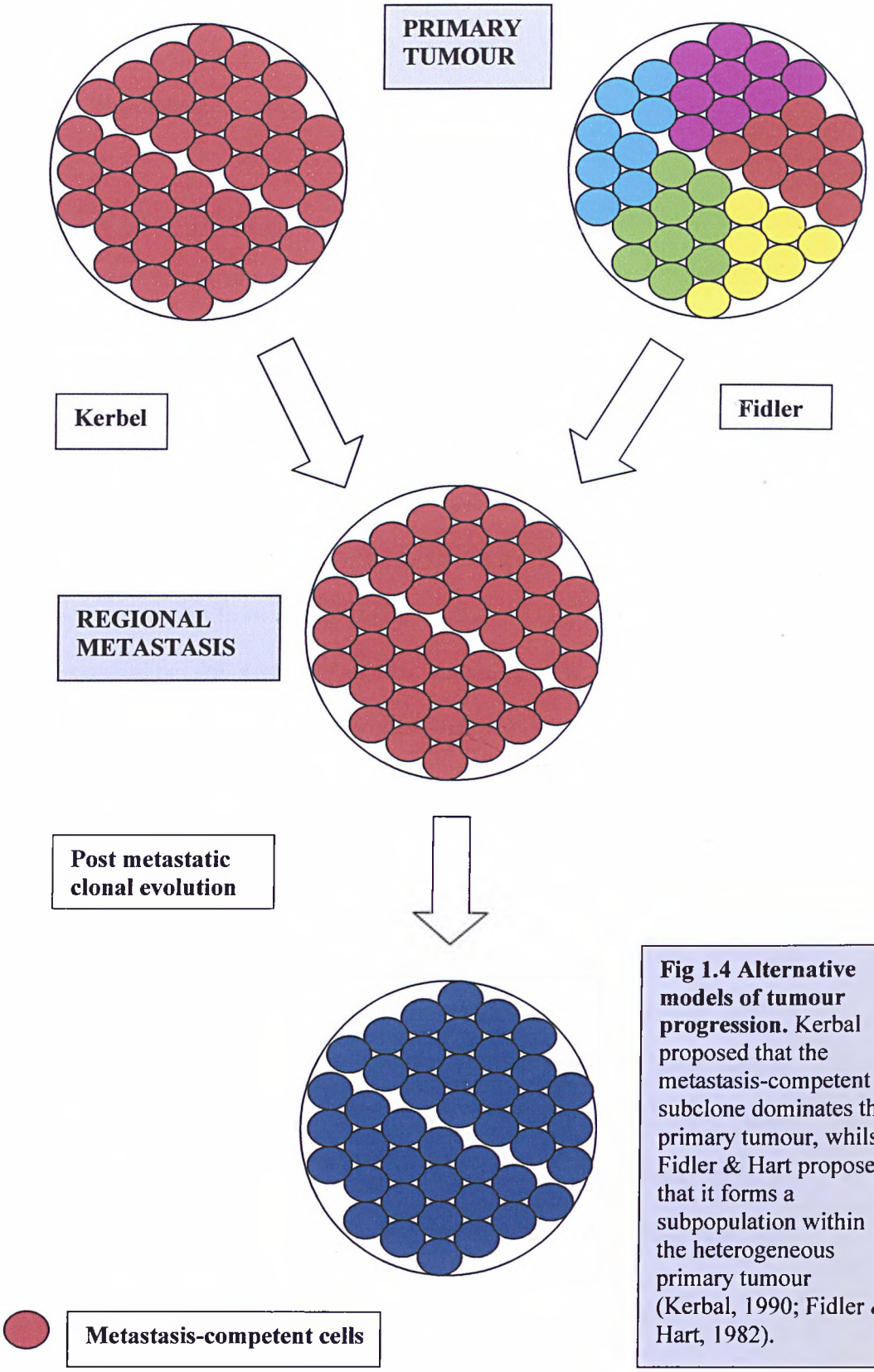


Fig 1.4 Alternative models of tumour progression. Kerbal proposed that the metastasis-competent subclone dominates the primary tumour, whilst Fidler & Hart proposed that it forms a subpopulation within the heterogeneous primary tumour (Kerbal, 1990; Fidler & Hart, 1982).

1.8 The genetics of the primary tumour

1.8.1 Chromosomal abnormalities

DNA ploidy is defined as the amount of genetic material within the nuclei of the cells. Normal human somatic cells contain 46 chromosomes (~ 7.18 pg of DNA) and are termed diploid (2n). The most frequent karyotypic change in HNSCC is tetraploidy (Shackney, 1989). Flow cytometry has been used to demonstrate aneuploidy (an abnormal DNA content greater than 2n) in a high proportion of HNSCCs. The mechanism of aneuploidy formation is believed to be unequal distribution of the chromosomes between daughter cells during mitosis. Support for this theory comes from studies in colorectal cancer showing that mutations of genes controlling the assembly of the mitotic spindle lead to unbalanced metaphases in diploid tumour cells with the resultant formation of aneuploid clones (Cahill, 1998). Several groups have correlated a diploid DNA content with increased patient survival in HNSCC (Zatterstrom *et al.*, 1991; Milroy *et al.*, 1997), whilst a significantly increased risk of local recurrence has been demonstrated in patients with aneuploid tumours (Kearsley *et al.*, 1991 and Hemmer *et al.*, 1996). It has been postulated that aneuploidy may provide tumour cells with a growth advantage by restoring copies of house-keeping genes that are lost through chromosome deletion or loss (Carey *et al.*, 1993). Thus aneuploidy would appear to be associated with the progression of HNSCC, although this crude measure of total quantity of cellular DNA sheds little light on the molecular mechanism of cancer progression.

Cytogenetics is the study of chromosomal abnormalities and rearrangements. Chromosomes are maximally condensed and separated from each other during the metaphase of mitosis. Classical cytogenetics involves the examination of metaphase spreads of chromosomes under the light microscope, after they have been stained with Giemsa solution to reveal the characteristic 'G' banding pattern (Czepulkowski, 2001). The majority of classical cytogenetic data pertains to haematological malignancies, where abundant numbers of dividing tumour cells can be obtained from the bone marrow and circulation. The observation of actively dividing tumour cells in solid malignancies, such as HNSCC, is relatively rare, which necessitates the establishment of short-term primary cultures to obtain metaphase spreads. The use of classical cytogenetics in HNSCC has, therefore, been limited by the difficulties of establishing short-term primary cultures from these tumours, with less than 30% of head and neck tumours growing in culture and yielding analysable metaphase spreads (Gollin, 2001). Comparative genomic hybridization (CGH) is a relatively new, powerful technique, which permits the rapid screening of the entire genome for changes in chromosome copy number (i.e. chromosome losses and gains), without the need to obtain metaphase spreads (see Section 1.9.3). In HNSCC, several studies have identified consistent regions of DNA over- or under- representation (Speicher, 1995; Bockmuhl, 1998). CGH appears to be slightly more sensitive than classical cytogenetics, but has otherwise tended to identify the same pattern of loss and gain aberrations (Mertens *et al.*, 1997; Gebhart and Liehr, 2000).

Overall, the non-random patterns of chromosomal abnormalities identified in head and neck cancer series are more complex than in the haematological

malignancies, and tend to be losses or gains of chromosomal material rather than the balanced translocations seen in leukaemia (Cowan, 1992). Studies have attempted to identify recurrent and consistent karyotypical abnormalities in tumours, rather than those arising purely by chance and having a neutral or coincidentally growth enhancing effect. A recent review of the classical cytogenetic data in HNSCC revealed consistent deletions of chromosomal material in over 30% of tumours studied at the following loci: 3p12-p24, 4q21-q31, 7q22-qter, 8pter-p21, 9p21-p24, 10q22-q26, 13q12-q24 14q and 18q21-q23 (reviewed by Gollin, 2001). Overall, fewer chromosomal gains were reported and at lower frequencies. Gain or amplification was noted at 3q, 5p, 7p, 8q13-24.3 and 11q13, implying the presence of proto-oncogenes at these sites. Karyotypic data has demonstrated extensive intra-tumour heterogeneity in HNSCC identifying multiple subclones arising from a common pre-cursor karyoype (Carey *et al.*, 1993; Jin *et al.*, 2000). The mechanism for the concomitant loss of one chromosome arm (e.g. 3p) and gain of the other (e.g. 3q) is believed to be isochromosome formation, whereby a radially symmetrical chromosome is formed by the duplication of one arm and simultaneous loss of the other (Carey *et al.*, 1993). Several of these non-random chromosomal abnormalities are consistent with findings in other malignancies. Loss of 3p was first implicated in small cell lung cancer (Whang-Peng *et al.*, 1982), loss of 13q12-24 in retinoblastoma (Cavenee *et al.*, 1983) and loss of 18q in colon carcinoma (Fearon *et al.*, 1990). Unlike ploidy data, cytogenetics highlights potential chromosomal regions of interest for the application of molecular genetic techniques and subsequent identification of culprit genes. Another advantage of cytogenetics, is that flow cytometry may erroneously identify a

near normal DNA content, whilst cytogenetic analysis of the same sample may reveal aneuploidy in the presence of multiple chromosomal deletions (Carey *et al.*, 1993).

In a large meta-analysis of cytogenetic data pertaining to eleven tumour types, HNSCC and breast cancer were the only two tumours that failed to demonstrate a characteristic chromosomal gain or loss that was not present in any of the other tumour types (Mertens *et al.*, 1997). In the case of HNSCC, this may reflect karyotypic complexity, general genetic instability and possible tumour sub-site diversity. Amplification of 11q13 and loss of 18q are the only cytogenetic abnormalities to have so far been correlated with poor prognosis in HNSCC (Meredith *et al.*, 1995; Akervall *et al.*, 1995; Van Dyke *et al.*, 1994). In one study of HNSCC, loss of material from 18q was identified more frequently in patients who died from their disease (Van Dyke *et al.*, 1994). These findings are consistent with molecular genetic findings, and are fully discussed in Sections 1.8.2 and 1.8.3 (Pearlstein *et al.*, 1998). An important observation is that several of the genetic changes (17p loss, 6p loss) discovered by molecular genetic methods and noted to be important in the progression of HNSCC, are not observed at high frequency by classical or molecular cytogenetic techniques. It may be that these molecular deletions are smaller than the level of resolution of the cytogenetic techniques (Gollin, 2001). Thus cytogenetic and molecular techniques are complementary.

1.8.2 Tumour suppressor genes

In their wild-type (WT) form, tumour suppressor genes encode proteins that tend to mitigate against tumour formation. They therefore function as recessive genes, whereby a cell need only retain one functional allele in order to effect tumour suppression. This understanding of the mechanism of action of TSGs is largely due to the statistical analyses carried out by Knudson (1971), on the incidence of both hereditary and sporadic cases of retinoblastoma. In hereditary retinoblastoma, a single additional somatic event in a cell that carries the inherited mutation is sufficient to give rise to the disease in the first few weeks of infancy, whilst two somatic events are required to produce a sporadic retinoblastoma in later life. That two independent genetic events are required to inactivate a gene mitigating against the development of cancer, became known as Knudson's 'two-hit' hypothesis. Thus a cell can carry a mutation affecting only one allele of a pair at a tumour suppressor locus, provided the other allele functions normally in encoding the tumour suppressing protein. However, this mutation will become apparent if a second genetic event disables the functional allele. Several mechanisms for revealing a recessive mutation have now been identified including deletion, point mutation and translocation (Cavenee *et al.*, 1983). More recently, epigenetic phenomena, such as hypermethylation of 5' CpG islands in the gene promoter region leading to transcriptional inactivation, have been implicated in TSG inactivation (Merlo *et al.*, 1995). The simplest way of revealing a recessive mutant allele is by deletion of the WT allele, resulting in hemizygosity at the mutated locus on the remaining allele. Indeed, deletion of specific chromosomal regions is one of the most common genetic events in solid

tumours, and it is inferred that these regions of non-random alleic loss in tumours represent the location of putative TSGs. This hypothesis underpins the commonly employed method of molecular detection of allelic losses by loss of heterozgosity (LOH) studies, which will be fully discussed in Section 1.9.5 (Lasko and Cavenee, 1991).

3p is a common site of chromosomal loss in many human cancers, and is deleted in around 60% of cases of HNSCC (Buchhagen *et* al., 1996; Li *et al.*, 1994; Partridge *et al.*, 1996). Re-introduction of 3p into oral tumours has been shown to suppress tumourigenicity in cell lines *in-vitro* (Uzawa *et al.*, 1995) providing strong evidence for the presence of TSGs on 3p. The common discrete regions of deletion are 3p14, 3p21, 3p22 and 3p25. The fragile histidine triad gene (*FHIT*) is a candidate TSG located at 3p14.2. One study demonstrated reduced expression of the FHIT protein in 66% (n=32) of oral squamous cell carcinomas (van Heerden *et al.*, 1999), supporting a specific tumour suppressor role for this protein in head and neck cancer.

Loss of chromosome region 9p21 is one of the most common chromosomal aberrations associated with human malignancies, and occurs in around 80% of HNSCCs (Cairns *et al.*, 1994; Reed *et al.*, 1996). This region contains the TSG *p16 ^{ink4a}*, the protein product of which is a critical effector of the G1/S cell cycle check point. P16 inhibits the ability of cyclin-dependent kinases to phosphorylate the retinoblastoma protein, thereby preventing cells from entering the S-phase of the cell cycle (Lukas *et al.*, 1995). Homozygous deletion and hypermethylation of the remaining 5' promoter are the predominant modes of

gene inactivation. Interestingly, LOH of 9p21 appears to be one of the earliest genetic events in tumourigenesis, and has been detected in histologically normal and dysplastic mucosa adjacent to invasive carcinomas (Lydiatt, 1998). This may have future implications for the diagnosis, and indeed definition, of early cancers and high risk pre-malignant lesions.

Loss of 13q14 has again been implicated as an early event in tumourigenesis, and has been demonstrated in over half of HNSCCs (Maestro *et al.*, 1996). This region of deletion includes the retinoblastoma gene (*Rb*), also known as the "master brake" of the cell cycle. In the resting G0 phase, the Rb protein binds to and inactivates the transcription factor, E2F. Phosphorylation of the Rb protein releases E2F, which is then able to diffuse into the nucleus and activate transcription of those proteins required for the cell to enter the G1 phase of the cell cycle. Surprisingly, despite frequent LOH at 13q, there does not appear to be consistent inactivation of the retinoblastoma TSG in HNSCC. It appears that a region telomeric to this locus at 13q14.3 is likely to contain additional, as yet unidentified, TSGs important in HNSCC (Yoo *et al.*, 1994).

Loss of heterozygosity of 17p has been demonstrated in well over half of HNSCCs, and is again believed to be an early event in tumourigenesis (Pavelic *et al.*, 1994). This region contains the p53 tumour suppressor gene (17p13), mutations of which are the most commonly detected genetic abnormality in human cancer. Interestingly, the reported mutation rate of this gene increases with the sensitivity of the genetic technique employed. Thus, CGH and classical karyotyping tend to detect genetic alteration at a lower frequency than LOH and

mutation analysis. Point mutation is a frequent mechanism of p53 inactivation, and so in isolation, is effectively missed by lower resolution techniques (El-Naggar *et al.*, 1995). It would appear that *p53* mutations are carcinogen-specific, as studies have consistently shown specific mutations in smokers and non-smokers with HNSCC (Brennan *et al.*, 1995a). P53 is a 53 kDa nuclear phosphoprotein, which has been described as the 'guardian of the genome'. It achieves tumour suppression through multiple complex interactions with many other proteins (e.g. p21/WAF1, BAX, Bcl-2), the overall effect of which is to prevent the propagation of acquired genetic abnormalities. In response to a genetic aberration, p53 induces G1 cell cycle growth arrest, before stimulating DNA repair or triggering apoptosis, depending on the extent of the abnormality. Mutation analysis of histologically normal resection margins in 25 HNSCC patients with a p53 mutation demonstrated a significantly higher local recurrence rate in patients with margins positive for p53 mutation (5/13 recurred) compared with patients whose margins were negative (0/12 recurred) (Brennan *et al.*, 1995b). The greater sensitivity of genotypical analysis over histopathological scrutiny has obvious diagnostic and therapeutic implications.

The long arm of chromosome 18 is lost frequently in HNSCC. Five "minimally lost regions" have been identified on 18q, in the regions 18q12, 18q21.1 and 18q21.1 – q21.3, 18q22.2 and 18q23 (Papadimitrakopoulou *et al.*, 1998; Takebayashi *et al.*, 2000). Several TSGs map to these regions of loss, including DCC, DPC4, MADR2, the protease inhibitor ovalbumin serpin genes, maspin (P15), SCCA1, SCCA2, PAI2 and headpin (PI13). The region affected by the loss at 18q21.1-21.3 appears to be distinct from the site of the *DCC* (Deleted in

Colorectal Carcinoma) gene suggesting the presence of additional TSGs at this locus (Rowley *et al.*, 1995). Importantly, loss of 18q is associated with a poor 2-year survival (p=0.008), as demonstrated by a study of 67 patients with HNSCC (Pearlstein *et al*, 1998).

1.8.3 Proto-oncogenes

Proto-oncogenes are genes which, when expressed in normal circumstances, tend to promote cell proliferation and growth, and encode proteins such as transcription factors, growth factors, signal transduction molecules and cell motility factors. When they are overexpressed in malignancy, they become known as oncogenes. Overexpression of only one allele is required to achieve increased function, due to their dominant mode of action. Whilst TSGs contribute to malignancy through inactivating genetic events (e.g. point mutation, deletion), the function of oncogenes must be preserved or even enhanced in malignancy. The molecular correlate of an activated oncogene is usually either a chromosomal breakpoint associated with DNA amplification or gain, or more rarely, a mutation within the oncogene, which then encodes a protein with even greater growth promoting properties (Heim and Mitelman, 1987). Activation of oncogenes appears to be a less frequent occurrence in solid tumours than inactivation of TSGs.

Consistent amplification of 11q13 has been identified in up to 60% of cases of HNSCC, and has been correlated with a poor prognosis in several studies (Akervall *et al.*, 1995; Meredith *et al.*, 1995). This region contains the putative

proto-oncogene PRAD 1 (or cyclin D1), whose over-expression correlates with amplification of 11q13 (Sidransky, 1995). Cyclin D1 enables the cyclin-dependent kinases cdk4 and cdk6 to phosphorylate the retinoblastoma protein, so liberating the transcription factor E2F, which diffuses into the nucleus and stimulates cell proliferation. Overexpression of both the cyclin D1 messenger RNA (mRNA) and the protein product itself have been demonstrated, supporting a specific role for cyclin D1 in head and neck cancer (Bartkova *et al.*, 1995). In HNSCC, cyclin D1 overexpression correlates with shorter time to recurrence, higher stage disease and lymph node involvement (Akervall *et al.*, 1997; Jones *et al.*, 1994; Michalides *et al.*, 1995; Michalides *et al.*, 1997). In a further study, chromosomal rearrangements at 11q13 were significantly associated with reduced 2-year survival (20% vs 60%, p=0.001) in patients with HNSCC (Akervall *et al.*, 1995).

The c-erbB proto-oncogene is located on chromosome 7p12, and codes for the epidermal growth factor receptor (EGFR). Amplification and gain of c-erbB have been detected in the majority of HNSCCs studied, and appears to be important in early tumorigenesis (Ishitoya *et al.*, 1989; Santini *et al.*, 1991; Uhlman *et al.*, 1996). Indeed, EGFR over-expression may be a potential biomarker of 'field cancerization'. Two studies have identified abnormalities in EGFR in histologically normal mucosa of HNSCC patients (Shin *et al.*, 1994; Grandis and Tweardy, 1993). Using immunohistochemistry, Shin *et al.* (1994) demonstrated higher levels of EGFR expression in the 'normal' mucosa surrounding the tumours of 36 patients with HNSCC than in the mucosa of healthy controls who had not been exposed to tobacco or alcohol. Likewise,

Grandis and Tweardy (1993), examined EGFR mRNA production in the histologically normal mucosa of HNSCC patients and also found that EGFR expression was increased (by an average of 29-fold) in the mucosa of HNSCC patients when compared with normal controls. This has implications for screening and the early detection of those at risk of subsequently developing HNSCC. This finding also calls into question the validity of equating histologically "normal" resection margins with disease eradication, following surgical resection of a HNSCC.

Overexpression of c-erbB2 (also known as neu and Her-2), another member of the EGFR family with tyrosine kinase activity, has recently shown much promise as a therapeutic target in carcinoma of the breast. Its overexpression in up to 30% of breast tumours, led to the development of an anti-c-erbB-2 monoclonal antibody (Herceptin™) (Schnitt, 2001; Pegram *et al.*, 2000). Administration of Herceptin™ blocks the over-expressed receptor molecules and so inhibits the growth stimulatory signals of c-erbB-2. Whilst c-erb-B2 overexpression has been detected in around 50% of HNSCCs most studies have failed to correlate this finding with a poor prognosis (Xia *et al.*, 1997; Craven *et al.*, 1992).

The *c-myc* proto-oncogene is located on chromosome 8q24, and most work has focused on its t(8;14) translocation in Burkitt's lymphoma (Adams *et al.*, 1993). Following dimerisation with another protein (max), myc forms an activated transcription factor, which regulates the expression of genes involved with DNA replication. Overexpression of the myc protein has been demonstrated in up to

half of primary head and neck cancers, and high levels of the myc protein have been correlated with reduced survival (Field *et al.*, 1989).

Interestingly, the mutation of the *ras* proto-oncogene, and subsequent overexpression of its protein product, demonstrates tumour subsite and geographical specificity. A high incidence of ras mutation has been consistently demonstrated in oral cancers in India (approximately 35%), but is found at low or undetectable levels (less than 5%) in the Western world, suggesting the possible existence of a specific mutagenic effect of chewing betel nut (Das *et al.*, 2000, Clark *et al.*, 1993).

1.8.4 A molecular and genetic progression model of HNSCC

A pioneering genetic progression model for colorectal tumourigenesis was proposed by Fearon and Vogelstein in 1987. Specific genetic alterations were assigned to each step of the well-established adenoma-carcinoma sequence in colorectal tumorigenesis (Fearon *et al.*, 1987; Fearon and Vogelstein, 1990). Califano *et al.* (1996) proposed a similar model for the progression of HNSCC, based on the analysis of the ten most commonly implicated chromosomal arms, as reported in the literature. They analysed areas of histologically normal tissue, dysplasia, carcinoma-in-situ, and invasive carcinoma, and later backed up their findings by microsatellite analysis of 'matched' normal, dysplastic and carcinomatous tissue from individual patients who were biopsied at different times (Califano *et al.*, 1996; Califano *et al.*, 2000). Mao and El-Naggar (1999) also proposed a model based on analysis of the published allelotype data, which

was largely in agreement with that of Califano *et al.* (1996). The salient features of both models have been combined in Figure 1.5, which demonstrates the cumulative increase in chromosomal aberrations in the progression from benign hyperplasia through to invasive carcinoma. Whilst loss of 4q, 6p, 8p and 14q have been consistently demonstrated in several studies, no specific TSGs pertaining to these chromosome arms have yet been implicated. Loss of 18q is included as this is a consistent finding in the published literature, and so features in the model of Mao and El-Naggar (1999), but was not analysed in the series of Califano *et al.* (1996). The other main difference between the models is in the exact order in which the genetic changes occur, but Califano *et al.* (1996) do stress that the order of loss was not the same for each of their tumours, and that it is therefore likely to be the accumulation, rather than precise order, of genetic events that determines tumour progression.

Knowledge of the approximate temporal relationships between genetic events has diagnostic and therapeutic implications, in terms of targeted screening to detect pre-malignant transformation and early detection of tumour recurrence. As Figure 1.5 shows, least is known about the molecular and genetic events underlying the transition between invasive squamous cell carcinoma and metastasis.

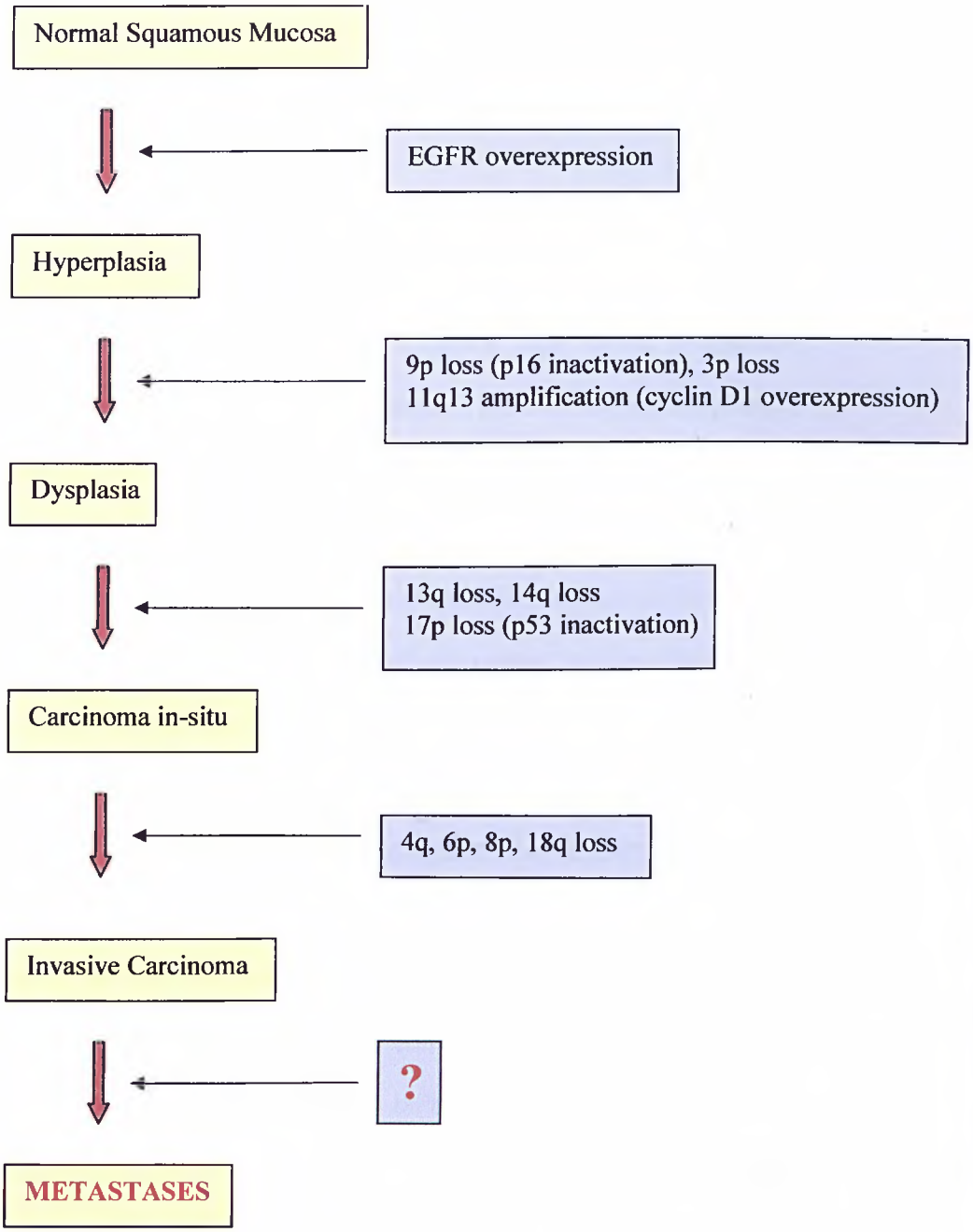


Figure 1.5

Tentative molecular and genetic progression model for head and neck squamous cell carcinoma. The histopathological sequence of events is shown on the left, with the underlying genetic or molecular events on the right. The temporal relationships between molecular events are inferred from the reported frequencies of aberrations in the different histological stages of malignant progression. (Adapted from Califano *et al.*, 1996 and Mao and El-Naggar, 1999).

1.9 The genetics of metastasis in HNSCC

1.9.1 Overview

It has been estimated that between four and seven rate-limiting genetic events are required for the development of the common epithelial cancers (Renan, 1993). Metastatic cancer cells represent "a higher stage of the progression cascade" (Welkoborsky *et al.*, 2000). As metastasis requires several independent steps beyond the acquisition of the malignant phenotype, no single gene is likely to be responsible (Kumar, 1992b). Properties as diverse as protease synthesis, surface adhesion molecule expression, histocompatibility antigen status and the ability to escape immune surveillance and NK cell activity will be controlled by multiple genes. In the genetic progression model proposed by Califano *et al.* (1996) the gap between invasive malignancy and metastasis may therefore represent several discrete genetic events. As the probability of a single cell acquiring all the necessary properties for metastasis by mutation and selection would appear to be very low, Ponta *et al.* (1994) have suggested a mechanism whereby a small number of mutations may be responsible for metastasis. They postulate that the metastatic genotype may be the result of the activation of a pre-existing genetic program for invasion, which may, for example, have been inactive since embryogenesis. The genetic event underlying metastatic progression may therefore be the expression or suppression of one or more critical genes that is capable of activating many other genes in a predetermined, co-ordinated fashion. As yet there is no defined molecular marker of metastasis.

1.9.2 Chromosomal Abnormalities

A large study of 429 oral cancer patients identified DNA aneuploidy as an independent prognostic factor for locoregional recurrence and a predictor of metastases to the neck (Hemmer *et al.*, 1999). Several studies have shown that whilst diploid head and neck carcinomas are only occasionally associated with lymph node metastases, patients with aneuploid lesions frequently present with simultaneous neck disease (Chen *et al.*, 1993; Saitao *et al.*, 1994, King *et al.*, 1995; Hemmer *et al.*, 1997). Multivariate studies have confirmed that aneuploidy in primary HNSCCs is an independent indicator of lymph node metastases (Suzuki *et al.*, 1994; Hemmer *et al.*, 1995). Again, ploidy studies tell us little about the mechanism of metastasis.

There have been very few studies comparing the specific chromosomal aberrations occurring in primary metastasising tumours and the corresponding lymph node metastases and non-metastasising tumours (Kujawski *et al.*, 1999a; Sun *et al.*, 1995; Welkoborsky *et al.*, 2000). One small series (n=20) demonstrated a significantly higher proportion of aneuploid cells and degree of DNA entropy (a measure of the variation of the DNA content of all cells) in node positive primary tumours compared to node negative tumours (Welkoborsky *et al.*, 2000). Subsequent CGH revealed that amplification of 11q13 (5/10 cases) and 22q (6/10 cases), and deletion of chromosome 18 (3/10 cases) occurred only in the node positive tumours and their metastases. Of these, gain of 22q does not appear in the molecular progression models discussed in Section 1.7.4, and so this may play a critical role in the progression from

primary malignancy to metastasis. Overall Welkoborsky *et al.* (2000) detected more chromosomal abnormalities in the lymph node metastases (average 7.8 affected chromosomes) than their matched primary tumours (average 4.6 affected chromosomes). Over-representations, which were more frequent in lymph node metastases, were identified on 1q, 8q and 17q. Their absence from the corresponding primaries implies either that the metastasis-competent subclone constitutes only a small proportion of the primary (and so its genome is effectively diluted and goes undetected) or that clonal evolution of the metastatic cells continues after they have metastasised. Sun *et al.* (1995) examined 12 primary tumours and their matched lymph node metastases. Although almost half (5/12) of the tumour pairs demonstrated genetic discordance at one or more microsatellite loci, chromosome 22 was not amongst the 15 chromosome arms studied. A further study, which used CGH to analyse 19 primary laryngeal carcinomas and their matched lymph node metastases, found no evidence of copy number gain at 22q, but did identify a significantly higher frequency of loss at 8p, 9q and 13 in the lymph node metastases (Kujawski *et al.*, 1999a).

1.9.3 CGH work in the laboratory

Comparative Genomic Hybridisation (CGH) is a powerful technique, which permits the rapid detection of DNA copy number changes throughout the entire genome in a single experiment. CGH is based on the competitive, co-hybridization of differentially labelled tumour and normal DNA samples from a patient to normal human metaphase spreads, thereby detecting areas of genetic under-representation or over-representation (Kallioniemi *et al.*, 1992). As no

prior knowledge of specific areas of interest within the genome is required, it forms a very useful genome-wide screening tool. The difficulties of establishing cell cultures from solid tumours, and the subsequent problems with artificial *in vitro* selection pressures, are by-passed as tumour metaphase spreads are not required. In a typical CGH experiment, tumour DNA is labelled by the incorporation of a green fluorochrome (FITC, Spectrum Green) and normal DNA with a red fluorochrome (TRITC, Spectrum Red, Rhodamine). The two, labelled, DNA samples are mixed and hybridised onto chromosome preparations generated from normal human lymphocytes (a much easier process than preparing chromosomes from a solid tumour). Images of the binding of the two sets of fluorochromes are captured using an epifluorescent microscope equipped with appropriate filters and a cooled, charged coupled device (CCD) digital camera. Image analysis of the signal intensities of the two fluorochromes, down the axis of each of the chromosomes, indicates regions of chromosomal imbalance in the test DNA. These chromosomal regions of amplified and deleted DNA are mapped directly onto normal chromosomes, allowing easy identification of the loci of regions of chromosomal loss and amplification. It should be stressed that this technique can only detect copy number changes (losses, deletions, gains, amplifications), and does not reveal translocations, inversions and other aberrations that do not change copy number. CGH is capable of resolving deletions as small as 10Mb, and amplifications as small as 2Mb. Copy number changes below these limits are undetectable.

A recent study applied CGH to 17 primary head and neck squamous cell carcinomas and their matched synchronous lymph node metastases (Personal

correspondence, Ashman *et al.*, 2000). A further 21 primary HNSCCs were also examined for copy number changes. Significant genetic discordance was identified between tumours and metastases at several loci. Gain of 22q and loss of 17q were detected in 5/17 (29%) LNMs, compared to none of their matched primary tumours or any of the 21 non-paired primaries. The 5 LNMs that showed gain of 22q were all derived from laryngeal or pharyngeal primary tumours. These results would tend to support a role for 22q gain in metastasis, and suggest that the metastasis-competent subclone does not make up the major population within the primary tumour, or that there is further clonal evolution after establishment of metastases. This finding is the foundation for the work in this thesis, the aims of which are outlined at the end of this chapter.

1.9.4 Gene abnormalities

Surprisingly few studies have looked at the genetic differences between primary tumours and their matched lymph node metastases. In one study, a high density cDNA (complementary DNA) microarray ("gene chip") was used to compare the mRNA expression of 7070 genes between primary and matched metastatic human melanoma cell lines (Clark *et al*, 2000). Only 16 of the genes studied were expressed at a higher level in the metastatic cell lines, and interestingly three genes, encoding fibronectin, thymosin β4 and RhoC, showed significantly increased expression in all of the metastases. Fibronectin, a component of the ECM, is capable of promoting cell migration, whilst thymosin β4 buffers monomeric actin, and may act to provide the lamellipodium (i.e. the advancing tip of a migrating tumour cell) with actin monomers, for rapid polymerisation

into actomyosin filaments. RhoC is believed to enhance actomyosin contraction, and was alone capable of transforming poorly metastatic cell into highly metastatic cells, following cell line transfection.

LOH analysis of colorectal carcinomas and matched liver metastases implicated chromosomes 13q and 14q as harbouring as yet unspecified metastasis-specific genes (Ookawa *et al.*, 1993). However, most LOH work in HNSCC implicates these two chromosomes earlier in tumorigenesis, and they appear between the stages of dysplasia and carcinoma *in-situ* in Califano *et al's* (1996) molecular progression model (see Figure 1.5).

The metastasis suppressor genes *Nm*-23 and *E-cadherin*, have mainly been studied at the protein level by immunohistochemistry (Reviewed by Yoshida *et al.*, 2000). *Nm-23* is a metastasis suppressor gene that resides on 17q, inactivation of which is consistently associated with metastases in breast and colorectal carcinomas (Hennessy *et al.*, 1991; Wang *et al.*, 1993). However, its role in head and neck cancer is not so clear, with a recent immunostaining study demonstrating a positive correlation between nm23 expression and tumour progression and poor outcome (Pavelic *et al.*, 2000). Loss of the cell-cell adhesion molecule E-cadherin, or inhibition of its function by monoclonal antibodies, has been associated with increased metastatic propensity in many tumour types, including head and neck squamous cell cancer (Ponta *et al.*, 1994). The role of matrix metalloproteinases will be discussed in Section 1.11.2.

Di Renzo *et al.*, (2000) showed that HNSCC cells that possessed a mutated MET oncogene were highly metastatic, compared to non-mutated clones from the same primary tumour. The MET proto-oncogene encodes the tyrosine kinase receptor for scatter factor, a cytokine that in normal circumstances stimulates epithelial cell motility and invasiveness during embryogenesis and tissue remodelling. This finding again supports the notion, that metastasis may be due to the inappropriate activation of a highly ordered pre-existing program for cell motility and invasion.

1.10 The role of chromosome 22 in the progression of HNSCC

1.10.1 Chromosome 22

Chromosome 22 is the first human chromosome to have been fully sequenced (Dunham *et* al., 1999). Although the second smallest of the human autosomes, comprising 1.6 – 1.8% of the genomic DNA, its long arm is relatively rich in genes. 22q is 34.49 Mb long and contains at least 545 genes and 134 pseudogenes. In contrast the short arm, which is 15.5 Mb in length, contains no protein encoding genes. Chromosome 22 is one of five acrocentric chromosomes, each of which shares substantial sequence similarity in the short arm, which encodes the tandemly repeated ribosomal RNA genes.

Alteration of gene dosage on part of 22q is responsible for a number of human congenital anomaly disorders including cat eye syndrome (CECR gene) and velocardiofacial / DiGeorge syndrome. Other regions associated with human

disease are the schizophrenia susceptibility locus and the sequences involved in spinocerebellar ataxia 10 (SCA10). The Philadelphia chromosome, a shortened chromosome 22 arising from a reciprocal translocation between chromosome 9 and 22 [t(9;22)(q34;q11)], is found in 95% of patients with chronic myeloid leukaemia (Nowell and Hungerford, 1960). This translocation results in transfer of the Ableson (*abl*) oncogene to the breakpoint cluster region (*bcr*) on 22q11. Transcription of this *bcr-abl* fusion gene leads to the synthesis of an abnormal tyrosine kinase protein. The *NF2* TSG also resides on chromosome 22, loss of both copies giving rise to neurofibromatosis type II, which is associated with bilateral acoustic neuromas.

1.10.2 Current status of chromosome 22 research

Welkoborsky *et al.* (2000) in their small series of 10 primary tumours of the oropharynx and hypopharynx and their matched lymph node metastases, identified overrepresentation of chromosome 22 as the most consistent aberration present in both the primary tumour and its corresponding LNM (6 out of 10 pairs). In the same study, gain of chromosome 22 was not detected in any of the 10 non-metastasising primary tumours studied. The genetic concordance between the primary tumour and metastasis suggests that the metastasis-competent subclone in the primary tumour was analysed, and that chromosome 22 may harbour a metastasis-enabling proto-oncogene. Interestingly, Ashman *et al.* (Personal correspondence, 2000) found no evidence of copy number change at this locus in the primary tumours that gave rise to metastases, suggesting that the metastasis-competent subclone was not sampled in their series. However,

amplification of chromosome 22q was not present in the non-metastasising primary tumours in either study, supporting the notion that chromosome 22q may harbour a metastasis-enabling gene.

In contrast, Bockmühl *et al.* (1998) performed CGH on 50 primary HNSCC specimens and observed amplification of 22q in 23 cases (46%). Likewise microsatellite marker analysis (see Section 1.10.3) of 22q in primary HNSCC specimens by another group revealed LOH in 19/50 informative cases (38%) (Poili-Frederico *et al.*, 2000). FISH analysis of 3 HNSCC cell lines also identified a common region of 22q amplification at 22q11.2-12, although the distinction between primary tumour and metastasis may be arbitrary when considering cell lines (Matsumura *et al.*, 2000).

Thus two apparently contrasting bodies of evidence exist for the role of a putative oncogene on 22q in HNSCC, one supporting a role during initial tumorigenesis, the other a role in allowing malignant cells to metastasise. A possible explanation for this apparent discrepancy is that Bockmühl *et al.* (1998) and Poili-Frederico *et al.* (2000) were actually sampling metastasis competent subclones from within their series of primary tumours. However, scrutiny of the papers also reveals non-metastatic primaries demonstrating gain of 22q, although the criteria for defining absence of metastases are not discussed. As discussed in section 1.5.7 the presence of a clinically N0 neck is too insensitive an indicator of nodal status. Ideally, pathological examination of a neck dissection specimen or an adequate period of loco-regional disease free-recurrence is necessary to confidently exclude the presence of neck metastases. The main aim of this thesis

was to address the issue of whether chromosome 22q has a specific role in initial tumorigenesis or metastasis, and to narrow the search for the putative oncogene in question.

1.11 The principles of microsatellite studies and allele imbalance

Cytogenetic techniques, such as CGH, have the advantage of examining the entire genome, but are of limited resolution. The minimum change detectable is in the order of 2,000,000 bases, whereas the highest resolution molecular genetic techniques, such as DNA sequencing and mutation analysis, are able to resolve changes at the one base pair level. Thus, cytogenetics helps to highlight which part of the genome is likely to contain the answer, and so points the way for subsequent molecular analysis (Cowan, 1992).

The analysis of allele imbalance is made possible by the presence throughout the genome of highly polymorphic, non-coding DNA sequences called microsatellites. These consists of small dinucleotide or trinucleotide units, repeated many times to give a length of DNA which is typically tens or hundreds of base pairs long. Microsatellites occur throughout the genome (approximately every 100,000 bps), and because they are highly polymorphic, they frequently differ in length between alleles, both within an individual, and between individuals. Primers are available, which map to either end of a particular microsatellite, and so through polymerase chain reaction (PCR, see Section 2.2) and subsequent resolution on an electrophoretic gel, are able to reveal the length of that microsatellite (Cawkwell *et al.*, 1993; Cawkwell *et al.*, 1994). If non-

tumour DNA from an individual yields two PCR products of different length at a particular microsatellite locus, it is said to be heterozygous, or informative for that marker. These products are represented graphically as 2 peaks following electrophoresis in an automated sequencer. If, however, the corresponding tumour DNA yields only one electrophoretic peak, the tumour is said to show loss of heterozygosity (LOH) or allele imbalance (AI). This implies that one of two things has occurred in the tumour DNA. More commonly, one allele, and therefore a variable length of the corresponding chromosome has been "lost" or deleted during tumourigenesis. Alternatively, one allele may have been amplified so many times, as to have effectively diluted out the presence of the other allele, such that it is not detected as a separate peak on electrophoretic resolution. The first implies the presence of a putative TSG, the second a putative oncogene. Thus, loss of heterozgosity is not necessarily synonymous with allele loss, although the two terms are frequently used interchangeably. The most likely explanation for this is that TSG inactivation appears to be far more common an event in head and neck tumourigenesis than proto-oncogene activation, and so the preferred term is 'allele imbalance'.

The resolution of microsatellite marker analysis depends on the number of markers analysed and their proximity to each other. Thus, if only one microsatellite is studied on a chromosome arm of interest, detection of AI in the tumour may imply loss of material anywhere in size between the length of the microsatellite itself through to loss of the entire chromosome. It is only through studying multiple markers on a chromosome arm that the affected area of AI can be narrowed down, and a minimum common area of loss identified. This concept

of examining LOH in tumours was popularised by Vogelstein's analysis of colorectal carcinomas, and has been termed allelotyping (Vogelstein *et al.*, 1989).

AI data have largely been in agreement with the findings of classical karyotyping studies, although the frequency of aberration has tended to be higher with AI studies. This reflects the potentially higher resolution of AI, and suggests that discrete regions of loss more commonly affect some chromosomal loci than gross chromosomal deletion. Important examples include 9p (AI – 70-80%; karyotypic data ~10%) and 17p (AI – 40-55%; karyotypic data 5-10%), sites of the p16 and p53 TSGs respectively (Califano *et al.*, 1996; El-Naggar *et al.*, 1995; Nawroz *et al.*, 1994; Ah See *et al.*, 1994; Field *et al.*, 1995a; Ransom *et al.*, 1996).

1.12 Putative proteins in metastatic progression

1.12.1 Overview

An alternative way of identifying which genes on chromosome 22 are responsible for metastatic progression is to study the expression of the protein products of the five hundred or so known genes that reside on its long arm. This would obviously be very labour intensive, and the information thus obtained would not necessarily tally with the level of gene expression. For example, overexpression of a particular protein may be due to post-translational modification of the protein itself, rather than an underlying polysomy or genetic amplification. Protein stabilisation is often achieved through glycosylation, and so protein overexpression as detected by immunohistochemistry may be due to a decreased

rate of protein degradation rather than an increased rate of protein synthesis. None the less, proteins are the final effectors of cell function and behaviour, and so studying their expression is a very useful technique. A gene database was used to identify the known genes on chromosome 22 and search for those known to have a role in cell growth, motility or invasion (www.gdb.com). The only such gene was matrix-metalloproteinase 11 (*MMP-11*), which, therefore, is a candidate gene for the potential role of chromosome 22 in metastasis.

1.12.2 Matrix-metalloproteinase 11

Matrix metalloproteinases (MMPs) are a family of zinc endopeptidases that play a key role in both physiological and pathological tissue degradation (Egeblad and Werb, 2002). Normally there is a delicate balance between both cell division and apoptosis and matrix synthesis and degradation, which is under the control of cytokines, growth factors and cell-matrix interactions. MMPs are instrumental in tissue remodelling in physiological processes such as embryogenesis and wound healing. Pathologically they are involved in the cartilage destruction seen in chronic inflammatory conditions such as rheumatoid arthritis, whilst increased matrix destruction is though to promote tumour cell invasion and metastasis. Collectively, the 21 mammalian MMPs so far discovered are capable of cleaving any component of the ECM. The historical classification according to substrate specificity into collagenases, gelatinases, stromelysins and matrilysins, has been superseded by sequential numbering of the MMPs.

Figure 1.6 shows a schematic diagram of MMP-11 (or stromelysin 3), a 47kDa protein encoded by a gene on chromosome 22q11.2 (www.gdb.com). The other two members of the stromelysin family have broad substrate specificities, and degrade proteoglycans, laminin, fibronectin and some collagens. MMP-11 degrades serine proteinase inhibitors, α_1 proteinase inhibitor and α_1-antitrypsin (Pei *et al.*, 1994). This novel mechanism of action therefore increases the stability of other proteases, and so indirectly leads to ECM degradation.

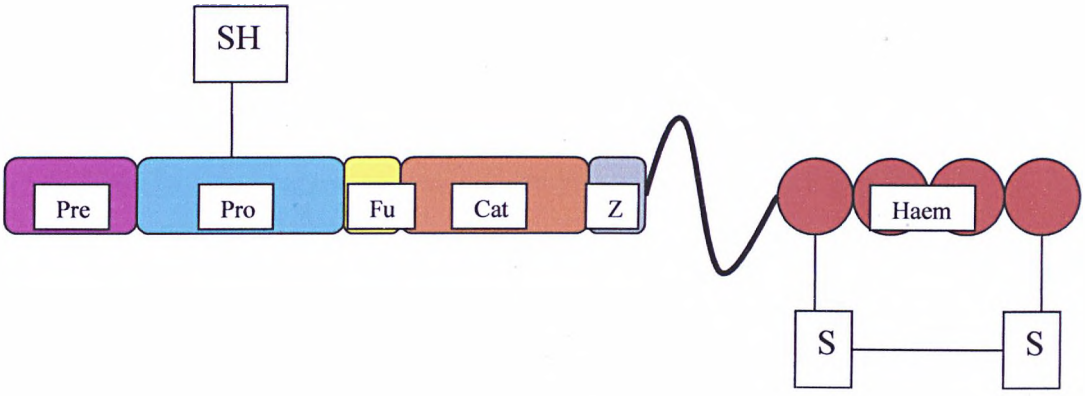


Fig 1.6-Schematic diagram of the structure of MMP-11

MMP-11 contains an amino-terminal sequence (Pre) that directs it to the endoplasmic reticulum, a propeptide (Pro) with a zinc-interacting thiol (SH) group that maintains it as an inactive zymogen and a catalytic domain (Cat) with a zinc binding site (Zn). MMP-11 also has a hemopexin-domain (Haem) that is connected to the catalytic domain by a hinge (~). The first and the last of the four repeats in the haemopexin-like domain are linked by a disulphide bond (S-S). The recognition motif (Fu) between the propeptide and catalytic domains allows intracellular activation by furin-like serine proteinases (adapted from Egeblad & Werb, 2002).

1.13 Aims of thesis

Preliminary CGH work in our laboratory has suggested that chromosome 22q may have a specific role in metastasis, in patients with HNSCC. This is an important finding as the high mortality in cancer is due mainly to tumour metastases. However, published literature suggests that 22q may have a role in initial tumorigenesis in HNSCC, rather than metastasis. This thesis is concerned with resolving this issue, and further narrowing the search for potentially important genes in metastasis.

The aims of this thesis were as follows:

1. To identify 28 primary laryngeal and pharyngeal squamous cell carcinomas with matched regional lymph node metastases.
2. To identify 26 non-metastasising primary squamous cell carcinomas of the larynx and pharynx.
3. To optimize the ABI Prism™ 310 Genetic Analyzer for AI studies in HNSCC.
4. To produce a high resolution AI map of the long arm of chromosome 22 for the 28 metastasising primary tumour DNA samples.
5. To use this information to test the following null hypothesis, "There is no difference in chromosome 22q status between normal and primary malignant tissue from patients with squamous cell carcinoma of the larynx or pharynx". This is the first step towards testing the theory that 22q has a specific role in metastasis, but not initial tumorigenesis in squamous cell carcinoma of the larynx or pharynx.

6. To conduct a gene database search, and use this information to identify "candidate" proto-oncogenes on 22q, which may be responsible for the role of 22q in metastasis.
7. To use immunohistochemistry to compare the level of expression of the protein products of these candidate genes between the metastasizing primaries, their matched lymph node metastases, and the non-metastasizing primaries.

CHAPTER 2

MATERIALS & METHODS

2.1 Sample collection and preparation

All general laboratory chemicals were obtained from Sigma, and all consumable plastics were obtained from Scientific Laboratory Supplies Ltd. (SLS) unless otherwise indicated. The full addresses of all suppliers are listed at the end of this chapter.

2.1.1 Identification of cases

Local Research Ethics Committee approval was granted to retrieve head and neck tumour specimens from the histopathology archives in Hull and East Yorkshire NHS Hospitals Trust (Hull, U.K.). Patients with histologically proven squamous cell carcinoma of either the larynx or hypopharynx were identified from the Head & Neck Cancer database, compiled by Professor N.D. Stafford, between 1995 and 2001. Details of 28 patients with histologically proven metastatic lymph node involvement, and 26 without lymph node involvement, are shown in Tables 2.1 and 2.2. All specimens had immediately been fixed in formalin following resection, and representative tissue blocks embedded in paraffin, catalogued and stored in the pathology archives, along with a haematoxylin and eosin (H&E) stained section of each block.

Patient	Age (sex)	Primary	T-stage	N-stage	Grade	F/U (mths)*	Recurrence*	Status*
1	71 (m)	Larynx (S)	T4	N1	Mod	60	N	A
2	68 (m)	Hypopharynx	T3	N2a	Poor	31	N	A
3	61 (m)	Hypopharynx	T3	N3	Mod	11	Reg	Dead
4	64 (m)	Hypopharynx	T3	N2b	Mod	62	Reg	A
5	67 (m)	Larynx (S)	T4	N1	Poor	3	N	Dead
6	68 (m)	Hypopharynx	T4	N1	Poor	56	N	Dead
7	49 (m)	Hypopharynx	T4	N2b	Poor	73	N	Dead
8	51 (f)	Hypopharynx	T4	N2b	Poor	17	Reg	Dead
9	58 (m)	Larynx (S)	T4	N1	Mod	28	N	Dead
10	58 (m)	Hypopharynx	T4	N2b	Poor	63	N	A
11	67 (m)	Larynx (S)	T4	N1	Poor	64	N	A
12	71 (m)	Hypopharynx	T4	N2b	Poor	33	N	A
13	56 (m)	Hypopharynx	T1	N2a	Poor	77	N	A
14	57 (m)	Larynx (G)	T4	N1	Mod	82	N	Dead
15	54 (m)	Hypopharynx	T4	N3	Poor	74	Lo	Dead
16	70 (m)	Larynx (S)	T4	N2b	Poor	38	Reg	A
17	48 (m)	Larynx (S)	T3	N2c	Poor	40	N	A
18	71 (m)	Hypopharynx	T1	N2b	Mod	21	N	A
19	48 (f)	Hypopharynx	T4	N3	Poor	26	Lo	Dead
20	78 (m)	Larynx (S)	T4	N1	Poor	6	N	Dead
21	52 (f)	Larynx (S)	T3	N1	Mod	80	N	Dead
22	62 (f)	Larynx (S)	T4	N3	Mod	6	N	Dead
23	55 (m)	Hypopharynx	T3	N2a	Poor	37	N	A
24	61 (m)	Larynx (S)	T3	N2b	Poor	25	Reg	A
25	56 (m)	Larynx (S)	T4	N2b	Poor	24	N	Dead
26	71 (m)	Larynx (S)	T4	N3	Mod	4	N	Dead
27	81 (m)	Hypopharynx	T1	N2c	Poor	23	N	A
28	55 (m)	Hypopharynx	T4	N1	Poor	89	Lo	Dead

Table 2.1 Clinico-pathological details of Node positive patients.
*Abbreviations: m, male; f, female; S, supraglottic larynx; G, glottic larynx; mod, moderate; F/U, follow up; mths, months; Lo, local; Reg, regional; A, alive. * On 31st August 2003. Patient 18, DNA extraction unsuccessful.*

Patient	Age (sex)	Primary	T-stage	Grade	F/U (mths)*	Recurrence*	Status*
29	72 (m)	Larynx (Sub)	T3	Anaplastic	82	N	A
30	70 (m)	Larynx (G)	T3	Poor	56	N	A
31	63 (f)	Larynx (S)	T4	Mod	63	N	A
32	65 (f)	Larynx (G)	T4	Mod	68	N	A
33	74 (m)	Larynx (G)	T3	Mod	56	N	A
34	80 (m)	Larynx (G)	T3	Poor	8	N	Dead
35	51 (m)	Larynx (G)	T3	Mod	23	Lo	Dead
36	60 (m)	Larynx (G)	T3	Mod	46	N	A
37	49 (m)	Larynx (S)	T3	Verrucous	43	N	A
38	63 (f)	Larynx (S)	T4	Mod	14	N	Dead
39	71 (m)	Larynx (S)	T2	Well	70	N	A
40	58 (m)	Larynx (Sub)	T4	Poor	19	N	Dead
41	61 (m)	Larynx (G)	T4	Poor	15	Lo	Dead
42	62 (m)	Larynx (G)	T3	Mod	87	N	A
43	51 (f)	Larynx (S)	T4	Poor	60	Reg	Dead
44	73 (m)	Larynx (S)	T4	Poor	33	Reg	A
45	59 (m)	Hypopharynx	T1	Poor	33	N	A
46	76 (m)	Hypopharynx	T4	Mod	89	N	A
47	74 (m)	Hypopharynx	T3	Mod	40	N	A
48	77 (m)	Larynx (G)	T3	Verrucous	30	N	A
49	64 (m)	Larynx (G)	T4	Verrucous	59	N	A
50	71 (m)	Hypopharynx	T4	Mod	5	N	Dead
51	83 (m)	Larynx (G)	T4	Mod	28	N	A
52	47 (m)	Larynx (G)	T4	Mod	59	N	A
53	90 (m)	Larynx (S)	T4	Mod	23	N	Dead
54	72 (m)	Larynx (S)	T4	Poor	29	N	A

Table 2.2 Clinico-pathological details of Node negative patients.
*Abbreviations: m, male; f, female; S, supraglottic larynx; G, glottic larynx; Sub, subglottic larynx; mod, moderate; F/U, follow up; mths, months; Lo, local; Reg, regional; A, alive. * On 31st August 2003.*

2.1.2 Clinico-pathological details

Table 2.1 contains the pertinent clinico-pathological details of the 28 patients selected for genetic analysis. All had histologically proven SCC of either the larynx or pharynx with regional metastasis to the neck. 24 of the patients were male, and 4 were female. The mean age at diagnosis was 61.7 years (range 48-81). The mean length of follow up, amongst surviving patients, was 43 months (range 23-77). Mortality data for the cohort are also shown.

Table 2.2 contains the pertinent clinicopathological details of the 26 patients with non-metastatic head and neck cancer selected for immunohistochemistry. All had histologically proven SCC of either the larynx or pharynx with no evidence of regional metastasis to the neck a minimum of 5 months after diagnosis. 22 of the patients were male, and 4 were female. The mean age at diagnosis was 67 years (range 47-90). The mean length of follow up, amongst surviving patients, was 54 months (range 5-89). Mortality data for the cohort are also shown.

2.1.3 Sample retrieval and specimen selection

All tissue blocks and their respective H&E stained sections were retrieved for each case. Examination of the H&E sections allowed selection of the most appropriate tissue blocks for further analysis. Where possible, blocks were selected which contained both malignant and histologically benign squamous epithelium, for the purposes of

immunohistochemistry. This was to enable a comparison to be made in the staining pattern between benign and malignant epithelium. Tumour and metastatic lymph node blocks with the highest malignant to normal cell ratio were selected for DNA extraction. Histologically normal blocks of tissue (usually negative lymph nodes) were identified for extraction of normal (control) DNA. Further selection criteria for blocks were a paucity of laryngeal cartilage, for ease of microtome sectioning, and easily identifiable foci of tumour, for ease of subsequent microdissection.

2.1.4 Section preparation

The selected blocks were cooled on ice, sectioned with a microtome (to 4μm thickness) and floated onto labeled microscope slides in a water bath at 40°C. For each block, one section was reserved for H&E staining and twelve for subsequent microdissection. The orientation of the sections was the same on each slide, to facilitate microdissection. The microtome was cleaned with xylene after each block, to avoid DNA contamination between different tumours. After draining excess water, the slides were then placed on a hotplate (Electrothermal Slide Drying Bench) at 70°C for 20 minutes, to encourage adhesion between the wax-embedded section and the slide.

2.1.5 Haematoxylin and eosin (H&E) staining

Haematoxylin is a basic dye that is blue in colour and predominantly stains nuclear structures (e.g. DNA), whilst eosin is an acidic dye that is pink in colour and mainly stains cytoplasmic structures (e.g. mitochondria).

Sections were deparaffinised in three successive changes of xylene (5 minutes each), rehydrated in 100% alcohol and then rinsed and immersed in tap water. Slides were stained in Harris haematoxylin for five minutes and excess stain removed under running tap water. Colour was then differentiated by repeatedly dipping the slides (x10) in 1% acid/alcohol (1% v/v HCl; 70% v/v ethanol), before rinsing and staining with 1% w/v eosin for two minutes. The slides were then rinsed under running tap water for one minute and dehydrated through three successive alcohols (100%) for thirty seconds each. Sections were briefly cleared in three changes of xylene, before applying Histomount (National Diagnostics) and cover slips.

2.1.6 DNA extraction

Genomic DNA was extracted using a Nucleon® BACC1 DNA Extraction Kit (SL-8501, Tepnel Life Sciences PLC). Sections were dewaxed in a warm (37°C) bath of xylene, and briefly dipped in two xylene baths at room temperature. Sections were then rehydrated by dipping in two changes of absolute alcohol followed by two changes of distilled water, and finally left in fresh distilled water.

H&E sections of patients' normal tissue were assessed to confirm that, histologically, the samples did not contain any malignant cells. All the sections for each case were then scraped off their slides with a scalpel blade (size 11 or 15) and transferred to a labeled screw-cap tube. A new scalpel blade was used for each case, to prevent cross-contamination.

The tumour H&E sections were examined, and the areas containing a histologically high proportion (at least 60% by area) of tumour cells were highlighted with a permanent marker pen. The highlighted H&E section was then used as a template to guide in the dissection of the unstained tumour sections. The tumour-rich areas were then scraped and transferred in the same way as the normal tissue.

340µl Reagent B (sodium dodecyl sulphate, Nucleon DNA Extraction Kit) and 3.4µl of proteinase K ($20mg.ml^{-1}$) were added to each tube to digest the tissue. The tubes were then inverted several times to mix the contents, before placing them on a rotator and incubating at 37°C for 48 hours.

2.1.7 DNA purification and precipitation

Deproteinisation of the cell lysate was achieved by adding 100µl 5M sodium perchlorate solution and mixing thoroughly. 600µl chloroform was added and mixed, to give a clear DNA containing phase and a whitish protein-containing "organic" phase. These two phases were then clearly demarcated by adding 150µl Nucleon™

Resin (Nucleon Silica Suspension kit) and spinning in a microfuge at 2000 rpm for 1 minute. The DNA-containing upper layer was removed, taking care not to disturb the resin layer, and transferred to a clean 1.5 ml polypropylene tube (Eppendorf). The contents were then examined in good light for the presence of brown resin. If present, these samples were again briefly spun and the contents transferred to a new Eppendorf and re-examined. Once deemed uncontaminated to the naked eye, DNA precipitation was carried out by adding 2 volumes (~1ml) of cold (-20°C) absolute ethanol, and 1µl glycogen (Gibco BRL®). The heavy glycogen molecule forms a nidus, onto which the DNA can precipitate within the aqueous phase. The mixture was then incubated at -80°C for 30 minutes, to encourage precipitation of the small DNA fragments commonly seen in fixed tissue. After thawing, samples were spun in the microfuge at 12,000 rpm for 15 minutes, to pellet the DNA. The ethanol supernatant was carefully discarded, and the remaining pellet further purified by adding 1ml 70% ethanol and again spinning and discarding, before allowing the DNA pellet to briefly air-dry. The DNA was then re-suspended in 50µl molecular biological grade water, and stored as "stock" DNA at 4°C.

2.1.8 Agarose gel electrophoresis

The "stock" DNA was resolved by agarose gel electrophoresis, to ensure success of the extraction technique. Tris-acetate EDTA (TAE) buffer (40mM Tris-acetate, pH7.5-7.8, 1mM EDTA) was used to make the gel and as a running buffer. A 2% w/v submarine agarose gel was prepared by adding 1g of Ultrapure agarose (Gibco) to 50mls of TAE

buffer. 2µl ethidium bromide was added, to intercalate with the DNA and allow its subsequent visualization under ultraviolet light. The gel was poured into the tank and wells cast with a comb (Flowgen Instruments), before completely immersing it in running buffer. For every 5µl of stock DNA, 1µl of 6X Gel Loading Buffer (0.25% w/v bromophenol blue, 30% v/v glycerol in Tris HCl pH 8.0) was added to each well, to prevent the DNA from floating away and to indicate the position of the leading edge of the sample along the gel. 1µg of a molecular weight ladder (Bioline) was also run on the gel, to permit an approximation of the size of the DNA fragments. The gel was then run at 80-100V for approximately 40 minutes, and photographed on a UV transilluminator using instamatic film (Polaroid).

2.2 The Polymerase chain reaction

2.2.1 Principles of the technique

The polymerase chain reaction (PCR) is a way of "amplifying" or making multiple copies of a piece of nucleic acid (Saiki *et al.*, 1986). One important application is the generation of large quantities of DNA suitable for molecular analysis, from relatively poor quality template sources such as archival paraffin-embedded tissue. The reaction revolves around a chosen pair of oligonucleotide primers, whose 5' ends flank a region of interest in the target DNA. A reaction, containing the oligonucleotide primers, a mixture of four deoxynucleotide triphosphates (dATP, dGTP, dCTP and dTTP), magnesium chloride, reaction buffer, a thermostable DNA polymerase and the double

stranded DNA template, is subjected to a repetitive series of temperature changes. These cycles involve a period of template denaturation (at ~ 95°C), primer annealing to DNA template (at ~ 55°C), and extension of the primers across the sequence between them (at ~ 72°C), which terminates on reaching the end of the primed strand. The newly synthesized extension products then serve as additional targets for subsequent cycles.

During denaturation, heat overcomes the covalent bonds holding the two strands of the DNA double helix together. This enables specific oligonucleotides (short single stranded lengths of DNA) to mark or "prime" either end of the section of DNA of interest. One of each primer pair may be labeled with a fluorochrome, if a fluorescence detection method is to be employed to detect the final product. The specificity of this annealing step reaction is again controlled by heat, the high reaction temperature overcomes non-specific binding of the primers and ensures that they only bind specifically to their complementary sequences on the sample DNA. A thermostable polymerase enzyme (Taq polymerase), extracted from a bacterium that exists naturally in a high temperature environment (Thermophilus *aquaticus*), then synthesises a copy of the nucleotide sequence between the primers, Following these 3 steps of DNA denaturation, primer annealing and sequence extension, the whole cycle is again repeated.

It can be seen that at the end of each cycle, the number of copies of DNA has doubled. In this way the amount of DNA increases exponentially with the number of cycles of

PCR. A typical 32 cycle PCR protocol will therefore amplify the initial number of copies of single stranded DNA by a factor of 2^{32}.

2.2.2 Reaction composition

All PCR mixtures were prepared in a sterile PCR hood, in a dedicated PCR laboratory. An ABI PRISM® True Allele™ PCR Premix solution (P/N 403061, Perkin-Elmer Applied Biosystems), consisting of AmpliTaq Gold® DNA polymerase, the four deoxynucleotide triphosphates (dATP, dTTP, dGTP, dCTP) and $MgCl_2$ buffer, was aliquoted and stored at -20°C. AmpliTaq Gold® is a "hot-start" Taq polymerase, which remains inactive until it has been heated to 95°C for 10 minutes, increasing operator control over the precise timing and duration of reactions. Likewise, the oligonucleotide primers were aliquoted before storing at -20°C, again to minimise the effects of repeat freeze-thawing cycles with each run of PCR reactions. The stock DNA was diluted to 1:10 for PCR use, with biological grade water (DNAase, RNAase free; Invitrogen).

Each PCR reaction was carried out in a final volume of 10µl consisting of the following final composition: 6µl True Allele Mix, 0.65µl oligonucleotide primer, 1µl template DNA and 2.35µl biological grade water.

2.2.3 Thermal cycling conditions

The aim of the PCR reaction was the synthesis of an optimal number of copies of the microsatellite regions of interest, for detection in the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems). The cycling parameters (the annealing and extension temperatures, the holding times for denaturation, annealing and extension, and the number of cycles) therefore required optimisation.

An empirical protocol was devised, based on the previous findings of Cawkwell et al. (Cawkwell *et* al., 1993; Cawkwell *et al.*, 1994; Cawkwell *et al.*, 1995) as well as the True Allele™ manufacturer's protocol (Perkin-Elmer). They had used primers and microsatellites of a similar size, as well as T*aq* DNA polymerase, which is known to catalyse DNA extension at an optimal temperature of 72°C. The cycle number was optimised for each DNA sample to ensure that the PCR products were detectable but not overamplified, as this caused the subsequent Genescan™ quantitation of peak height to be inaccurate and therefore unusable.

All reactions were carried out in a PCR machine (2400, Perkin-Elmer, Warrington) and the thermal cycling conditions for a typical PCR were as follows:

- Initial denaturation at 95°C for 10 minutes, to activate the AmpliTaq Gold®.
- 32 cycles of:

  denaturation at 95°C for 30 seconds

  annealing at 55°C for 1 minute

Extension of the products occurred at 72°C as they were heated up towards the denaturation temperature after annealing of the primers. As the length of the products was so short (~ 100-200 bps), it was not necessary to "hold" the samples at 72°C. PCR products were then stored at 4°C, until required for analysis.

2.3 Selection of microsatellite markers

Fine deletion mapping was performed for chromosome 22q, using a set of microsatellite markers that were approximately equally spaced along the long arm of chromosome 22. Only dinucleotide repeats were used as they produce alleles within a narrow size range, thereby avoiding preferential amplification problems. They are also more common than other microsatellites and are a well established research tool. Commercially available markers from Perkin-Elmer Applied Biosystems, which are grouped into low, medium and high resolution linkeage mapping sets, with mean resolutions of 20, 10 and 5 centimorgans (cM) respectively, were used (Perkin-Elmer Applied Biosystems). A total of 9 markers were selected from these three sets, to give an optimal spread across 22q. These markers had been selected from the 1996 Genethon linkeage map (Dib *et al.*, 1996). This human genome linkeage map was constructed using a bank of genotyping data from 8 large families. This CEPH (Centre d'Etudes Polymorphisme Humain) collaboration of over 100 laboratories, enabled the relative positions of these microsatellite markers to be determined by statistical linkeage analysis (Weissenbach *et al*, 1992; Gyapay *et al.*, 1994). The location of each of the markers selected is shown against an ideogram of chromosome 22 (Fig 2.1).

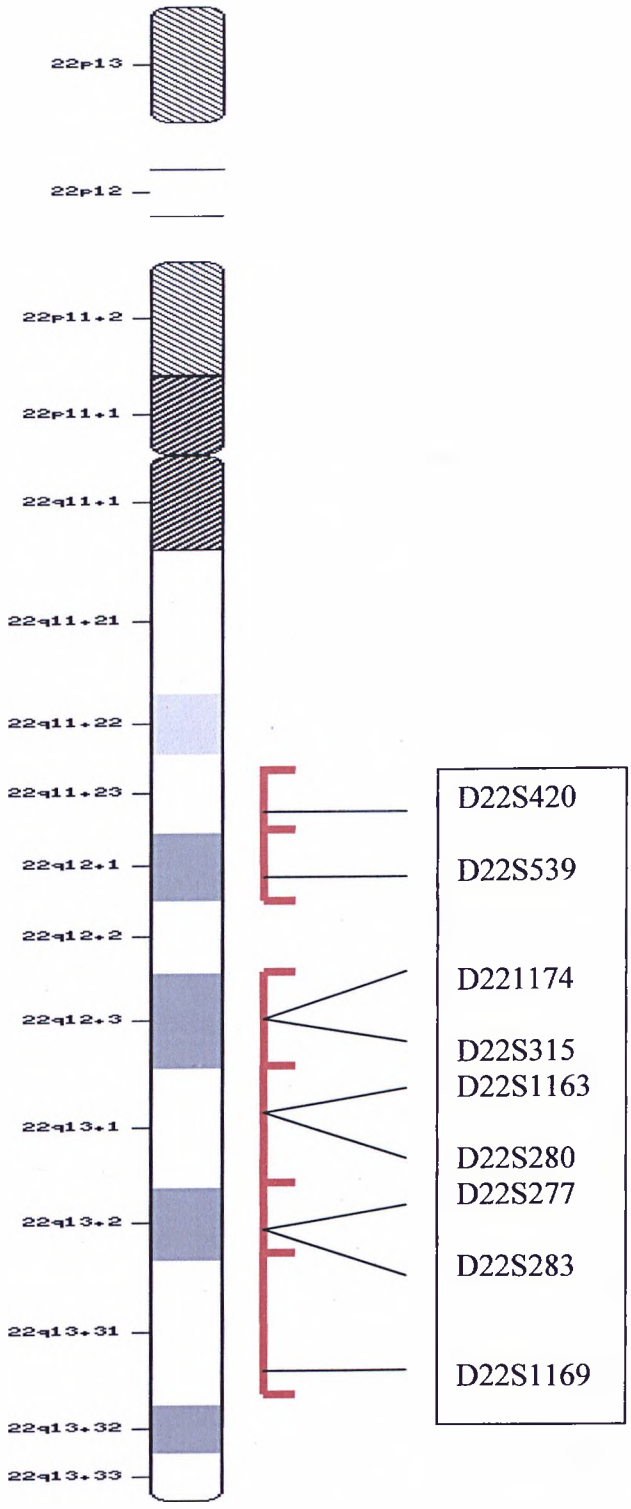


Figure 2.1 An ideogram of chromosome 22 illustrating the region to which each microsatellite marker maps.

Table 2.3 shows the properties of each marker. Microsatellite marker nomenclature starts with a "D" then the chromosome number, followed by "S" and then the unique marker number (e.g. D22S420). The fluorescent dyes, [FAM], [HEX] and [NED], emit blue, green and yellow fluorescence respectively, although the colour of the peak seen on the monitor depends on which matrix is used (see Section 2.4.4). The internal size standard is labeled with [ROX], which appears as red on the electrophoretogram. Heterozygosity refers to the proportion of the population expected to be heterozygous for that particular marker. A high degree of heterozygosity is desirable when selecting a microsatellite marker, as homozygous individuals obviously cannot be analysed for LOH, and so are discounted as "non-informative" cases. The allele size range is the size range, in base pairs, of all the different alleles for each marker. Co-loading of different markers with overlapping allele size ranges is only possible if they are labeled with different fluorescent dyes, so that they can be distinguished from each other during analysis. Table 2.3 illustrates three ways of describing the locus of each marker. Each marker is described in terms of its G band location. The relative distance between markers is given a value in centimorgans (cM), and is based on the linkage analysis studies used to construct the Genethon map (Dib *et al.*, 1996). A unit of measure of recombination frequency, one centimorgan is equal to a 1% chance that a marker at one genetic locus will be separated from a marker at a second genetic locus due to crossing over in a single generation. The final column gives the physical distance of each marker in kilobases from the p terminus. This information was obtained from the Cedar website, and is based on the findings of the Human Genome Project (ftp://cedar.genetics.soton.ac.uk).

Locus	Dye	Het	ASR (bp)	Dist (cM)*	Band**	Pter-(kb)**
D22S420	Hex	0.77	156-172	10.0	q11.23	25.470
D22S539	Ned	0.58	203-221	5.5	q12.1	29.377
D22S1174	Ned	0.82	126-152	1.1	q12.3	36.105
D22S315	Fam	0.78	184-214	n/a	q12.3	36.678
D22S1163	Fam	0.75	147-163	3.8	q13.1	40.648
D22S280	Hex	0.82	216-228	4.9	q13.1	41.841
D22S277	Ned	0.85	165-201	2.0	q13.2	43.123
D22S283	Ned	0.89	132-160	n/a	q13.2	43.626
D22S1169	Fam	0.78	69-85	-	q13.31	49.705

Table 2.3 **Microsatellite markers**

Note that the locus of each marker can be described in terms of the relative distance between markers in centi-Morgans (number refers to the distance between that marker and the one in the row below), as a Q band location or as the number of base pairs from the tip of the short arm of chromosome 22p. See text for further explanation.
Abbreviations: Het, heterozygosity; ASR, allele size range; dist, distance; cM, centi-Morgans; kb, kilobases; pter, p-terminus; n/a, not available.

* *http://www.perkin-elmer.com*
** *ftp://cedar.genetics.soton.ac.uk*

Gaps remained near the centre of 22q (22q12.2), at the centromeric end of 22q (22q.11.1, 22q11.21, 22q11.22) and at the telomeric end (22q.13.32, 22q.13.33).

2.4 Genetic analysis

2.4.1 Fluorescent PCR

In fluorescent PCR, one of the pair of oligonucleotide primers is labeled with a fluorescent dye prior to performing a conventional amplification reaction. This allows subsequent fragment detection, following electrophoresis, by a fluorescence based DNA analysis system, such as the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems).

2.4.2 Overview of the ABI® PRISM 310 Genetic Analyser

This automated machine performs capillary electrophoresis on fluorescently labeled DNA, for the purposes of sequencing and PCR fragment analysis. The major advantages of this system over conventional microsatellite analysis techniques are that it uses fluorescence instead of radioactivity, and polymer-filled capillaries instead of polyacrylamide gel. It is therefore safer (no radioactivity) and less labour intensive (no gels to prepare).

A single capillary connects a cathode electrode to an anode electrode. Dye-labeled DNA fragments (e.g. fluorescent microsatellites) migrating through the polymer filled capillaries make the electrical connection. This separates fragments according to size, which are then excited by a laser beam. A spectrophotometer then collects and separates the fluorescent light emitted by the dyes. A camera then records the quantity of fluorescence at each wavelength. This information is converted into digital data, which is then processed by the data collection software. The Genescan® (Applied Biosystems) analysis software analyses the resulting peaks, and so is able to accurately determine the size of fragments, in base pairs, as well as semi-quantitatively compute the amount of product, according to the relative intensity of the fluorescence (peak height).

2.4.3 Fragment mixture composition

A mastermix of Genescan-350™ ROX size standard (Applied Biosystems, see Section 2.4.4) and deionised formamide was made. Mastermix and PCR product were then added to a 0.5ml microtube, to give a final mixture composition of 1µl PCR product, 0.5µl Genescan-350™ ROX and 12µl deionised formamide. The tube was sealed with a rubber bung, before pulsing on a centrifuge. The contents were denatured at 95°C for 5 minutes in a Techne™ Genius thermal cycler (SLS) and the tube then rapidly transferred to ice to prevent reannealing.

2.4.4 Fragment analysis

The ABI PRISM® 310 Genetic Analyzer was used in Genescan® mode for allele peak analysis (rather than Sequence® mode, which is used for "calling bases"). Up to 48 microtubes could be loaded at a time, each of which could hold several different fragment mixtures ("co-loading"). This versatility is due to the analyser's ability to recognise and process signals from four different fluorescent dyes simultaneously. Also, some of the PCR fragments varied greatly in length, and so 2 microsatellites labeled with the same dye could be run together, if there was no overlap between the size ranges for their respective microsatellites.

In addition, each microtube contained a size standard (Genescan-350™ ROX), consisting of DNA fragments of known length and fluorescence. Genescan-350™ ROX enables DNA fragments between 35 and 350 base pairs long to be sized, and consists of a mixture of the following sized fragments: 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340 and 350 base pairs. This enabled the computer software to calibrate each run, and so calculate the size of each DNA fragment that passed the laser beam. The same general principle applies with capillary electrophoresis; that small DNA fragments move more quickly than large ones through the polymer. As the voltage applied between cathode and anode was constant, the software was able to accurately compute the size of fragments that were different to those in the ROX size standard, on the basis that the time taken for a fragment to reach the laser beam was inversely proportional to its molecular weight, which in turn is proportional to its size, in base pairs.

The cathode end of the capillary is suspended above the autosampler, a mechanical tray that holds the microtubes. The anode end of the capillary is immersed in a genetic analyser buffer. On initiation of a run, the autosampler presents the first microtube to the cathode end of the capillary. A potential difference is then applied between the electrodes, which draws a portion of the sample into the capillary; a process known as electrokinetic injection. This potential difference is then maintained for the duration of the run and is responsible for fragment separation by electrophoresis. The contents of the capillary are held at a constant temperature throughout. Thus separated by size, measuring fluorescence will permit calculation of the quantity of each fragment. The smallest fragments are the first to reach the Argon-ion laser beam. This shines through a small window in the capillary and excites the fluorescent tags. The resulting fluorescence is collected, via a series of lenses, by a spectrophotometer. This focuses the separate wavelengths onto a charge-coupled device (CCD) camera, which records the intensity of fluorescence. The data collected, stored and analysed by the Genescan® Collection Software (ABI), includes the colour, height and area of each fluorescent peak and the fragment size to which it corresponds in base pairs. As mentioned earlier the time taken for the fragments to reach the laser beam is proportional to fragment size, whilst the height and area of each peak is proportional to the quantity of each PCR product. The matrix is a virtual filter, which allows one set of fluorescent peaks to be analysed at a time, by subtracting out the signal within each dye's detection range that is due to fluorescence from other dyes (Figure 2.2).

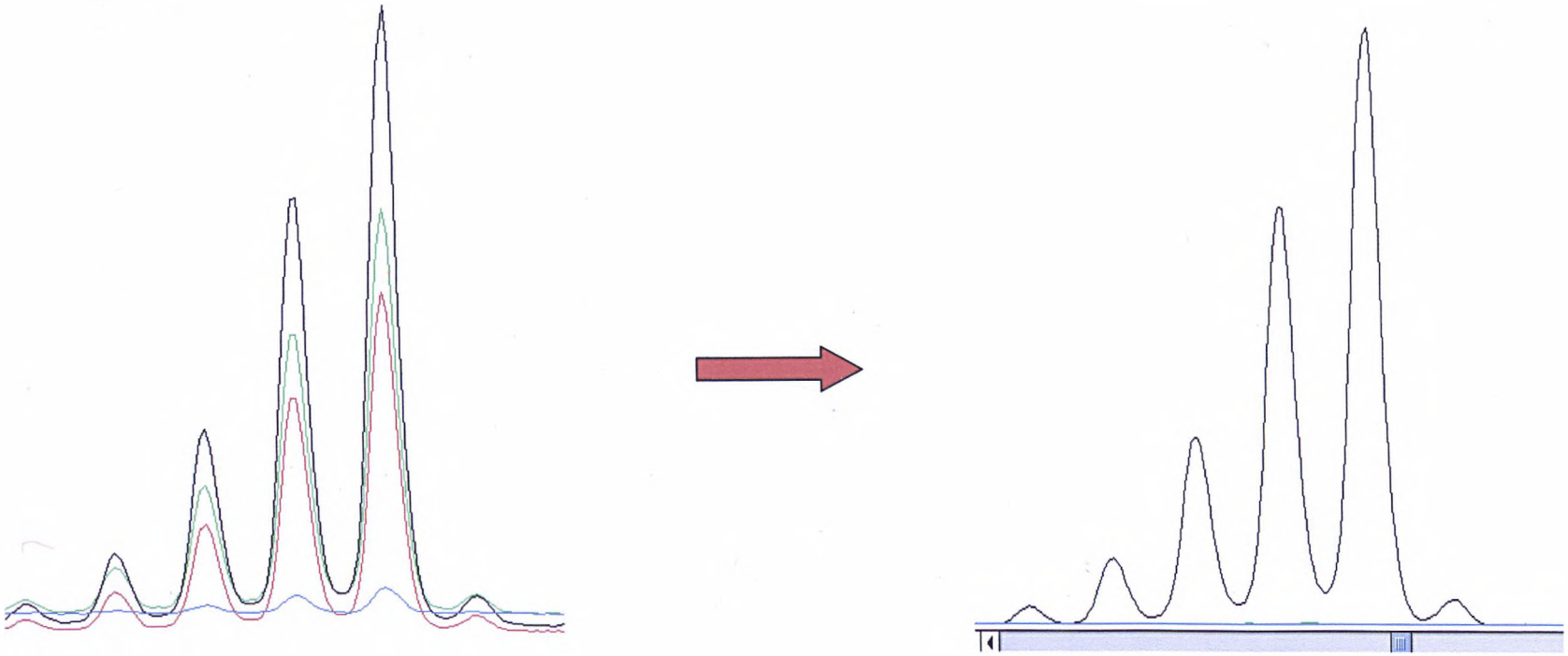


Figure 2.2 **The matrix principle.** Each PCR fragment actually emits a spectrum of fluorescence. The matrix is a virtual filter, which extracts all but the most intense of these wavelengths. This allows each PCR fragment to be represented as a mono-fluorescent peak.

The matrix needs to be re-calibrated each time one of the physical parameters of the analyser is altered (e.g. dyes used, polymer type or concentration).

2.4.5 Electrophoretic parameters

The GS STR POP4 program was used, and has the following characteristics: potential difference: 5 mV; electrokinetic injection time: 5 seconds; polymer: POP4™ (performance optimised polymer at a concentration of 4%, ABI); duration of run: 24 minutes; holding temperature: 60°C. As the analyser can hold up to 48 microtubes, the time taken to complete processing when loaded to capacity is in the order of 19 hours (48 x 24 mins).

2.4.6 Genescan® analysis

Details regarding the interpretation of peaks, with examples, are discussed in section 3.2. Analysis started with checking the series of peaks produced by the internal size standard. If these corresponded with the expected pattern, then this suggested that the run had been technically successful and that micro-injection and electrophoresis had taken place. The scale on the y-axis was then adjusted, so that it was appropriate for the height of the series of peaks to be analysed. The tallest peak within a series represented the most abundant allele, and the Genescan® analysis software highlighted its size (in base pairs) as well as the peak height and area under the peak. If heterozygous, the same information was highlighted and recorded for the second allele peak. Obviously

homozygotes were rejected from further analysis as, by definition, they cannot be used to assess allele imbalance. These data were stored in the computer hard-drive, and printed off for subsequent calculation of the allele imbalance ratio and verification of the result by the supervisor.

2.4.7 Calculation of allele ratios

The degree of AI was then quantified for the heterozygous samples, by calculating the allele ratio of paired normal and tumour samples. Worked examples of calculations are shown in Figures 3.8 and 3.9. The ratio of alleles was calculated for each normal and tumour sample, and then the tumour ratio was divided by the normal ratio: T1:T2 / N1:N2, where T1 and N1 are the area values of the shorter length allele product peak for the tumour and normal sample respectively, and T2 and N2 are the area values of the longer length allele product peak for the tumour and normal samples respectively. In cases where the resulting allele ratio was greater than 1.00, the reciprocal was taken (i.e. 1 / [T1:T2 / N1:N2]) to give a result in the range 0-1. An allele imbalance ratio of <0.5 was taken to indicate significant allele imbalance, or loss of heterozygosity (Cawkwell *et al.*, 1993). This is based on the theoretical basis that tumours containing no normal contaminating cells and showing complete allele loss would give a ratio of 0. However, the relatively crude method of separating tumour and normal cells in this study resulted in inevitable contamination with normal cells. Cawkwell *et al.* 1993, estimate that some tumour extracts in their series contained up to 50% normal cells, giving an allele ratio of up to 0.5 in tumours demonstrating complete allele loss. The

emerging technology of laser capture microdissection of tumour specimens may considerably reduce this threshold ratio in the future (Emmert-Buck *et al*, 1996).

2.5 Immunohistochemistry

2.5.1 General principles

Immunohistochemistry (IHC) is a detection method for the *in-situ* localisation of antigens of interest within a tissue section. Monoclonal antibodies to the antigen are raised by inoculation of a suitable recipient (e.g. rabbit or goat) with an epitope that is specific to that antigen. The resulting monoclonal antibody is purified and under optimal conditions will specifically bind antigen in tissue sections. A number of subsequent steps then amplify this specific binding between antigen and antibody, and finally cause a localized colour change that can be detected under the light microscope. The principles of this technique are shown in Figure 2.3.

The first step in working with archival sections is to deparaffinise the slides and rehydrate the tissue sections. Formalin fixation of tissue at the time of surgery can conceal antigen binding sites of potential interest, and so these epitopes must be "unmasked" or "retrieved" prior to incubation with antibody. Several techniques are available, utilizing heat, chemicals or enzymes, for overcoming the hydroxyl bonds that cross-link formaldehyde molecules between adjacent proteins.

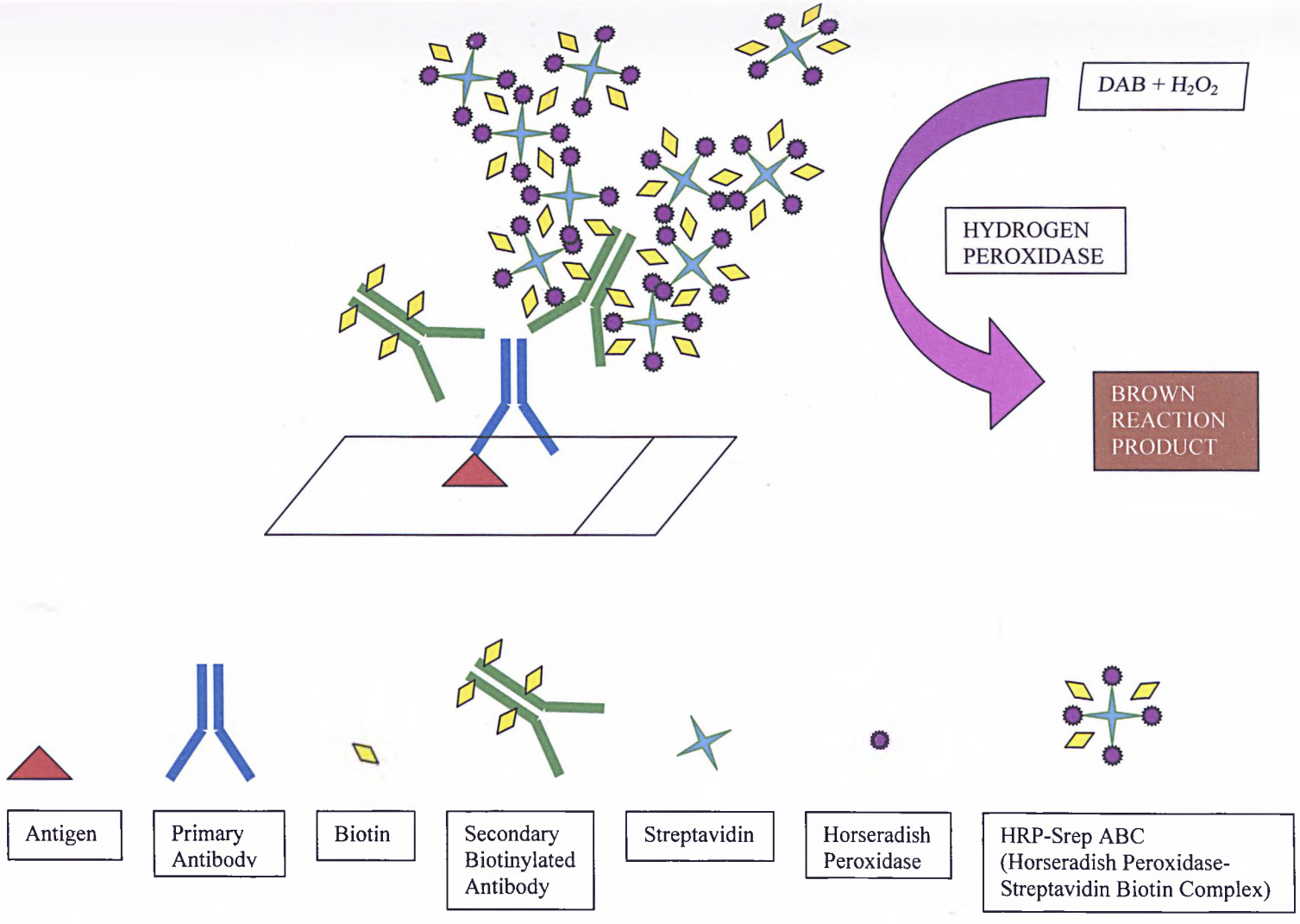


Figure 2.3 Schematic diagram of the principles of immunohistochemistry

DAB: 3,3'-Diaminobenzidine; H_2O_2: Hydrogen peroxide.

Endogenous tissue peroxidase activity must then be quenched with hydrogen peroxide, as the final chemical reaction that allows visualization of the site of bound primary antibody, is peroxidase dependent. Non-specific tissue binding sites (e.g. positively charged, hydrophobic and electrostatic binding sites) are then blocked with blocking serum or casein, to help eliminate non-specific or background staining.

The tissue sections are then incubated with primary monoclonal antibody. Prior "optimization" of the antibody defines the optimal antibody concentration and incubation time, so that maximal specific binding of antibody to antigen can occur, in the absence of non-specific binding. Biotinylated secondary antibody, which has been raised against immunoglobulins from the species that produced the primary antibody (e.g. anti-rabbit or anti-goat antibodies), is then applied. The secondary antibody is usually polyclonal, as it consists of a mixture of antibodies that recognize multiple binding sites on the primary antibody. An amplification step then exploits the high affinity of avidin for biotin (over 1 million times higher than that between most antibodies and their corresponding antigens, *Hsu et al*, 1981). Avidin (or streptavidin) is a large glycoprotein molecule derived from egg white that has binding sites for up to 4 biotin molecules. Biotin is a low molecular weight vitamin derived from egg yolk that has one high affinity binding site for avidin. The signal is amplified by adding HRP-StrepABC (horseradish peroxidase-streptavidin-biotin complex) to the tissue sections, after washing away unbound secondary antibody. This partially biotinylated molecule has most of its biotin binding sites free to bind to biotinylated secondary antibody as well as other HRP-StrepABC molecules. In this way, large

macromolecules rich in enzyme (horseradish peroxidase) are laid down wherever there is primary antibody bound to antigen. Signal is then visualized by adding hydrogen peroxide as the enzyme substrate, in the presence of the chromagen 3, 3'-diamino-benzidine tetrahydrochloride (DAB), which turns from colourless to brown.

Positive and negative controls are a critical part of IHC, and reflect the sensitivity and specificity of the staining, respectively. A sample of tissue known to express the particular antigen under investigation was used as a positive control (in this case breast carcinoma). A further breast carcinoma slide was used as a negative control, in which the primary antibody of interest was replaced by TBS. Both positive and negative controls were run with each batch of immunostaining.

2.5.2 Primary Antibody

Immunostaining was utilised to study the expression of MMP-11 in head and neck tumour specimens. A mouse monoclonal antibody (anti-MMP-11 (Ab-2), Cat # IM86), which had been raised against recombinant protein corresponding to the active form of human MMP-11, was used (Oncogene Research Products).

2.5.3 Protocol Optimisation

The established immunostaining protocol used in the Cell & Molecular Medicine Laboratory of the University of Hull was adopted as the basic framework for the final

protocol. A Vector unmasking and avidin / biotin blocking kit was used to unmask antigen and block non-specific binding sites (Vector Laboratories Ltd.). The secondary detection kit used was the StreptABC Complex / HRP Dako Duet kit (K0492, Dako), which provides the biotinylated secondary antibody, streptavidin-HRP complex, and DAB substrate. The only step of the protocol that required optimization for this particular antibody was the primary antibody concentration. All reactions were carried out at room temperature.

The manufacturer's recommended primary antibody concentration of 1 in 50 was tested on 20 head and neck specimens with controls. This was found to give consistently weak, and therefore possibly insensitive, staining, even on repeating the run. Concentrations of 1 in 40 and 1 in 30 were therefore attempted, with 1 in 40 giving optimal staining. This was intense and well localized, with no evidence of background staining, and was taken to be the corollary of maximal sensitivity and specificity.

2.5.4 Final immunostaining protocol

Tissue sections had been cut at 4μm and floated onto Superfrost* Plus slides, as detailed in section 2.1.4, and dried overnight at 37°C. Sections were racked and submerged in warm xylene for 10 minutes, before dipping in two further xylene baths for 10 seconds each. After de-waxing, slides were dipped in three successive ethanol baths (each 100%) for 10 seconds and rinsed in running tap water for 1 minute to rehydrate the sections. Endogenous peroxidase activity was then quenched by

incubating the sections in hydrogen peroxide in methanol (8ml of 30% w/v hydrogen peroxide in 400ml methanol). After rinsing in tap water, heat-induced epitope retrieval was carried out by boiling the slides in 1% v/v Vector Antigen Masking Solution in distilled water (1.5l final volume; Vector Laboratories Ltd.) for 3 minutes in a pressure cooker. Slides were cooled by rapidly transferring to TBS (Tris Buffered Saline pH7.5: 15mM NaCl; 40mM Tris(HCl)) for 5 minutes. Slides were mounted onto Sequenza coverplates whilst immersed in TBS, and placed in Sequenza racks (Shandon Scientific). The reservoirs were filled with TBS to rinse the slides and to identify any "fast flowers" (i.e. added TBS leaves the reservoir very quickly) due to trapped air bubbles between slide and coverplate. Such slides were re-assembled under TBS to exclude air, and re-checked for fast flow. In the first antigen blocking step, 100µl of 1x casein in TBS was added to each section for 10 minutes to block non-specific binding sites (Vector Laboratories). Slides were then rinsed in TBS for 5 minutes, before blocking endogenous biotin with 3 drops of avidin solution from the Vector blocking kit for 15 minutes. After a further rinse in TBS, endogenous avidin was then blocked with 3 drops of the biotin solution from the kit, again for 15 minutes, before rinsing twice in TBS for 5 minutes. Sections were then incubated with 100µl primary antibody, diluted to 1 in 40 in 0.2x casein in TBS, at room temperature for 2 hours. Primary antibody was omitted from the negative controls, and substituted for 100µl 0.2x casein in TBS. Slides were then rinsed twice with TBS for 5 minutes, to remove all unbound primary antibody. Biotinylated secondary antibody was then applied (100µl reagent C: biotinylated anti-mouse antibody; Strep ABComplex kit, Dako Cytomation) for 30 minutes at room temperature. After rinsing in TBS, sections were incubated with HRP-

StrepABC (horse radish peroxidase – conjugated Streptavidin biotin complex) for 30 minutes. This had been complexed 30 minutes before use and diluted in TBS (1 in 100) according to the manufacturer's instructions (reagents A and B; StreptABComplex / Dako Duet kit). Sections were again washed in TBS and transferred to racks. Immunoreactivity was visualised by adding H_2O_2 as enzyme substrate, in the presence of 0.05% 3,3'-diaminobenzidine (DAB) (3 mls DAB; 15 drops hydrogen peroxide {30% w/v}; 400mls TBS). Sections were monitored under the microscope for approximately 5 minutes, before rinsing off excess solution under running tap water for 2 minutes. Slides were then immersed in copper sulphate (0.5% w/v copper sulphate in 0.9% w/v saline) for 5 minutes to enhance staining. After rinsing in running tap water for 30 seconds, sections were counterstained in freshly filtered Harris haematoxylin (BDH) for 20 sections, to allow visualization of the cell nuclei. Slides were rinsed in running tap water for 30 seconds and differentiated by 10 dips in acid-alcohol (1% v/v HCl in 70% v/v ethanol) followed by a further rinse in rinse in running tap water for 30 seconds. Slides were then differentiated in Scott's tap water solution (0.2% w/v potassium bicarbonate, 2% w/v magnesium sulphate) to enhance the contrast between the DAB and the counterstain, before finally rinsing in running tap water for 10 seconds. The slides were then dehydrated through three successive baths of ethanol (100%) for 10 seconds each. Sections were briefly cleared in 2 changes of xylene, before applying Histomount and cover slips.

2.6 Suppliers names and addresses

Applied Biosystems

850 Lincoln Centre Drive, Foster City, California 94404, USA

www.appliedbiosystems.com

Bioline Ltd.

16 The Edge Business Centre, Humber Road, London, NW2 6EW, U.K.

BDH Laboratory Supplies

Broom Road, Poole, Dorset BH13 1TP, U.K.

Dako Ltd.

Denmark House, Angel Drove, Ely, Cambridgeshire CB7 4ET, U.K.

Flowgen Instruments

Broad Oak Enterprise Village, Broad Oak Road, Sittingbourne, Kent, ME9 8AQ, U.K.

Gibco BRL

Life Technologies Ltd., Lincoln Road, Cressex Industrial Estate, High Wycombe, Bucks, HP12 3XJ, U.K.

Invitrogen

PO Box 2312, 9704 CH, Groningen, The Netherlands.

National Diagnostics

Unit 4 Fleet Business Park, Itlings Lane, Hessle, East Riding of Yorkshire, HU13 9LX, U.K.

Oncogene™ Research Products

650 Albany Street, Boston, MA 0218, USA

Perkin Elmer

Warrington, Cheshire, U.K.

Scientific Laboratory Supplies Ltd.

Units 26-27, Wilford Industrial Estate, Ruddington Lane, Wilford, Nottingham NG11 7EP, U.K.

Sigma Chemical Company Limited

Fancy Road, Poole, Dorset, BH12 4QH, U.K.

Shandon Scientific

Shandon, Lancashire, U.K.

Tepnel Life Sciences PLC

Scotscroft Building, Towers Business Park, 856 Wilmslow Road, Didsbury, Manchester, M20 2RY, U.K.

Vector Laboratories Ltd.

16 Wulfric Square, Bretton, Peterborough, PE3 8RF, U.K.

CHAPTER 3

GENETIC ANALYSIS

RESULTS

3.1 Introduction

The aim of this chapter was to test the following null hypothesis, "There is no difference in chromosome 22q status between normal and primary malignant tissue from patients with squamous cell carcinoma of the larynx or hypopharynx". This is the first step towards testing the theory that 22q has a specific role in metastasis, but not initial tumorigenesis in squamous cell carcinoma of the larynx or hypopharynx.

This was achieved by producing a high resolution AI map of the long arm of chromosome 22 for the 28 node positive primary tumours of the larynx and hypopharynx selected for study (Table 2.1). Details of the methodology used and the nine microsatellite markers are to be found in Chapter 2. Briefly, after extracting normal and primary tumour DNA from tissue samples, PCR was carried out using fluorescent primers for the nine microsatellite markers of choice. Following electrophoresis, detection and quantification of the fragments was then performed using an automated DNA sequencer (ABI PRISM® 310 Genetic Analyser). The calculation of allele ratios was discussed in Section 2.4.7, and a ratio ≤ 0.5 was taken to indicate AI.

The success or otherwise of DNA extraction was confirmed by agarose gel electrophoresis (Fig 3.1). Nine microsatellites were studied in 54 DNA samples (27 normal and 27 tumour DNA samples as there was failure of tumour DNA extraction in one patient) giving a total of 486 sets of peaks for subsequent verification and analysis. In practice far more data was generated, as various problems were encountered that required some analyses to be repeated. The next section discusses

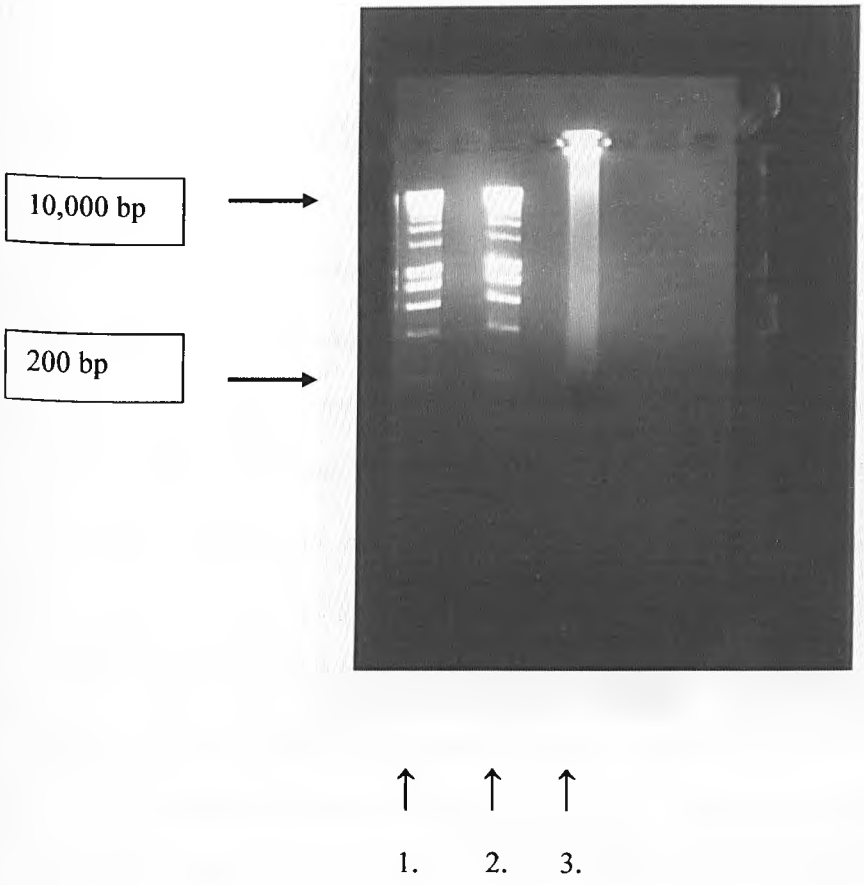


Figure 3.1 **Photograph of a 2% agarose gel**

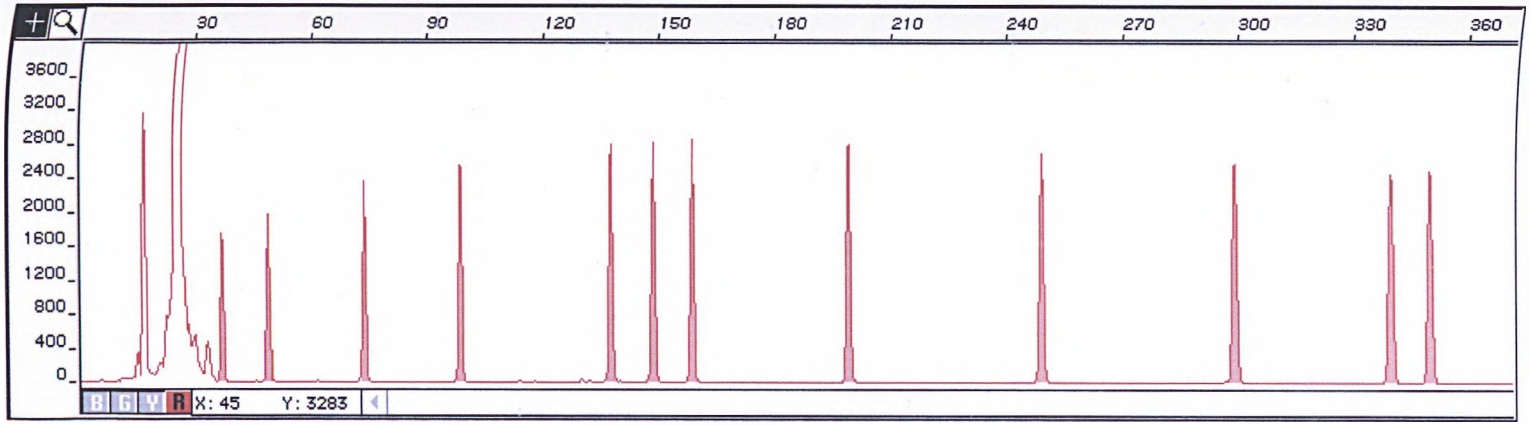
Lanes 1 and 2: Molecular weight ladder (Hyperladder 1, Bioline); Lane 3: extracted DNA sample (undigested stock DNA). This confirms that DNA extraction has been successful, the majority of it remaining close to the well due to its high molecular weight.

the way in which the data were interpreted, the problems encountered, and the solutions used to overcome these problems.

3.2 Data interpretation and troubleshooting

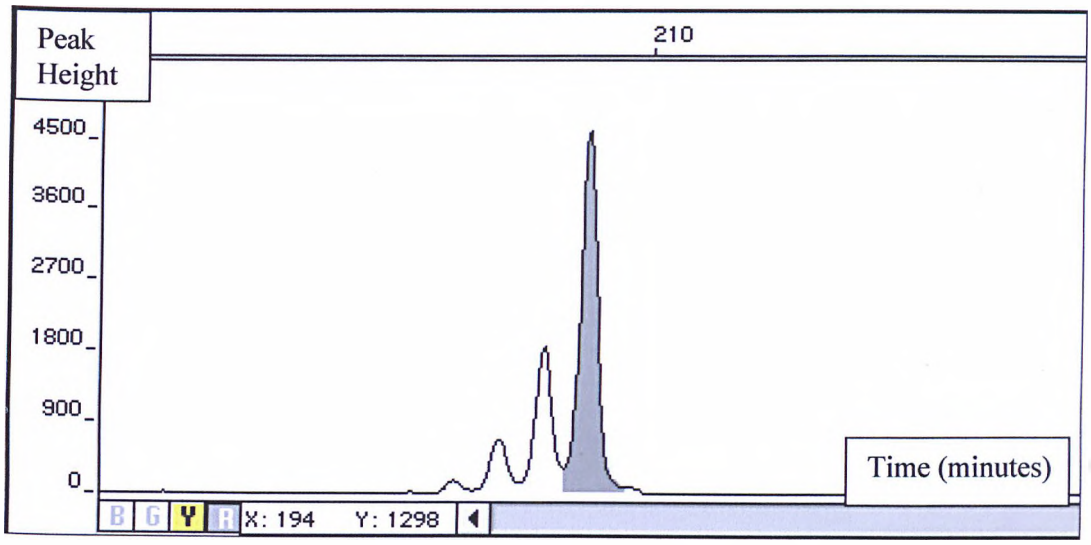
The Genescan® software generates a cross-sectional image (a "peak") for each fluorescent PCR product. This visual representation of the results, or electrophoretogram, greatly aids their interpretation. In addition each product is automatically quantitated in terms of peak height and area, and the product size is calculated in base pairs, using the internal size standard as a reference. Figure 3.2 shows the series of peaks generated by the Genescan-350™ ROX internal size standard, whilst Figures 3.3 and 3.4 illustrate the typical pattern of peaks seen in a homozygous and heterozygous control sample, respectively. The tallest peak was taken to represent the only allele in a homozygous sample, and the smallest allele (smallest number of base pairs) in a heterozygous sample. A second, shorter peak was seen in heterozygotes, which represents the larger (greatest number of base pairs) allele. The second allele peak was always a multiple of 2 base pairs greater in length than the first allele peak, owing to the fact that all the microsatellites studied were dinucleotide repeats. The bigger of the two alleles always produces a shorter peak, due to the less efficient amplification of longer fragments by PCR, effectively reducing the amount of product for electrophoresis.

Stutter peaks are shorter than the allele peaks that they immediately precede and are always in multiples of 2 base pairs shorter in length (Figures 3.3 and 3.4). They are believed to be due to the *Taq* polymerase enzyme falling off the fragment before



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
R, 16	10.79	38.04	1762	10804	2942
R, 18 •	11.28	50.00	1999	11452	3074
R, 20 •	12.29	75.00	2392	13065	3350
R, 21 •	13.14	100.00	2610	14229	3582
R, 23 •	14.47	139.00	2875	15794	3945
R, 24 •	14.80	150.00	2893	16081	4035
R, 25 •	15.10	160.00	2868	16448	4118
R, 26 •	16.41	200.00	2818	17955	4474
R, 27 •	17.86	250.00	2720	19020	4870
R, 28 •	19.51	300.00	2595	21219	5319
R, 29 •	20.64	340.00	2456	21910	5628
R, 30 •	20.97	350.00	2493	22680	5718

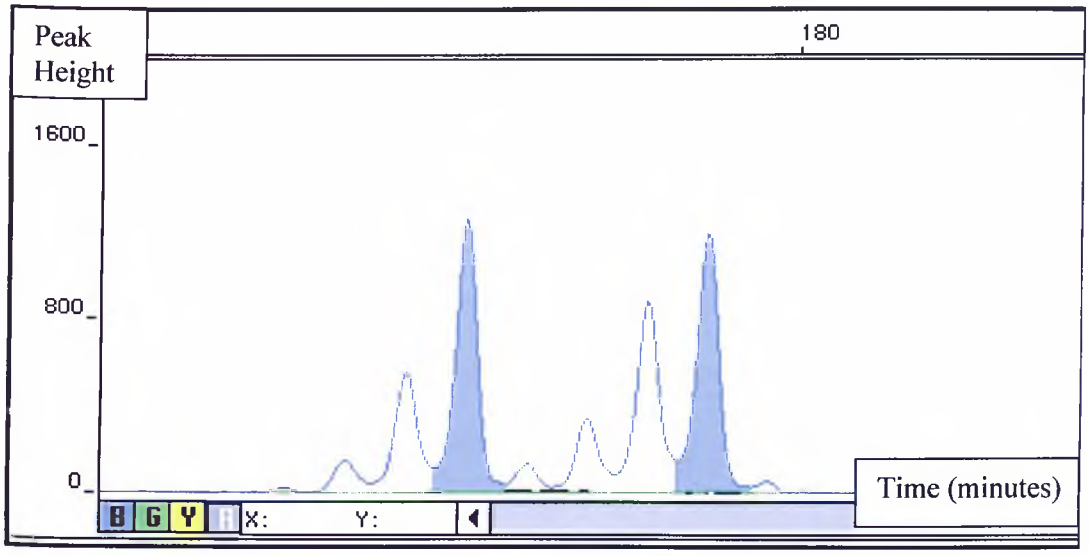
Figure 3.2 **The Genescan-350™ ROX internal size standard (Applied Biosystems).** This is the size ladder that is included with each sample, and allows the analyser to accurately calculate the size of test fragments (see Section 2.4.4).



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
Y, 47	16.96	200.49	174	1428	4625
Y, 48	17.02	202.58	703	5590	4642
Y, 49	17.09	204.79	1890	15022	4660
Y, 50	17.15	206.88	4672	35288	4677

Patient 2, control DNA, marker D22S539

Figure 3.3 A Homozygous sample of control DNA.
The single allele is highlighted and is 206 bps in length. It is preceded by multiple stutter peaks, each multiples of 2 bps shorter than the allele. This sample was non-informative. The size of the fragments is rounded down to the nearest whole even number, given that the microsatellite markers are known to be dinucleotide, or tandem, repeats.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
B, 50	15.17	165.48	151	1269	4135
B, 51	15.22	167.31	558	4450	4151
B, 52	15.29	169.25	1274	8914	4168
B, 53	15.35	171.30	136	1048	4186
B, 54	15.41	173.24	340	2830	4203
B, 55	15.47	175.06	892	6889	4219
B, 56	15.54	177.00	1222	8732	4236

Patient 9, control DNA, marker D22S277

Figure 3.4 A Heterozygous sample of control DNA.
Two allele peaks are present, both preceded by stutter peaks. Note, the second allele is larger (176 bps compared to 168 bps), but the peak slightly shorter (1222 units compared to 1274 units).

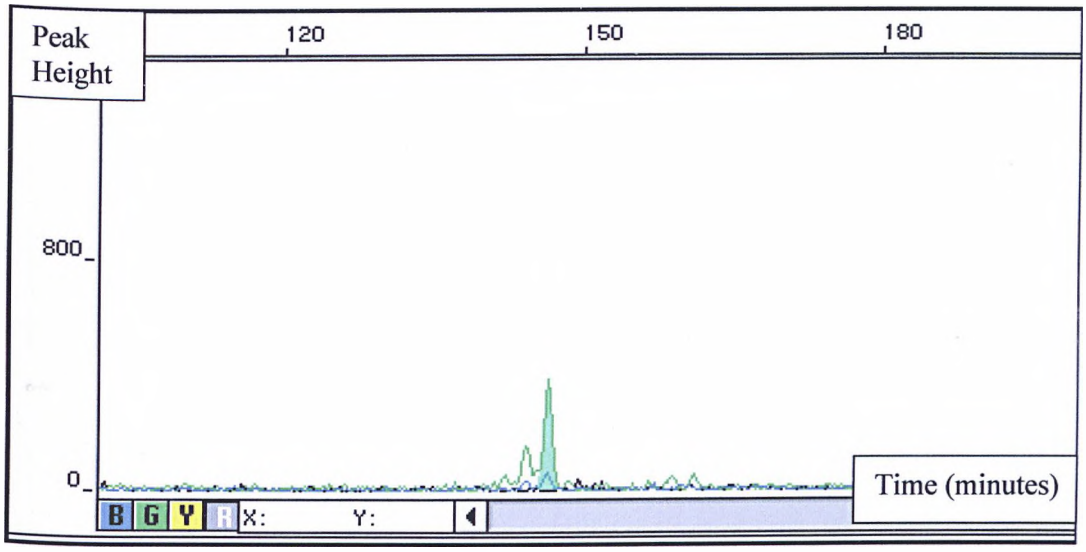
completing amplification. A slightly shorter peak immediately following an allele is known as a "plus A" artifact. This is one base pair greater in length than the actual allele, and occasionally causes ambiguity when the 2 peaks are of near equal height. However the single base pair size difference clinches its identity, and should prevent the actual allele being mistakenly identified as a stutter peak. The "plus A" artifact occurs as a result of the tendency of the *Taq* polymerase to add a non-templated nucleotide (usually an adenine) to the 3' end of double stranded DNA (User manual, ABI PRISM® 310 Genetic Analyzer). Therefore, a peak following the only allele in a homozygous sample represents a "plus A" artifact, whereas a peak following the smaller of the two alleles in a heterozygous sample may represent a "plus A" artifact (if 1 bp greater), or be a stutter peak preceding the second allele (a multiple of 2 bps greater).

Rarely there was a complete absence of peaks. If the internal size standard peaks (and any co-loaded sample peaks) were also absent, this suggested there had been a technical problem with that run, such as incorrect sample tray loading or a failure of electrokinetic injection. A re-run was therefore performed from the same batch of PCR product. If, however, the internal size standard peaks (and any co-loaded sample peaks) were satisfactory, this implied that the PCR had failed for that batch, and so the PCR was repeated for that particular marker with stock DNA.

Peak height (represented by the y-axis on electrophoretograms) is displayed in arbitrary units of fluorescence, which are proportional to the amount of PCR product.

On occasions where the allele peaks were less than 500U in height, they were deemed potentially unreliable due to too little PCR product (Figure 3.5). Analysing too little product runs the risk of missing smaller peaks altogether, and of exaggerating the effect of Genescan® errors in peak height estimation. PCR was repeated, and if necessary, the number of cycles increased (e.g. from 32 to 40) to increase the amount of product. The height of peaks that were too tall (overamplified) could not be accurately measured, and so re-runs were performed using an appropriate dilution of the PCR product, or a shorter electrokinetic injection time (Figure 3.6). The dilutions of PCR product were based on cumulated experience and frequently several re-runs at different concentrations were required to obtain a satisfactory result. Appropriate dilutions ranged from 1:2 to 1:40, and some samples required as many as 5 progressive dilutions to yield peaks that were suitable for analysis. The optimal height window for peaks was between 500 and 6000U. Occasionally, very broad peaks were observed, which gave spurious results for the area under the peak. A reduction in fragment length resolution is a recognized problem towards the end of the life-span of the capillary (User manual, ABI PRISM® 310 Genetic Analyzer). Repeating the run after changing the capillary produced satisfactory results.

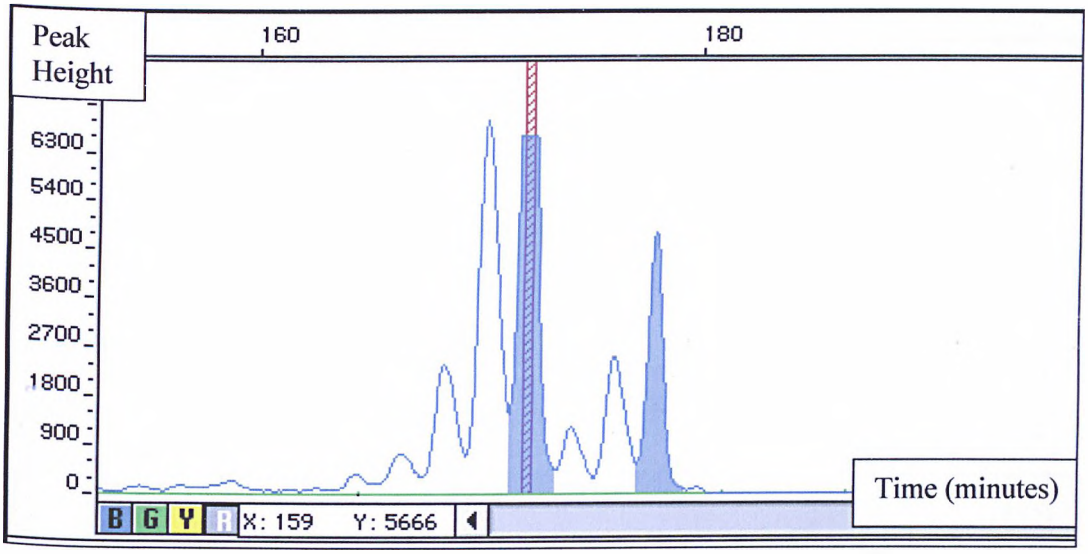
Rarely, novel alleles were detected in the tumour DNA; a phenomenon known as microsatellite instability (MI) (Figure 3.7). Although of interest in its own right, the finding of MI precluded the detection of AI. MI is recognised as a change in allele size(s) in tumour DNA from the constitutional allele pattern observed in the corresponding non-tumour DNA sample, and will be discussed more fully in Section 3.3.2.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
G, 18	14.75	142.13	55	410	4023
G, 19	14.82	144.18	158	1086	4040
G, 21	14.88	146.36	400	2627	4058

Patient 23, control DNA, marker D22S283

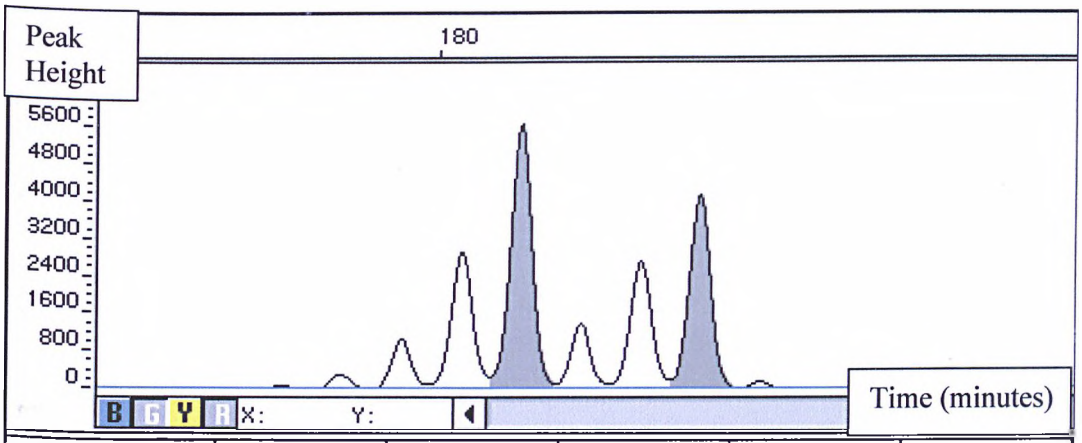
Figure 3.5 Height of allele peak too low for analysis (<500 units). PCR was repeated (increased number of cycles) to increase the quantity of product. Subsequent analysis confirmed that this sample was, indeed, homozygous.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
B, 81	16.04	166.39	762	10364	4374
B, 82	16.11	168.27	2404	26337	4392
B, 83	16.18	170.25	6927	70267	4411
B, 84	16.25	172.44	6640	74823	4432
B, 85	16.31	174.11	1243	13535	4448
B, 86	16.38	175.99	2537	26069	4466
B, 87	16.45	177.86	4844	36656	4484

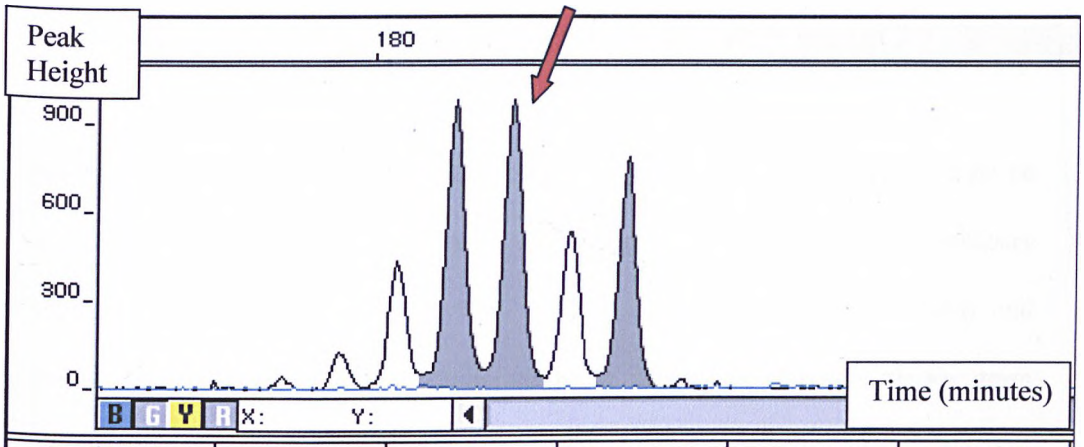
Patient 22. tumour DNA. marker D22S277

Figure 3.6 Overamplification problems.
The red hatched area indicates that the quantity of product has exceeded the scale, and so the height of the peak cannot be accurately assessed. The PCR product was diluted and re-run through the analyser.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
Y, 62	15.69	182.64	5712	39021	4277
Y, 65	15.87	188.35	4184	28582	4327

Patient 6, control DNA, marker D22S277



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
Y, 60	15.58	182.65	994	6427	4247
Y, 61	15.63	184.50	988	6267	4263
Y, 63	15.76	188.32	797	4959	4296

Patient 6, tumour DNA, marker D22S277

Figure 3.7 Microsatellite Instability.
Note the two alleles in the control DNA are 182 and 188 bps in length, whilst the tumour DNA has an additional novel allele peak at 184 bps (red arrow).

3.3 Results

DNA extraction was relatively straightforward and had to be repeated in just 8 instances (of a total of 56 extractions). Patient 18 was excluded as agarose gel electrophoresis revealed failure of DNA extraction after three attempts.

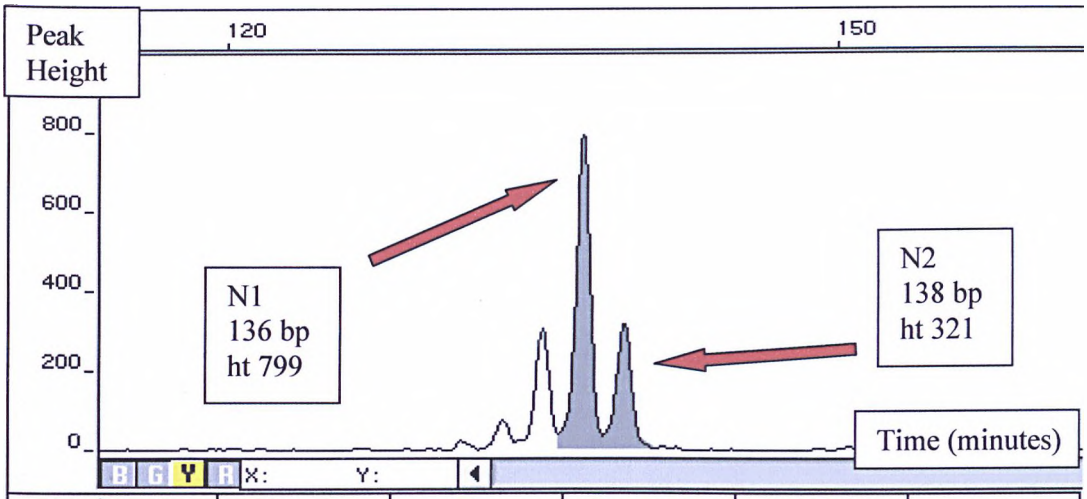
The success or otherwise of the PCR could only be assessed following attempted fragment quantification. The potential pitfalls have been discussed in Section 3.2, and the optimal thermal cycling parameters for each marker were discovered through cumulated experience from other markers' parameters and 'trial and error'. The main problem encountered was that of too little product for analysis, which was remedied by increasing the cycle number from 32 to 40, in steps of 2.

By far the most challenging area was fragment quantification. Each sample required an average of 3 attempts at electrophoresis (range 1-7) to produce a satisfactory electrophoretogram. Thus around 1500 electrophoretograms were produced and subsequently analysed. Even though the basic principles of electrophoretogram analysis have been discussed, the interpretation of a significant proportion was potentially subject to error. For this reason, all peaks were 'second-read' by a supervisor (LC) and equivocal cases discussed, or occasionally re-run through the sequencer.

3.3.1 AI Results

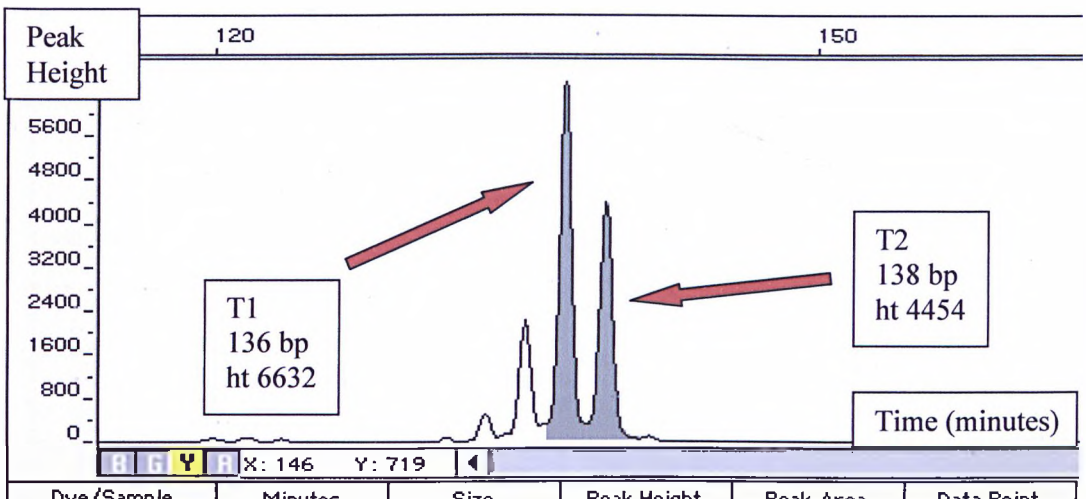
Typical electropherograms of non-AI and AI are shown in Figures 3.8 and 3.9. The presence or absence of AI in tumour DNA was established by comparing the ratio between the heights of its two constituent alleles, with the equivalent ratio in the corresponding sample of control DNA. The method for calculating the AI ratio was discussed in Section 2.4.7. Non-AI was often obvious from the similar appearance of the two sets of peaks, and was defined by an AI ratio of > 0.5. Conversely, AI was defined by an AI ratio ≤ 0.5, manifesting as a much greater discrepancy between the size of the tumour alleles compared with the control ones.

The results of AI analysis are summarized as an AI map in Figure 3.10, whilst Appendix 1 lists the actual allele imbalance ratios for each tumour and marker. Table 3.1 shows that the observed rate of heterozygosity was very similar to that claimed by the manufacturers for each marker. Overall, 76.5% of cases were heterozygous, 22.2% homozygous and 1.2% demonstrated MI. The vast majority of primary tumours studied, retained heterozygosity at informative loci on chromosome 22. The overall rate of AI across all the markers was 8.2%, which is around the expected background AI rate of around 10%. Reference to Figure 3.10 shows the seemingly random pattern of AI, with no evidence of a common region of overlap. Table 3.2 shows that the AI rates for each individual microsatellite marker range from 0 to 25%. Only 4 of the patients studied (11, 20, 24 and 28) demonstrated potentially significant overall AI frequencies of 33%, 22%, 44% and 22% respectively.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
Y, 32	14.51	133.49	79	590	3955
Y, 33	14.57	135.35	312	2368	3972
Y, 34	14.63	137.34	799	5738	3990
Y, 35	14.70	139.36	321	2463	4008

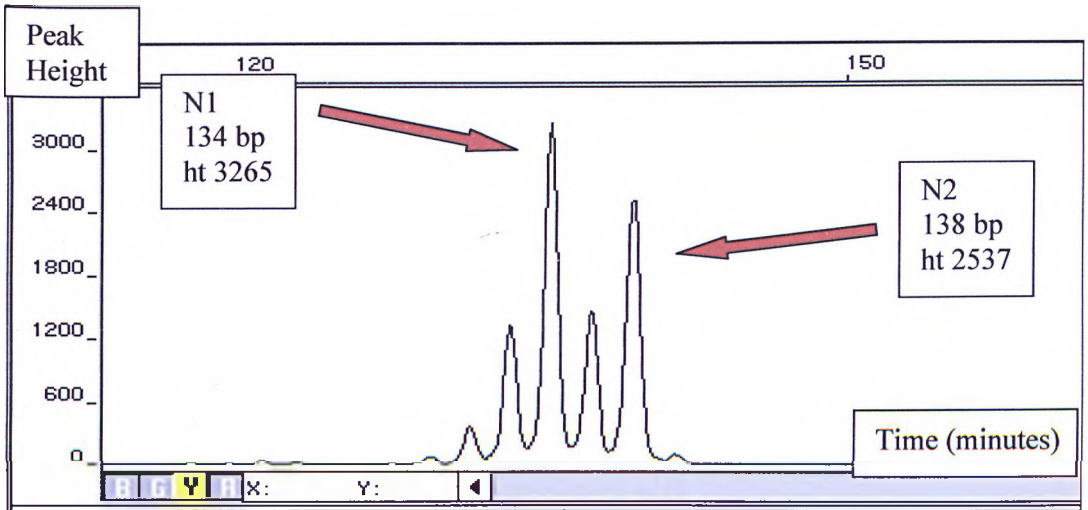
Patient 5, control DNA, marker D22S1174



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
Y, 60	14.51	133.42	531	3216	3957
Y, 61	14.58	135.42	2251	14093	3975
Y, 63	14.64	137.32	6632	40434	3992
Y, 64	14.71	139.36	4454	27032	4010

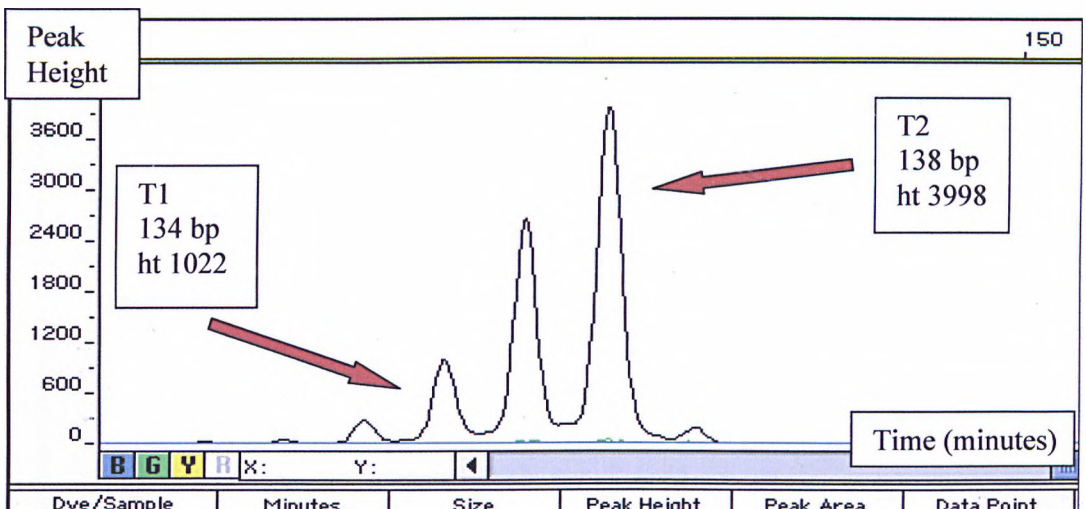
Patient 5,tumour DNA, marker D22S1174

Figure 3.8 An example of non-allele imbalance (non-AI).
The ratio in this case is 6632:4454 / 799:321 = 0.60



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
Y, 35	14.57	131.46	374	2819	3972
Y, 36	14.63	133.41	1360	9840	3990
Y, 37	14.70	135.37	3265	23447	4008
Y, 38	14.77	137.35	1476	10745	4026
Y, 39	14.83	139.36	2537	18463	4044

Patient 11, control DNA, marker D22S1174



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
Y, 78	14.64	133.81	286	1786	3993
Y, 79	14.71	135.79	1022	6758	4011
Y, 80	14.78	137.78	2672	17787	4029
Y, 81	14.84	139.83	3998	25339	4047
Y, 82	14.91	141.97	199	1246	4065

Patient 11, tumour DNA, marker D22S1174

Figure 3.9 An example of allele imbalance.
The ratio for this case is 1022:3998 / 3265:2537 = 0.20.
The first allele peak is absent in the tumour DNA (134bp); the residual peak at T1 is due to contamination of the tumour DNA sample with normal patient DNA.

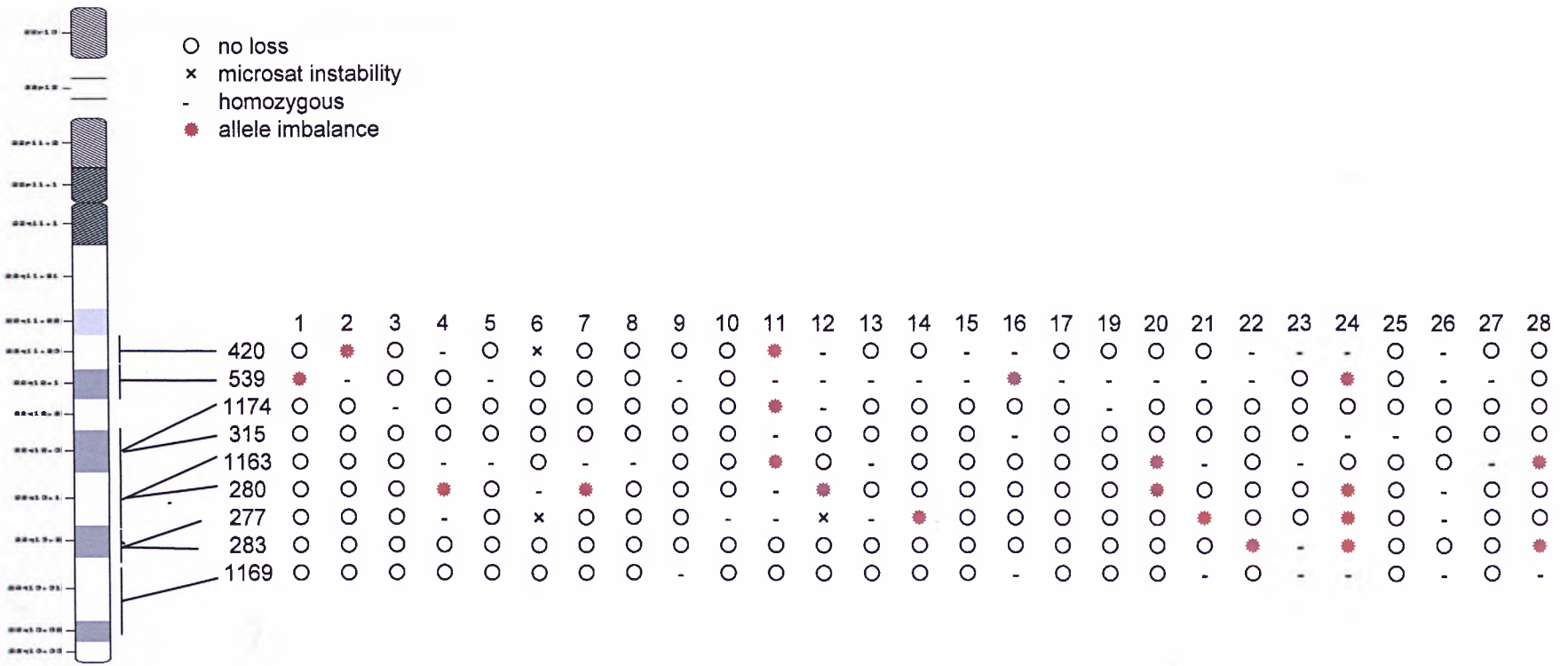


Figure 3.10 AI map of chromosome 22q

Marker	Manufacturer's Het	Observed Het
D22S420	0.77	0.70
D22S539	0.58	0.52
D22S1174	0.82	0.93
D22S315	0.78	0.89
D22S1163	0.75	0.74
D22S280	0.82	0.89
D22S277	0.85	0.81
D22S283	0.89	0.96
D22S1169	0.78	0.74

Table 3.1 **Frequency of heterozygosity: manufacturer's versus observed.**
Abbreviations: Het, heterozygosity.

Marker	Location	Frequency of AI % (AI / informative cases)
D22S420	q11.23	11 (2/18)
D22S539	q12.1	25 (3/12)
D22S1174	q12.3	4 (1/24)
D22S315	q12.3	0 (0/23)
D22S1163	q13.1	16 (3/19)
D22S280	q13.1	20 (5/24)
D22S277	q13.2	15 (3/20)
D22S283	q13.2	11 (3/26)
D22S1169	q13.31	0 (0/20)

Table 3.2 **Frequency of AI for each microsatellite marker.**

3.3.2 Microsatellite Instability Results

Microsatellite instability (MI) is defined as the expansion or contraction of one or both alleles in tumour DNA compared with the control DNA. It is therefore recognized as a change in allele size(s) in tumour DNA from the constitutional allele pattern observed in the corresponding nontumour DNA sample. The phenomenon of MI was first observed in colorectal tumorigenesis, and was found to be associated with an improved prognosis (Ionov *et al.*, 1993). It has subsequently been recognized in other malignancies, including squamous cell carcinoma of the head and neck (Field *et al.*, 1995b).

MI is believed to be due to mutation in DNA mismatch repair genes (For review, see Baker & Cawkwell, 2000). Four such genes have been identified in humans, *h*MSH2, *h*MLH1, *h*PMS1, *h*PMS2, and encode enzymes that are believed to act as a complex. Together they repair DNA mismatches and unpaired DNA loops that may occur as a result of slipped strand mispairing during the replication of DNA. Mutation in any one of these genes results in failure of DNA mismatch repair, which leads to the generation of incorrectly replicated DNA strands containing inserted or deleted bases. These strands appear as novel alleles in the tumour DNA electrophoretogram, as shown in Figure 3.6.

MI was scored in all of the specimens analysed by a demonstration of a shift of one or both of the alleles in the tumour DNA specimen as compared with the normal DNA specimen. The shift was indicated by either an addition or deletion of one or more repeat units. In contrast to allele imbalance, MI can be detected in both

heterozygous and homozygous samples, and so all loci were examined. Analysis of 9 markers in 27 tumour samples revealed only 3 cases of MI. Interestingly, patient 6 demonstrated MI at two separate loci, whilst the marker D22S277 was responsible for two cases of MI (see Figure 3.10).

3.3.3 Clinicopathological correlations

No significant correlation was found between AI on chromosome 22 and clinicopathological features (Table 3.3 and 3.4). In particular, there was no correlation between any AI on chromosome 22 and patient age, T-stage, degree of differentiation, tumour recurrence or death. Likewise, no significant difference was detected in the frequency of allele imbalance between tumours arising from the larynx, and those arising from the hypopharynx.

Parameter	AI			
	Yes (%)	No (%)	Test	*p* value
Mean age (yrs)	64	57	-	-
Laryngeal primary	8 (62)	5 (38)	Fisher's exact	0.170 (NS)
Stage T3/T4	13 (100)	12 (86)	Fisher's exact	0.259 (NS)
Poorly differentiated	8 (62)	11 (79)	Fisher's exact	0.293 (NS)
Recurrence	4 (27)	4 (29)	Fisher's exact	0.615 (NS)
Death	6 (46)	9 (64)	Fisher's exact	0.288 (NS)

Table 3.3 **Correlation of any AI on chromosome 22q with clinico-pathological details.**

Abbreviations: yrs, years; AI, allele imbalance; NS, not significant.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	19	20	21	22	23	24	25	26	27	28
420	O	●	O	-	O	×	O	O	O	O	●	-	O	O	-	-	O	O	O	O	-	-	-	O	-	O	O
539	●	-	O	O	-	O	O	O	-	O	-	-	-	-	-	●	-	-	-	-	-	O	●	O	-	-	O
1174	O	O	-	O	O	O	O	O	O	O	●	-	O	O	O	O	O	-	O	O	O	O	O	O	O	O	O
315	O	O	O	O	O	O	O	O	O	O	-	O	O	O	O	-	O	O	O	O	O	O	-	-	O	O	O
1163	O	O	O	-	-	O	-	-	O	O	●	O	-	O	O	O	O	O	●	-	O	-	O	O	O	-	●
280	O	O	O	●	O	-	●	O	O	O	-	●	O	O	O	O	O	O	●	O	O	O	●	O	-	O	O
277	O	O	O	-	O	×	O	O	O	-	-	×	-	●	O	O	O	O	O	●	O	O	●	O	-	O	O
283	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	●	-	●	O	O	O	●
1169	O	O	O	O	O	O	O	O	-	O	O	O	O	O	O	-	O	O	O	-	O	-	-	O	-	O	-
T	4	3	3	3	4	4	4	4	4	4	4	4	1	4	4	4	3	4	4	3	4	3	3	4	4	1	4
N	1	2	3	2	1	1	2	2	1	2	1	2	2	1	3	2	2	3	1	1	3	2	2	2	3	2	1
M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Grade*	2	3	2	2	3	3	3	3	2	3	3	3	3	2	3	3	3	3	3	2	2	3	3	3	2	3	3
Site	L	H	H	H	L	H	H	H	L	H	L	H	H	L	H	L	L	H	L	L	L	H	L	L	L	H	H
Rec	-	-	R	R	-	-	-	R	-	-	-	-	-	-	Lo	R	-	Lo	-	-	-	-	R	-	-	-	Lo
Status	A	A	D	A	D	D	D	D	D	A	A	A	A	D	D	A	A	D	D	D	D	A	A	D	D	A	D

Table 3.4 **Summary of the deletion status of each tumour compared with clinico-pathological data.**
L=Larynx H=Hypopharynx Rec=Recurrence R=Regional Lo=Local A=Alive D=Dead
* 1=well differentiated 2=moderately differentiated 3=poorly differentiated
O no loss × microsatellite instability - homozygous ● allele imbalance

3.4 Discussion

3.4.1 Discussion of allele imbalance results

The markers D22S539 and D22S280 demonstrated the highest frequencies of AI, at 25% and 20% respectively. Although these rates are of only borderline significance, a search was made for putative genes mapping to the regions of these two markers. Both D22S539 (22q12.1) and D22S280 (22q13.1) are telomeric of the *MMP-11* gene, but together they flank the *NF2* TSG (22q12.3). However, scrutiny of Figure 3.10 reveals that only one patient (patient 24) demonstrated AI at both of these loci, with no evidence of AI at any of the 3 loci between them. This makes it very unlikely that a common region of overlap, involving the *NF2* TSG and flanked by the markers D22S539 and D22S280, is implicated in the malignant progression of the group of tumours studied. Also the markers D22S315 and D22S1174 which both map to 22q12.3, showed no significant AI, again discounting a role for the *NF2* TSG in this cohort of tumours. The Gene Database (www.gdb.org) was searched for genes that mapped to within 5% (approximately 1.7 Mb) of these two loci. This produced 17 genes for D22S539, including a topoisomerase (TOP1P2), but none that could be classified as proto-oncogenes. D22S280 is in a more gene-rich region, with 39 genes mapping to within the 5% of genetic material around this marker. These included *PDGFB* (platelet-derived growth factor beta), *CSF2RB* (colony stimulating factor 2 receptor beta) and *CYP2D6* (cytochrome P450, subfamily IID, polypeptide 6). The first two are putative proto-oncogenes, in that amplification of their protein products could potentially enhance cell growth. The important role of angiogenesis in the progression of HNSCC was discussed in

Section 1.4.3. Two studies in HNSCC have found an association between PDGF and microvessel density, suggesting that this may be an important angiogenic mediator in HNSCC (Alcalde *et al.*, 1997; Giatromanolaki *et al.*, 1998). There has been much interest in the potential role of gene polymorphisms in detoxification enzymes. The CYP2D6 family of enzymes is involved in the detoxification of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-butanone. Current data on the association of cytochrome P450 oxidase status with tobacco smoke-related cancer, including HNSCC, are conflicting (Oude *et al.*, 1998; Sato *et al.*, 1999). A recent study of 187 patients with HNSCC and 139 controls found no significant association between *CYP2D6* polymorphisms and HNSCC (Gronau *et al.*, 2003). As there are many detoxification enzymes there may be functional redundancy of some, and studying just one or two in isolation may not give a complete picture of an individual's carcinogen detoxification status. A comprehensive literature search revealed no significant associations between HNSCC tumorigenesis and *CSF2RB* expression. At 20% AI, these regional proto-oncogenes are of doubtful significance in the progression of the tumours studied.

3.4.2 Comparison of allele imbalance results with published literature

Table 3.4 shows details of all eight published studies that have looked at AI of 22q in head and neck squamous cell cancers to date. Only four of the studies gave subsite-specific data. Where the same marker has been studied, the AI frequencies from this work are compared with the published ones. Overall, AI at a rate greater than background (AI in greater than 10% of tumours) has been identified at more than half of the microsatellite marker sites examined in primary head and neck

Author	Tumour type	No. tumours	No. loci	Loci	% AI in study	% AI in thesis
Poli-Frederico *et al.*, 2000	Not Specified	50	4	D22S421 D22S277 D22S446 D22S280	28 10 8 4	 15 20
Field *et al.*, 1995	Not Specified	80	1	Not Spec.	0	
Ah See *et al.*, 1994	Not Specified	28	1	22q11.2-qter (CYP2D)	16	
Narwoz *et al.*, 1994	Not Specified	29	1	IL2RB	29	
Ransom *et al.*, 1996	OralCavity(40), Hypopahryngeal(19), Laryngeal(17), Other(2)	78	1	Not Spec.	18	
Reis *et al.*, 2002a	Oral Cavity(37), Hypopharyngeal(31), Laryngeal(32)	100	7	D22S929 D22S446 D22S421 D22S315 D22S280 D22S277 D22S274	14(La) 4 44(La) 24(La) 2 4 15(Or)	 0 20 15
Reis *et al.*, 2002b	Oral Cavity(40)	40	2	D22S274 D22S277	25 ?	 15
Miyakawa *et al.*, 1998	Oral Cavity(33) Primary/metastases(5) (Not Specified)	33	13	D22S264 D22S420 D22S315 D22S430 D22S268 D22S273 D22S281 D22S278 D22S283 D22S272 D22S282 D22S274 D22S294	9 5 0 10 14 4 16 3 13 16 4 40 9	 11 0 11

Table 3.5 A summary of all the published literature on AI of 22q in HNSCC. The findings of this thesis are shown in red for comparison, in cases where the same marker has been studied.
(% AI, i.e. the % of informative cases that demonstrated AI)

squamous cell carcinomas on chromosome 22 (Ah See *et al.*, 1994; Narwoz *et al.*, 1994; Field *et al.*, 1995a; Ransom *et al.*, 1996; Miyakawa *et al.*, 1998; Poli-Frederico *et al.*, 2000; Reis *et al.*, 2002a; Reis *et al.*, 2002b). However the occurrence of AI at just above the background rate may not necessarily be significant, and in most series AI must be demonstrated in at least 30% of tumours before it is deemed significant.

D22S421 (22q11.2-q12.3) was the most frequently affected region in two of the studies, with AI identified in 44% of exclusively laryngeal tumours in one study, and 28% of tumours in the other (Reis *et al.*, 2002a; Poli-Frederico *et al.*, 2000). Unfortunately D22S421 was not studied in this thesis, due to its lack of commercial availability at the start of this project. However, D22S421 is in a 0.5Mb common region with D22S315, which was studied in this thesis. Interestingly, there is conflicting evidence in the literature about AI at D22S315 (22q12.3), which maps to the same region as the *NF2* TSG. AI at this locus may be due to allele loss, resulting in inactivation of one copy of the *NF2* TSG. Reis *et al.* (2002a) demonstrated AI in 24% of laryngeal carcinomas, whilst Miyakawa *et al.* (1998) demonstrated a complete lack of AI in their series of oropharyngeal tumours. Although 24% is of borderline significance, it again highlights the potential pitfalls of considering all head and neck squamous cell carcinomas together as one homogeneous group, as the implication is that the *NF2* region may be important in the progression of laryngeal carcinoma, but is unlikely to play a role in oropharyngeal carcinoma. As discussed earlier, the work in this thesis identified no significant AI at this locus, in either the laryngeal or hypopharyngeal tumours, casting doubt on the significance of the findings of Reis *et al.* (2002a).

Miyakawa *et al.* identified significant AI (40%) at the telomeric end of 22q (D22S274) in their series of oral cavity tumours, a finding common to both ovarian and sporadic colorectal carcinomas (Englefield *et al.*, 1994; Yana *et al.*, 1995). Interestingly, significant AI was identified, albeit at a lower frequency (25%), in the oral cavity tumours of Reis *et al's* (2002a) study, to the exclusion of the laryngeal and hypopharyngeal tumours. This region of chromosome 22q was incompletely covered by the markers used in this thesis, again due to lack of commercial availability.

Matsumura *et al.* (2000) identified a common region of DNA amplification at 22q11.2-12 in HNSCC by quantitative FISH analysis on 3 head and neck cell lines. This suggests the presence of a proto-oncogene on the centromeric portion of 22q, a region that encompasses the MMP-11 gene (22q11.2). However studies utilizing mFISH have shown that cell lines are genotypically different to their tumour of origin, and it is difficult to know closely they resemble the *in-vivo* situation (Bahia *et al.*, 2002). The mechanism of selection in culture is clearly different to that which a growing tumour or metastasis must face in the body, and so those cells that thrive in culture may be genotypically different to those that successfully grow and metastasise *in-vivo*. Nevertheless, Matsumuara *et al.* (2000) have postulated that *PRKM 1* (protein kinase, mitogen-activated 1, also called P41MAPK or ERK2) may act as a proto-oncogene in these cell lines, as it maps most closely to the region of amplification. PRKM 1 has been shown to function as an important mediator of signal transduction by growth factor receptors (Gonzalez *et al.*, 1992).

Just one study has examined AI in primary head and neck tumours (n=5) and their matched LNMs (Miyakawa *et al.*, 1998). Only one of the five pairs (20%) studied demonstrated a difference in AI at one of thirteen microsatellite loci on 22q. AI was present in the metastasis at D22S268 but not the corresponding primary tumour; this marker maps to the MMP-11 region (22q11.2) of chromosome 22. Interestingly a recent study, which compared the gene expression profiles of 50 primary hepatocellular carcinomas and their matched metastases, revealed no significant difference in genetic profile between the primary tumours and their matched metastases (Ye *et al.*, 2003). Primary tumours that had not metastasised, however, had a gene-expression profile that was quite different to that of primary tumours that had undergone metastasis. This implies that metastatic tumours have a similar gene expression signature to their parent tumour, whereas metastasis-free tumours are distinct from metastatic primary tumours. A 'supervised machine learning' classification algorithm (also known as a 'compound covariate predictor') was used, in conjunction with the genetic profile results, to define a gene set that could predict, with 90% accuracy, which primary tumours were likely to metastasise. The most consistently upregulated gene in metastatic hepatocellular carcinoma was found to be osteopontin on chromosome 4q, which encodes a secreted cytokine that is also overexpressed in metastatic breast cancer. These findings, however, do not preclude the possibility that in head and neck cancer, the primary and matched LNMs are genetically distinct from one another. Whilst this work remains to be done, Chapter 4 addresses the issue at an immunohistochemical level, and identifies differences in protein expression between primary tumours and their matched LNMs.

The findings of this thesis corroborate the findings of Reis *et al.* (2002a) that AI on 22q does not contribute to the genesis of primary pharyngeal carcinoma, but, unlike Reis *et al.* fail to support a role for 22q in the development of primary laryngeal carcinoma. As only 15% (4/27) of the patients studied demonstrated significant LOH on chromosome 22, these results would tend to mitigate against a specific universal role for chromosome 22 in the acquisition of the malignant phenotype.

3.4.3 Discussion of microsatellite instability results

Analysis of 9 markers in 27 tumour samples revealed only 3 cases of MI, two of which were in the same patient (patient 6). This suggests that the tumours in this study did not arise primarily through defective mismatch repair. This low level of MI contrasts with another study, in which MI was detected in 28% (7/25) of HNSCC specimens (Field *et al.*, 1995b). These samples had been analysed with at least 10 markers (range 10-38) and evidence of microsatellite alteration in two or more markers was considered diagnostic of MI. Presumably this is a reasonable allowance for the intrinsic susceptibility of an intact DNA mismatch repair system to error. By the same measure, therefore, only 1 patient out of 28 (4%) demonstrated MI in our study.

It is difficult to account for the lack of MI in our panel of tumours, compared with the findings of Field *et al.* (1995b). We examined fewer loci, which may reduce the pick-up rate of MI. On the basis of their findings, Field *et al.* (1995b) have suggested that MI is associated with HNSCC in non-smokers. All of the patients whose tumours were examined in our study were smokers, which may have had

some impact on the results. It has also been argued that microsatellite instability is a more common event in trinucleotide than dinucleotide repeats (Wooster *et al.*, 1994). Thus, the low level of MI in this study may also be partly attributable to the fact that all of the microsatellite markers examined were dinucleotide repeats.

3.4.4 Suggestions for future work

The results in this chapter support the null hypothesis that there is no difference in chromosome 22q status between paired normal and tumour samples. Obviously this null hypothesis will hold true, both if chromosome 22 does and doesn't play a role in metastasis in HNSCC. The results also imply that microsatellite instability, and therefore defective mismatch repair, is not one of the significant mechanisms by which head and neck cancer arises in smokers.

A future direction for this work would include covering the "missing" markers which were unavailable when this work was performed, to give a complete AI map for 22q. Also, attempting to estimate the actual degree of contamination with normal cellular DNA would help validate the definition of allele imbalance as an AI ratio ≤ 0.5. The current definition is, to some extent, arbitrary, and is based on the untested assumption that there is less than 50% contamination with normal cellular DNA. If the actual contamination rate were as high as 60%, then over twice the number of loci in this study would have been classified as demonstrating allele imbalance, giving an overall AI rate of 17%, instead of 8%. Use of the laser capture microscope for tumour dissection would also help in this respect, and would minimise the actual degree of contamination with control DNA.

CHAPTER 4

IMMUNOHISTOCHEMISTRY

RESULTS

4.1 Introduction

This thesis has so far examined the role of chromosome 22 in the progression of head and neck cancer at the genetic level, by assessing AI at key microsatellite loci. Immunohistochemistry can also be used to assess the contribution of various genes to malignant progression, as it measures protein expression, which is the end product of gene activation. Clearly the information obtained by these two techniques is different, but complementary, as the findings of one may help support or refute the findings of the other. MMP-11 was identified from the gene database as a candidate proto-oncogene that may contribute to any role that chromosome 22 may have in stimulating metastatic progression. This was selected for analysis as it has previously been shown to be an important mediator of tumour invasion in other malignancies, such as breast cancer (Basset *et al.*, 1990). The aim of this chapter was to assess the expression of MMP-11 in the node negative and node positive primary head and neck cancer specimens, as well as in the positive lymph nodes themselves. Comparison of the levels of expression in these three groups would allow the following hypothesis to be tested: there is no difference in MMP-11 expression between node negative and node positive primary tumours, but MMP-11 expression is increased in lymph node metastases. This hypothesis assumes that MMP-11 has a specific role in enabling malignant cells to metastasise, and is not implicated in the general process of malignant transformation.

All 26 node negative and 28 node positive primary tumours (see Tables 2.1 and 2.2), and their corresponding lymph node metastases, were stained for the presence of the MMP-11 protein, as described in Section 2.5.

4.2 Positive and negative controls

MMP-11 is the only MMP gene which has been found to be regularly overexpressed in invasive breast carcinomas without being upregulated in benign lesions (Basset *et al.*, 1990). Three samples of breast carcinoma tissue and three head and neck cancer specimens were used as positive and negative controls with all three tumour groups stained. These were critical during optimisation of the antibody concentration, and subsequently as a means of quality assurance for the final optimised run. All the negative controls were graded as negative (i.e. absent staining), reflecting the high specificity of the technique. The positive controls demonstrated intense and well localised staining, with virtually absent background staining, reflecting both the high sensitivity and specificity of the technique. Duplication of the experiments at the optimal antibody concentration successfully confirmed the reproducibility of the technique.

4.3 Grading of immunostaining

Staining was graded independently by two observers (the author and Mr. P. Nix). Observers were blinded as to whether the primary tumours were node negative or positive, but it was impossible to blind when grading the lymph nodes. The initial intention had been to grade benign epithelium, malignant epithelium and stroma, but only malignant epithelial grading is presented. There was very little, if any, benign epithelium in the sections studied, and certainly not enough to be graded confidently. Stromal staining for MMP-11 was very scant and infrequent in the tumour sections, and obviously the lymph node metastases contained neither stroma

nor benign epithelium. Thus, malignant epithelial cells only were assessed, and were only regarded as positive if they showed unequivocal brown reaction product in the cytoplasm. As staining was uniform across the sections (i.e. homogeneous), it was possible to select representative areas for assessment. The immunostaining within these areas was estimated under intermediate power light microscopy (x200). A semiquantitative scoring system was used, according to the proportion of malignant epithelial cells (0-9%: -, 10-50%: +, >50%: ++) staining positive. Positive immunostaining is defined by scores of + (low positive) or ++ (high positive; see Figure 4.1). The –ve range of 0-9% is to allow for the occasional finding of a small quantity of non-specific, or "background" staining. This grading system is similar to that of other groups studying MMPs (Autio-Harmainen *et al.*, 1993; Charous *et al.*, 1997; Kurahara *et al.*, 1999), although Yoshizaki *et al*, (1997) graded their specimens according to the intensity of the DAB signal, rather than according to the proportion of cells staining positive.

Where consensus was not obtained after initial grading, the entire batch of slides, alongside the H&E slides, was reviewed and independent grading repeated. There was a very high degree of inter-observer concordance, with only around 15% of cases requiring review. In 5% of cases disagreement persisted over whether there was low or high expression of MMP-11. Joint scrutiny of the slides in these cases resolved the issue satisfactorily.

All statistical analyses were carried out using the SPSS v.10 statistical package. Categorical data were analysed using Fisher's Exact test, and results were regarded as significant when $p \leq 0.05$.

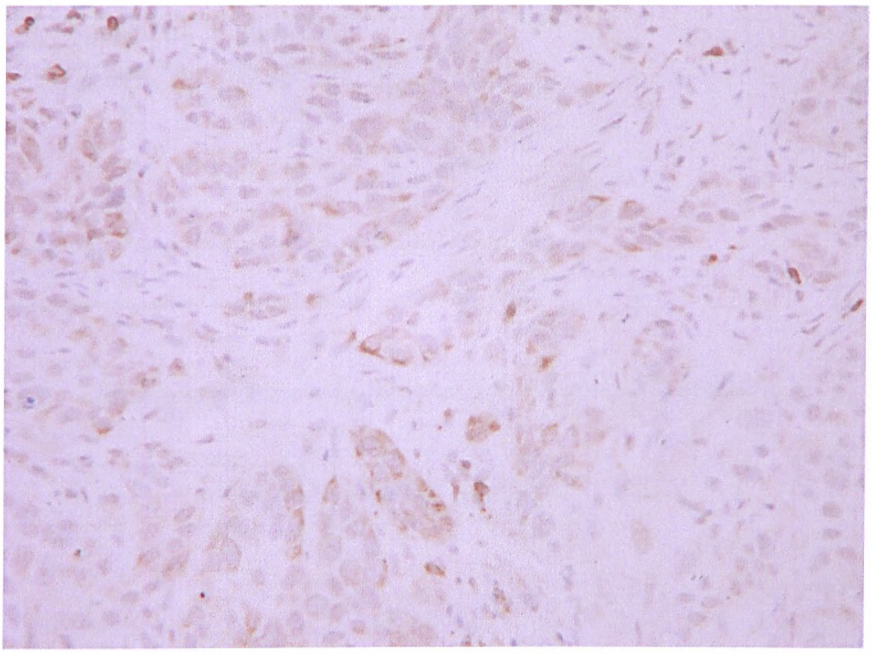


Figure 4.1 **Intermediate power photomicrograph of laryngeal tumour stained for MMP-11 (x200).**
Note the cytoplasmic location of the MMP-11 in the malignant epithelium (brown DAB). This section was graded as ++

4.4 Results

Table 4.1 summarises the immunostaining results. All experiments were performed in duplicate, to confirm their reproducibility. The results are depicted graphically in Figures 4.2 using a three tier grading system (specimens graded into high, low or negative expression categories) and 4.3 using a two tier grading system (specimens graded as positive or negative).

Staining	Node – ve primaries	Node +ve primaries	LNs	Total
-	15	20	10	45
+	9	8	11	28
++	2	0	7	9
Total	26	28	28	82

Table 4.1 **Summary of the immunostaining results for MMP-11**

-	*≤9%*	*positive staining*
+	*10 - 50%*	
++	*>50%*	

4.4.1 Immunostaining results

The majority of node negative (15/26; 58%) and node positive (20/28; 71%) primaries showed no staining for MMP-11. Low levels of expression were observed in 9/26 (35%) node negative, and 8/28 (29%) node positive cases, with high levels of expression seen in 2/26 (8%) node negative cases only. There was no significant difference in staining for MMP-11 between the node negative and node positive primaries, using the two tier grading system (i.e. tumours graded as negative or positive expressers; p= 0.22, Fisher's exact test).

However, the majority of lymph node metastases demonstrated positive staining for MMP-11, with low levels of expression observed in 11/28 (39%) cases and high levels in a further 7/28 (25%) cases. Overall 64% of lymph node metastases were positive for MMP-11 expression, compared with just 35% of node negative and node positive primaries. This difference in MMP-11 expression was statistically

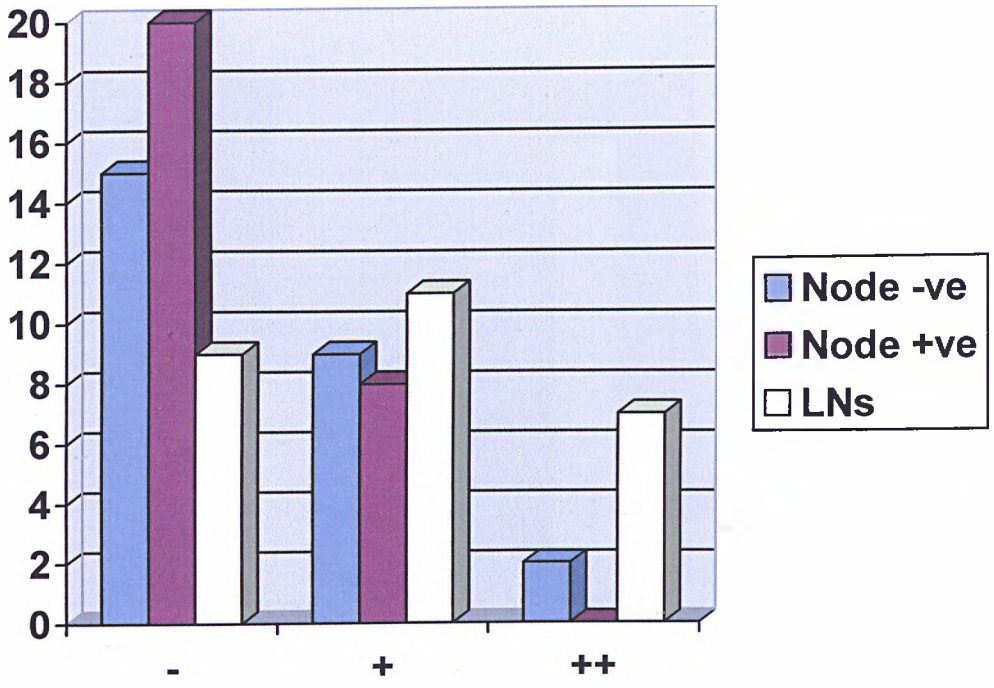


Figure 4.2 **Bar chart illustrating the difference in MMP-11 status using a three-tier system of grading (i.e. -, + or ++)**

-	*≤ 9%*	
+	*10 - 50%*	*positive staining*
++	*> 50%*	

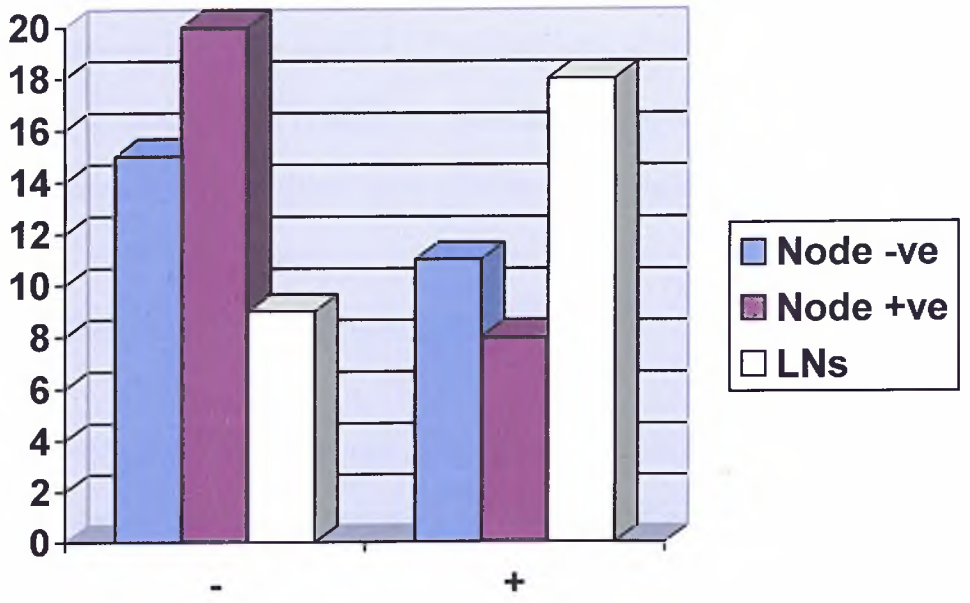


Figure 4.3 **Bar chart illustrating the difference in MMP-11 status using a two-tier system of grading (i.e. either - or +)**

\- ≤ 9% }
\+ *6 - 100%* } *positive staining*

significant (p=0.01, Fisher's exact test). Table 4.2 shows the MMP-11 status of each primary tumour and its matched lymph node metastasis, highlighting the 10/28 primary tumours that were negative for MMP-11, but gave rise to metastases that expressed detectable levels of MMP-11. Interestingly, patient 20 expressed MMP-11 in the primary tumour, but not in the corresponding lymph node metastasis.

4.4.2 Clinico-pathological correlations

Clinico-pathological details, as well as the AI status of each primary tumour, are also included in Table 4.2. Within the node negative and node positive primaries, there was no significant correlation between MMP-11 status and clinicopathological features (T-stage, histological differentiation, tumour recurrence and death; Tables 4.3a & b). Likewise, there was no significant correlation between MMP-11 status in lymph node metastases and the clinicopathological features of its corresponding primary tumour, or N-stage (Table 4.3c).

4.4.3 Comparison with allele imbalance data

There were only 5 instances of allele imbalance at 22q11.2 (see Section 3.3.1) in the primary tumours, which are shown against the MMP-11 results in Table 4.2. Interestingly, there was no relationship between MMP-11 status and allele imbalance at 22q11.2 in the primary tumours (p=0.40, Fisher's exact test).

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Prim T	+	-	-	+	-	-	-	+	-	-	-	-	+	-	-	-	+	-	-	+	+	-	-	-	-	+	-	-
LNM	+	+	++	+	+	++	-	++	-	-	++	+	-	++	+	+	+	+	-	-	++	+	+	-	-	-	-	++
A.I.	Y	Y	N	N	N	N	N	N	N	N	Y	N	N	N	N	Y	N	-	N	N	N	N	N	Y	N	N	N	N
T	4	3	3	3	4	4	4	4	4	4	4	4	1	4	4	4	3	1	4	4	3	4	3	3	4	4	1	4
N	1	2	3	2	1	1	2	2	1	2	1	2	2	1	3	2	2	2	3	1	1	3	2	2	2	3	2	1
Grade*	2	3	2	2	3	3	3	3	2	3	3	3	3	2	3	3	3	2	3	3	2	2	3	3	3	2	3	3
Site	L	H	H	H	L	H	H	H	L	H	L	H	H	L	H	L	L	H	H	L	L	L	H	L	L	L	H	H
Rec	-	-	R	R	-	-	-	R	-	-	-	-	-	-	Lo	R	-	-	Lo	-	-	-	-	R	-	-	-	Lo
Status	A	A	D	A	D	D	D	D	D	A	A	A	A	D	D	A	A	A	D	D	D	D	A	A	D	D	A	D

Table 4.2 **Summary of the MMP-11 status of each primary tumour and matched lymph node metastasis compared with clinico-pathological data.**

L=Larynx H=Hypopharynx Rec=Recurrence R=Regional Lo=Local A=Alive D=Dead
A.I.=Allele imbalance detected at 22q11.2 in the primary tumour Y=Yes N=No
* 1=well differentiated 2=moderately differentiated 3=poorly differentiated

\- 0-9%
\+ *10-50%*
++ *>50%*

Grading of immunostaining, according to proportion of cells staining positive.

\- / + / ++ *Signify difference in staining between primary tumour and lymph node metastasis.*

Parameter	MMP-11		
	Yes (%)	No (%)	*p* value
Laryngeal primary	8/11 (73)	14/15 (93)	0.19 (NS)
Poorly differentiated	5/11 (45)	4/15 (27)	0.28 (NS)
Recurrence	2/11 (18)	3/15 (20)	0.53 (NS)
Death	4/11 (36)	4/15 (27)	0.46 (NS)

Table 4.3a

Parameter	MMP-11		
	Yes (%)	No (%)	*p* value
Laryngeal primary	5/8 (62)	8/20 (40)	0.21 (NS)
Poorly differentiated	4/8 (50)	15/20 (75)	0.19 (NS)
Recurrence	2/8 (25)	6/20 (30)	0.39 (NS)
Death	4/8 (50)	11/20 (55)	0.36 (NS)

Table 4.3b

Parameter	MMP-11		
	Yes (%)	No (%)	*p* value
Laryngeal primary	8/18 (44)	5/10 (50)	0.38 (NS)
Poorly differentiated	11/18 (61)	8/10 (80)	0.24 (NS)
Recurrence	6/18 (33)	2/10 (20)	0.32 (NS)
Death	9/18 (50)	6/10 (60)	0.35 (NS)

Table 4.3c

Tables 4.3 (a-c) **Correlation of MMP-11 status of a) node negative primary tumours b) node positive primary tumours, and c) lymph node metastases with clinico-pathological details.**

Abbreviations: yrs, years; NS, not statistically significant

4.5 Discussion

As discussed in Section 1.12.2, MMP-11 is a member of an important group of enzymes, which degrade extracellular matrix components, and are postulated to have a role in malignant progression. The gene encoding MMP-11 resides at 22q11.2 (Basset *et al.*, 1990). The CGH finding, that chromosome 22q is amplified in a proportion of HNSCC metastases but not their corresponding primary tumours, and the fact that MMP-11 had previously been shown to be an important mediator of tumour invasion in other malignancies, led to the hypothesis that increased expression of MMP-11 may be implicated in the establishment of regional metastases. A monoclonal antibody was used to assess the level of expression of MMP-11 in node negative and node positive primary tumours, as well as in the latter's corresponding lymph node metastases. Expression of MMP-11 was observed in all three groups, but was present in 64% (18/28) of lymph node metastases, compared to 35% (19/54) of node negative and node positive primaries. The higher level of expression in lymph node metastases was found to be statistically significant ($p < 0.01$).

The role of MMP-11 in cancer progression has been most widely studied in carcinoma of the breast. MMP-11 is particularly attractive as a candidate proto-oncogene, as it is the only MMP gene that has consistently been shown to be absent from normal breast tissue and to be present in the majority of invasive breast carcinomas (Reviewed by Curran and Murray, 1999).

One study positively correlated MMP-11 gene expression with increased local invasiveness in a series of over 100 samples of HNSCC (Muller *et al.*, 1993). Surprisingly, expression was detected in stromal fibroblasts immediately surrounding tumour cell islands of invasive head and neck SCCs, and it was this that correlated with increased invasiveness. Likewise its increased expression in several breast cancer series has been shown to be in stromal cells surrounding cancer cells, rather than the neoplastic cells themselves (Reviewed by Curran and Murray, 1999). This has lead to the suggestion that neoplastic cells may be able to stimulate the stromal cell production of MMP-11, either in a paracrine fashion or by a cell-to-cell contact mechanism (Curran and Murray, 1999). Human breast cancer cells have been shown to be capable of direct activation of the promoter region of the MMP-11 gene (Ahmad *et al.*, 1997). It may be that malignant cells stimulate benign stromal cells to synthesise and secrete MMP-11, so paving a way for the advancing tumour.

These studies imply that the location of the MMP-11 gene on chromosome 22 may be irrelevant if it is only expressed by stromal cells and not by the neoplastic cells themselves. However, it is by no means uniquely expressed by stromal cells, and there are several reports in the literature of both benign and malignant epithelial expression in many different tissue types, including breast cancer (Thorns *et al.*, 2003; Wasenius *et al.*, 2003; Nakopoulou *et al.*, 2002). Indeed, one study demonstrated an association between poor prognosis and combined stromal and epithelial expression of MMP-11 in breast cancer (Nakopoulou *et al.*, 2002).

The MMP-11 detected by the work in this thesis was predominantly located in the malignant epithelium itself, rather than the stroma. This contrasts with the large head and neck series of Muller *et al.* (1993) discussed above. This may be due to the different antibodies used in the two studies. Muller *et al.* raised a rabbit polyclonal antibody to the 25 COOH-terminal amino acid residues of active MMP-11 (47kDa). In contrast, a commercial mouse monoclonal antibody was used in this thesis, which recognises both a pro- (60kDa) and active (47kDa) form of the MMP-11 enzyme. Thus, the work in this thesis may have recognised the inactive form of the enzyme in the cytoplasm of malignant epithelium, before it was secreted and activated. In contrast, Muller *et al.* (1993) may only have detected the enzyme in the stroma following secretion and activation.

The exact mechanism of action of MMP-11 is poorly understood. As its name suggests, it is an enzyme with proteolytic activity. Uniquely among the MMPs, however, it is not capable of digesting any of the components of the extracellular matrix. Indeed, the natural substrate of this protease remains unknown. In 1994, Pei *et al.*, demonstrated that MMP-11 was capable of degrading serine proteinase inhibitors, α_1 proteinase inhibitor and α_1-antitrypsin (Pei *et al.*, 1994). They proposed this as a novel mechanism of action, whereby MMP-11 increases the stability of other proteases, and so indirectly leads to ECM degradation. More recent work, however, in mice, has shown that MMP-11 promotes tumorigenesis through the inhibition of apoptosis, leading to increased tumour cell survival (Boulay *et al.*, 2001). It is thought that MMP-11 might inhibit apoptosis by releasing IGFs (insulin-like growth factors), which can act as survival factors. A further mechanism of action of MMP-11 has been proposed, whereby one of the

cleavage products of α_1-antitrypsin decreases the sensitivity of tumour cells to natural killer cells (Kataoka *et al*, 1999). Thus, MMP-11 may promote tumour progression through increasing the stability of other MMPs, inhibiting apoptosis and modulating the immune response to cancer. A further potential mechanism of action is suggested by the observation that an increased number of neutrophils and macrophages infiltrate tumours in *MMP-11*-null mice compared with wild-type mice, indicating that MMP-11 inhibits a chemoattractant for these cells (Boulay *et al*, 2001).

Potential clinical interest in members of the MMP family stems from the discovery of naturally occurring tissue inhibitors of MMPs (TIMPs), and the subsequent development of synthetic MMP inhibitors (e.g. Marimastat™). Marimastat™ is a peptidomimetic agent which mimics the cleavage sites of MMP substrates, and so competitively blocks the active sites of MMPs. A trial for advanced pancreatic cancer, intended to detect differences in survival between patients treated with various doses of Marimastat™ and conventional chemotherapy, failed to detect increased survival for the Marimastat™ treated groups. However the highest dose of the drug was as effective as the conventional chemotherapy, with the added advantage of being better tolerated (Bramhall *et al*, 2001).

Other trials of MMP inhibitors have been less fruitful, and this group of drugs is now regarded as less promising than originally hoped (Egeblad & Werb, 2002). However, MMP inhibitors have so far only been compared with cytotoxic drugs and tested in patients with late-stage tumours. This methodology may be flawed, as MMP inhibitors are cytostatic rather than cytocidal, and may be important earlier

on in cancer progression. Thus inhibition of MMP activity might not be expected to make a significant difference in late stage cancers, if their role is in the initial spread of the primary and establishment of regional and distant metastases. Instead of using MMP inhibitors to treat inoperable tumours with established metastases, they might be better used in patients without metastases in whom the primary tumour has been successfully removed, but who are at high risk of subsequently developing metastases. In support of such an approach, mice treated with Batimastat™ before and after removal of a primary mammary carcinoma had a 100% survival and no metastases, whereas all control animals died with extensive metastases (Eccles *et al*, 1996).

Finally, the last part of the results section compared the MMP-11 status with the allele imbalance data for the node positive primary tumours. Interestingly, there was no relationship between MMP-11 status and allele imbalance at 22q11.2 in the primary tumours ($p=0.40$, Fisher's exact test). This implies that A.I. at 22q11.2 may not be the usual mechanism of MMP-11 upregulation in these tumours, although it must be remembered that this region was incompletely covered by the markers used in this thesis, raising the possibility that relevant allele imbalance went undetected. Other possible mechanisms of protein upregulation include promoter sequence alteration, post-transcriptional stabilization of mRNA and post-translational stabilization of the protein product. By increasing either the rate of transcription, or the half-life of either the mRNA or the protein product itself, protein upregulation may be achieved. In the absence of allele imbalance, and therefore amplification of DNA, any one of these could have been responsible for the increased expression of MMP-11 in the node positive primary tumours.

CHAPTER 5

FINAL DISCUSSION

The presence or absence of metastases is the most important disease-specific predictor of survival in patients with head and neck squamous cell cancer. Therefore, short of preventing head and neck cancer, patients would benefit most from the development of methods to prevent metastasis.

This thesis set out to determine what role, if any, chromosome 22q may have in the establishment of regional metastases. Previous CGH and AI studies have suggested that 22q may have a role in initial tumorigenesis, whilst unpublished CGH findings from our department suggested the possibility of a metastasis-specific role for 22q in head and neck cancer. The work in this thesis represents an attempt to resolve this issue. The null hypothesis, that no significant allele imbalance would be found at 22q in primary tumours that have given rise to metastases, was borne out by this work. This would tend to refute a specific role for allele imbalance of chromosome 22 in initial tumorigenesis. Whether the lymph node metastases themselves demonstrate AI remains to be seen, and is the focus of continuing work. The near absence of microsatellite instability, is compelling evidence that defective mismatch repair was not the underlying mechanism of tumorigenesis in this cohort of head and neck cancers.

The second part of this thesis looked specifically at the expression of one of the 545 protein products of the genes on chromosome 22q; namely MMP-11. This was selected for analysis as it has previously been shown to be an important mediator of tumour invasion in other malignancies, such as breast cancer. The MMP-11 gene (22q11.2) encodes one of a family of secreted proteinases, between them capable of degrading all the components of the extracellular matrix, which may, in the context

of malignancy, be a proto-oncogene. Immunohistochemical analysis of tumour sections revealed that MMP-11 was significantly more abundant in metastatic tumour cells (positive staining in 64% cases), than in their matched primaries or node negative primary tumours (positive staining in 35% cases overall; $p<0.01$).

The limitations of the techniques used in this thesis must be discussed, in order to appreciate the significance of these findings. The problem of researchers considering head and neck cancers as one homogeneous group was discussed in the introduction. In this study, only laryngeal and hypopharyngeal tumours were studied, in an attempt to minimise the impact of tumour subsite as a confounding factor. However, the work of Reis *et al*, published after completion of this work, suggests that laryngeal and pharyngeal carcinomas may be genetically different (Reis *et al.*, 2002). In their study of AI on chromosome 22q in 100 tumours, they demonstrated a striking dichotomy between the 32 laryngeal and 31 pharyngeal carcinomas studied, with a high frequency of AI in the laryngeal tumours and virtually none in the pharyngeal tumours. Another group performed CGH on 75 HNSCCs, and found different patterns of copy number aberration in the various head and neck subsites (Huang *et al.*, 2002). However, when analysed separately, no significant difference was observed in either the frequency of AI or the level of MMP-11 expression between the laryngeal and pharyngeal tumours studied in this thesis. This may be due to the relatively small cohort studied in this thesis, with fewer than half the number of tumours studied by comparison.

Our study of chromosome 22 was based on the CGH finding that 29% (5/17) of LNMs demonstrated DNA amplification along the length of 22q, compared with

none of their primary head and neck tumours of origin. Whilst this suggests a potential role for 22q in metastasis, it is clearly not the whole story, as 71% of metastases exhibited no amplification. It is possible that smaller segments of DNA amplification were missed, as CGH can only detect amplifications of at least 2Mb. It should be noted that CGH can only screen genomes for fairly gross changes in copy number (amplifications or deletions), and so many subtle mechanisms for increasing or reducing gene expression may be missed. These include small amplifications and deletions, point mutations and epigenetic phenomena such as hypermethylation of promoter regions and chromatin remodeling. CGH is also unable to detect copy number changes due to differences in ploidy. The small size of the cohort will also have increased the risk of a type II error. Thus, whilst the finding of 22q gain in 5 of 17 metastases compared to none of the corresponding primary tumours is statistically significant ($p=0.02$; Fisher's Exact test), there is a 2% probability that this finding is due to chance alone. Interestingly, a similar CGH study, that examined 34 primary tumours and their matched LNMs and was published after completion of this work, demonstrated no amplification of 22q in the LNMs (Bockmuhl *et al.*, 2002). Instead they found that gains involving chromosomes 3, 11, 7 and 1, as well as losses involving chromosomes 8, 11, 10 and 14, tended to be associated specifically with metastases, and so were more likely to be implicated in an extended progression model of HNSCC.

Microsatellite marker analysis similarly suffers from limitations. In contrast to the large copy number changes detected by CGH, this high resolution technique examines segments of DNA that are in the order of 100 base pairs in length. Consequently, the 9 markers used in this study only examined around 1kb of the

34Mb that make up 22q. Whilst the resolution of this technique is 1bp (hence the ability to detect microsatellite instability) each set of primers only examines a tiny fraction of the DNA for AI. If a reasonable spread of markers is used across a chromosome arm, and significant AI is identified in "neighbouring" markers, it is inferred that the intervening (but unexamined) DNA segment is also affected. In this way minimal common areas of overlap are identified as probable regions of amplifications or gains. This was not applicable in our study as no significant AI was identified. In calculating the allele ratios, a threshold of 0.5 was taken, to make allowance for the crude method of isolating tumour tissue. The precision of LCM (laser capture microdissection) would have been useful in this context as it increases resolution from the tissue level to that of individual cell populations (Mills *et al.*, 2001). Ideally, we might have performed experiments in duplicate (Cawkwell *et al.*, 1994) or triplicate (Reis *et al.*, 2002) to confirm reproducibility of results.

Unlike CGH and AI studies, immunohistochemistry gives information about whether or not a given gene is expressed. The presence of a gene, or even amplified quantities of a gene, does not necessarily mean that it is being expressed. Northern blotting and real time quantitative PCR detect mRNA, and so can confirm that a gene has been transcribed. Immunohistochemistry and Western blotting, however, detect the end protein product of gene expression. Whilst not quantitative, immunohistochemistry has the added advantage of revealing the cellular localization of the relevant protein. Thus, immunohistochemistry was used to assess the expression of MMP-11 in primary tumours (both metastasising and non-metastasising) and LNMs. Interestingly, MMP-11 was detected in the cytoplasm of

the malignant cells themselves, in contrast to other studies that have predominantly detected it in the stroma surrounding malignant cells. This may be due to the use in this study of an antibody that detects the pro-form of the enzyme, which may be detecting the inactive form of the enzyme before it is secreted into the extracellular space by malignant cells.

An inevitable limitation of IHC is that it captures only a single point in time of what is a continuous physiological or pathological process. This is of particular relevance to this thesis, as it has been suggested that cell migration is regulated by cycles of MMP activity, rather than by continuously high activity (Egeblad & Werb, 2002).

The problem of using both of these techniques together, as "complementary" techniques, is that the findings of one may not necessarily complement the findings of the other. If immunohistochemistry identifies increased expression of a particular protein, microsatellite analysis will only substantiate this if the mechanism of increased protein expression is gene amplification. There are, however, many other mechanisms of protein upregulation. Alteration of the promoter sequence can increase the rate of transcription, whilst stabilisation of the mRNA molecule can increase translation. Likewise stabilisation of the protein product itself, by increasing its half-life, leads to accumulation of the protein in the cell. Post-translational glycosylation is one such mechanism, and will resemble gene amplification when detected by immunohistochemistry.

The three important findings of this thesis on head and neck squamous cell carcinoma are that allele imbalance of chromosome 22q does not appear to play a

role in development of the primary tumour, defective mismatch repair is not a significant mechanism of tumorigenesis, and overexpression of MMP-11 may be instrumental in the formation of metastases. If on-going studies demonstrate significant allele imbalance of 22q11.2 in metastases that seemingly acquire MMP-11 expression, this would support a critical role for MMP-11 as a proto-oncogene in the metastatic progression of head and neck cancer. This may be one of the important steps in extending the progression model proposed in Section 1.8.4. Interestingly, however, the primary tumours studied in this thesis that showed increased MMP-11 expression, did not demonstrate allele imbalance at 22q11.2. This suggests that A.I. at 22q11.2 may not be the usual mechanism of MMP-11 upregulation, although it must be remembered that coverage of the MMP-11 region by the markers used in this thesis was incomplete. This does not necessarily undermine the potential importance of MMP-11 in the formation of metastases, and indeed highlights one of the limitations of proposing a purely genetic model for malignant progression, given all the post-translational and epigenetic phenomena that potentially may contribute to tumour evolution. It may be that a truly holistic model of tumour progression should be capable of incorporating data from genetic, RNA, epigenetic and protein studies. Future work will indicate whether allele imbalance at 22q11.2 is associated with overexpression of MMP-11 in metastatic carcinoma cells.

As for the direction of future work, there is now enough evidence to suggest that tumours arising from different subites within the head and neck differ in their aetiologies, genetics and biological behaviour (Huang *et al.*, 2002). Future work should therefore select a cohort of tumours from a single subsite within the head

and neck to minimise the effect of this variability as a confounding factor. The relatively small number of cases in this study increases the risk of both types I and type II errors, and a larger cohort should therefore be selected for future study. Lack of commercial availability of some microsatellite markers resulted in an incomplete allele imbalance map, with 28% of the length of 22q not screened for allele imbalance. Markers can be synthesised "to order" commercially, which would allow the gaps in this study to be filled. These measures would improve the validity of the results in this thesis, before proceeding to allele imbalance studies on the lymph node metastases. It would also be interesting to repeat the immunostaining studies with an antibody that only detects the active form of MMP-11, to see whether this produces a different pattern of protein distribution. The potential benefit of treatment with MMP inhibitors also needs to be investigated further, particularly in patients with operable primary tumours who are at a high risk of subsequently developing metastases. If a cytostatic effect were demonstrated, following initial assessment of their effect on head and neck tumour cell lines, there would then be a scientific rationale for a randomized controlled trial of this therapy in patients with locally advanced, node negative laryngeal or hypopharyngeal cancer.

So what is the potential value in studying the genetics of cancer, and what clinical relevance does it have? The increasing availability of DNA microarrays (or biochips) is likely to make them an integral part of the molecular assessment of cancers in the future, as a means of more accurately predicting tumour behaviour. The principle of labeled DNA probes remains the same with microarrays, but technological advances have made it possible to miniaturise this process, such that

thousands or even tens of thousands of specific RNA or DNA sequences can be detected simultaneously on a small glass slide only 1-2 cm^2. This obviously permits analysis of gene expression on a completely different scale, and generates huge quantities of data. It is hoped that it will identify characteristic patterns of gene expression, which will have diagnostic, therapeutic or prognostic value. Such gene-expression signatures may be more useful than the identification of single chromosomal aberrations, as the sophisticated process of metastasis may be more dependent upon combinations of genetic alteration, rather than single gene phenomena. As for gene therapy, at its simplest level it is defined as the use of nucleic acids as drugs. Attempts at manipulating the genetic and chromosomal aberrations responsible for cancer have largely been hampered by the difficulties of specifically directing therapy at the malignant cells themselves. The development of safe and effective viral and non-viral vectors is currently the focus of intense research.

A generally held principle is that "prevention is better than cure". Global abstinence from tobacco products, and moderation of alcohol intake, would, of course, significantly reduce the incidence of head and neck cancer. As long as this prospect remains unrealistic, research into the development of novel therapeutic approaches, such as the emerging field of gene therapy, remains not only an academic challenge, but possibly our only hope of reducing the mortality of head and neck cancer.

APPENDICES

Appendix 1: The TNM classification for laryngeal and hypopharyngeal tumours

T-stage

Common to all sites

TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma-in-situ

Supraglottic Larynx

T1	Tumour limited to one subsite of supraglottis without fixation of hemilarynx
T2	Tumour invades more than one adjacent subsite or site outside supraglottis without fixation of hemilarynx
T3	Tumour limited to larynx with fixation of hemilarynx and/or invades post-cricoid area and/or pre-epiglottic tissues
T4	Tumour invades adjacent structures

Glottic Larynx

T1	Tumour limited to glottis (T1a one vocal cord, T1b both vocal cords)
T2	Tumour extends to supraglottis or subglottis or impairs vacal cord mobility
T3	Tumour limited to larynx with hemilarynx fixation
T4	Tumour invades beyond larynx

Subglottic Larynx

T1	Tumour limited to subglottis
T2	Tumour extends to glottis
T3	Tumour limited to larynx with hemilarynx fixation
T4	Tumour invades beyond larynx

Hypopharynx

T1	Tumour confined to one subsite of hypopharynx and <2 cm
T2	Tumour invades > 1 subsite or an adjacent site, or measures 2-4 cm, and without fixation of the larynx
T3	Tumour > 4cm or with fixation of hemilarynx
T4	Tumour invades adjacent structures

N-stage

N0	No regional lymph node metastases
N1	Metastasis in single ipsilateral node and < 3cm
N2a	Metastasis in single ipsilateral node, 3-6 cm
N2b	Metastasis in multiple ipsilateral nodes and all < 6 cm
N2c	Metastasis in bilateral or contralateral nodes and all < 6cm
N3	Metastasis > 6cm

M-stage

MX	Presence of distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Appendix 2: Stage grouping in HNSCC (UICC, 1997)

Stage group	T	N	M
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T1	N1	M0
	T2	N1	M0
	T3	N0, N1	M0
IV	T4	N0, N1	M0
	Any T	N2, N3	M0
	Any T	Any N	M1

	Microsatellite Marker								
Pt	D22S420	D22S539	D22S1174	D22S315	D22S1163	D22S280	D22S277	D22S283	D22S1169
1N	1.68	1.16	1.95	1.74	1.50	1.72	1.78	1.74	1.46
1T	2.12	2.56	1.01	2.30	1.91	1.44	2.21	2.38	1.97
R	0.79	0.45	0.51	0.76	0.78	0.84	0.81	0.73	0.74
2N	1.21	HOM	2.11	2.35	1.72	1.33	1.76	1.99	1.37
2T	8.06	-	1.30	1.94	2.80	1.44	1.36	1.81	2.42
R	0.15	-	0.62	0.83	0.61	0.92	0.77	0.91	0.57
3N	1.46	1.65	2.84	2.59	2.24	1.69	2.38	4.42	1.57
3T	1.50	1.50	1.78	2.01	1.83	1.63	1.96	3.52	1.33
R	0.97	0.90	0.62	0.78	0.81	0.96	0.82	0.80	0.84
4N	HOM	1.22	1.48	1.64	1.80	1.66	HOM	1.79	1.45
4T	-	0.67	2.88	2.89	4.34	0.80	-	1.40	0.77
R	-	0.54	0.51	0.57	0.41	0.48	-	0.78	0.53
5N	1.99	HOM	1.51	2.18	HOM	1.25	1.74	2.53	1.61
5T	1.60	-	1.49	1.74	-	1.65	1.49	1.74	1.31
R	0.80	-	0.99	0.80	-	0.75	0.86	0.68	0.81
6N	1.54	1.74	1.06	1.79	2.69	HOM	1.37	2.66	1.59
6T	MI	1.53	1.72	1.59	2.55	-	MI	2.55	1.81
R	-	0.88	0.62	0.89	0.95	-	-	0.96	0.88
7N	1.15	1.32	1.66	1.93	HOM	2.52	2.23	1.30	1.81
7T	1.36	1.63	1.50	1.74	-	1.21	1.29	1.71	1.25
R	0.85	0.81	0.90	0.90	-	0.48	0.57	0.76	0.69
8N	1.56	1.40	1.53	1.27	HOM	2.09	1.91	1.58	2.58
8T	1.76	1.80	1.11	1.88	-	2.47	1.07	2.18	2.33
R	0.89	0.78	0.73	0.68	-	0.85	0.56	0.72	0.90
9N	1.68	HOM	1.00	3.32	1.57	1.97	2.31	1.31	HOM
9T	1.82	-	1.62	5.06	1.07	2.00	1.76	1.92	-
R	0.92	-	0.62	0.65	0.68	0.98	0.76	0.68	-
10N	1.66	1.32	1.53	1.54	1.55	1.34	HOM	1.63	1.41
10T	1.62	1.12	1.77	1.61	1.23	1.52	-	1.45	1.64
R	0.98	0.85	0.86	0.95	0.79	0.88	-	0.89	0.86
11N	1.66	HOM	1.29	HOM	1.29	HOM	HOM	1.92	1.39
11T	0.83	-	0.25	-	0.55	-	-	1.12	2.42
R	0.50	-	0.19	-	0.42	-	-	0.58	0.57
12N	HOM	HOM	HOM	2.32	2.73	1.69	-	1.85	1.81
12T	-	-	-	2.17	2.88	0.85	MI	1.13	1.95
R	-	-	-	0.94	0.95	0.50	-	0.61	0.93
13N	1.62	HOM	1.57	2.10	HOM	1.46	HOM	2.02	1.08
13T	1.61	-	1.59	2.64	-	1.43	-	1.67	2.06
R	1.00	-	0.98	0.79	-	0.98	-	0.82	0.52
14N	1.63	HOM	1.96	1.73	1.54	1.56	1.65	1.91	2.19
14T	1.80	-	1.13	1.54	1.79	2.76	0.87	1.87	3.67
R	0.90	-	0.58	0.89	0.86	0.57	0.53	0.98	0.59
15N	HOM	HOM	1.49	1.70	1.88	1.66	1.76	2.07	1.46
15T	-	-	1.56	1.62	1.72	1.69	1.68	2.55	1.46
R	-	-	0.96	0.95	0.91	0.98	0.95	0.81	1.00

Appendix 3: Individual AI ratios for all microsatellite markers and tumours

	Microsatellite Marker								
Pt	D22S420	D22S539	D22S1174	D22S315	D22S1163	D22S280	D22S277	D22S283	D22S1169
16N	1.79	2.01	1.45	HOM	1.73	1.46	2.33	1.92	HOM
16T	1.90	4.53	1.16	-	1.79	2.09	1.55	1.47	-
R	0.94	0.44	0.80	-	0.96	0.70	0.67	0.77	-
17N	HOM	HOM	1.22	1.78	1.50	1.60	1.58	2.11	1.47
17T	-	-	1.54	1.48	1.58	1.38	2.10	2.42	1.54
R	-	-	0.79	0.83	0.95	0.86	0.75	0.87	0.95
18N	-	-	-	-	-	-	-	-	-
18T	-	-	-	-	-	-	-	-	-
R	-	-	-	-	-	-	-	-	-
19N	1.65	HOM	HOM	1.77	1.27	1.62	1.06	1.63	1.29
19T	1.89	-	-	2.29	1.65	1.99	1.15	1.80	1.40
R	0.87	-	-	0.77	0.77	0.81	0.92	0.91	0.92
20N	1.66	HOM	1.49	1.67	1.20	1.49	1.52	1.67	1.46
20T	1.10	-	0.92	1.24	3.78	0.66	2.70	2.54	2.53
R	0.66	-	0.62	0.74	0.31	0.44	0.56	0.66	0.58
21N	1.62	HOM	1.49	1.67	HOM	1.32	1.43	1.87	HOM
21T	1.47	-	1.93	1.72	-	0.76	3.44	1.14	-
R	0.90	-	0.77	0.97	-	0.58	0.41	0.60	-
22N	HOM	HOM	1.60	2.35	2.28	1.76	1.52	1.16	1.46
22T	-	-	1.09	3.16	3.69	1.01	2.88	2.56	2.61
R	-	-	0.68	0.74	0.61	0.56	0.52	0.45	0.55
23N	HOM	1.78	1.58	1.81	HOM	1.58	1.59	HOM	HOM
23T	-	2.52	1.96	1.77	-	2.39	1.95	-	-
R	-	0.70	0.81	0.98	-	0.66	0.82	-	-
24N	HOM	1.47	1.47	HOM	1.54	1.51	1.43	1.49	HOM
24T	-	0.43	2.44	-	2.87	0.46	0.44	0.43	-
R	-	0.29	0.60	-	0.54	0.30	0.31	0.29	-
25N	1.65	1.59	1.56	HOM	1.54	1.57	1.72	3.96	1.44
25T	1.58	1.91	1.64	-	1.41	1.60	1.81	2.25	1.37
R	0.96	0.83	0.95	-	0.92	0.98	0.95	0.57	0.95
26N	HOM	HOM	1.30	1.76	1.52	HOM	HOM	1.37	HOM
26T	-	-	1.08	2.12	2.03	-	-	1.23	-
R	-	-	0.83	0.83	0.75	-	-	0.90	-
27N	1.53	HOM	1.57	1.47	HOM	1.53	1.44	3.09	1.86
27T	1.51	-	1.52	1.58	-	1.58	1.48	2.15	1.94
R	0.99	-	0.97	0.93	-	0.97	0.97	0.69	0.96
28N	1.52	0.99	1.37	4.20	2.79	1.82	1.67	3.97	HOM
28T	2.01	1.63	1.48	3.53	1.37	2.06	1.69	1.82	-
R	0.76	0.60	0.93	0.84	0.49	0.88	0.99	0.46	-

Key: Pt, patient number; R, AI ratio; HOM, homozygous; MI, microsatellite instability. Allele imbalance is shown in red.

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