Gene expression analysis of head and neck cancer development

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A thesis submitted to the University of Glasgow in part fulfilment for the degree of Doctor of Philosophy

April 2005

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Abstract

Squamous cell carcinoma of tissues of the head and neck (HNSCC) continues to be a major cause of morbidity and mortality in many parts of the world, and survival is not improving. Much research has been devoted to the identification of molecular markers characteristic of the development and progression of HNSCC. Alterations in many molecular markers in HNSCC have been demonstrated, including genes related to proliferation, differentiation, adhesion and others such as those that control invasion and metastasis. Some of these single markers may indicate poor prognosis or risk of recurrence, but none has been found to be robust enough for clinical use.

Limitless replicative potential, or immortality, has been identified as one of the six basic "hallmarks" of cancer. Clinical follow-up data from our HNSCC cultures, and other studies, show a correlation between the ability of HNSCCs to establish as cell lines in culture (i.e. become immortal) and poorer patient survival. In addition, 40% of primary cell cultures derived from dysplastic premalignant oral lesions are immortal in culture. Thus, the acquisition of immortality and invasion may be independent events. Previous work has demonstrated a specific pattern of four biological markers associated with oral SCC or dysplasia immortality in cell culture: namely p16 and RARβ loss, p53 mutation, and telomerase activation. The changes in p16, p53 and hTERT were found in the dysplasia biopsies from which the cultures were derived, however the *in vitro* changes in RAR-*β* expression were not reproduced in the biopsy tissues.

Microarray analysis was performed on 32 head and neck keratinocyte cultures using Affymetrix U133A/B genechips. The panel of cultures included normal cells, mortal and immortal cultures of dysplastic keratinocytes and mortal and immortal cultures from carcinomas, all grown to a standard protocol.

The overall GEP revealed that many of the well-established HNSCC molecular markers associated with motility and invasion were up-regulated in the mortal cells, particularly in the mortal carcinomas. Immortal HNSCC cells showed elevated expression of cell-cycle markers and loss of differentiation markers. In addition, a small number of common changes in gene expression in all the

carcinomas, regardless of replicative fate, were identified. This included several transcription factors.

A series of 49 novel gene expression changes consistently associated with immortality in dysplastic keratinocytes and SCCs were identified. The list included genes involved in cell cycle control, signalling, cellular metabolism and maintenance of cellular structure. Validation of the expression of these genes by western blot demonstrated that, in general, the protein expression of genes agreed with the RNA expression level from the microarray data. However, some heterogeneity was evident.

The mortal and immortal gene expression signatures were validated by IHC in the tumours from which the cultures were derived. The tumours that gave rise to immortal cell cultures demonstrated a relatively uniform pattern of staining in relation to the novel markers of immortality. However, those tumours which gave rise to mortal cultures exhibited significant heterogeneity of gene expression pattern, with areas characteristic of both the mortal and immortal phenotype present.

The patterns of gene expression characteristic of mortal and immortal cells may reflect the differing origin of these populations, relating to different mechanisms involved in the generation of HNSCC subtypes. The differences may also indicate that the mortal and immortal cells utilise different mechanisms of invasion. In some cases, mortal and immortal cells exist in adjacent areas within the same tumour, but it is not clear whether the immortal HNSCCs derive from the adjacent mortal cells. However, the presence of intermediate features *in vivo* and *in vitro* raises the possibility that inter-conversion between the two HNSCC types may be possible, with mortal neoplastic keratinocytes gaining the immortal phenotype

These novel markers give us further insight into the mechanisms and importance of keratinocyte immortalization. Surrogate markers of immortality could therefore be valuable for assessment of prognosis and therapy if confirmed in larger *in vivo* studies.

Acknowledgements

There are a great number of people to whom I am immensely indebted and without whom this thesis would never have come to fruition. At BICR, my supervisor, Professor Paul Harrison requires special thanks for his patience, encouragement and all the "just thinking out loud" sessions, which were an endless source of challenge and food for thought. The intellectual input and dynamism of Ken Parkinson, my advisor, requires acknowledgment. What initially seemed to me to be sheer information overload is now beginning to make some sense! To my lab mentor, Janis Fleming, glowing praise for her patience and dedication in teaching me all the molecular biology I know, particularly when nothing seemed to be working. For technical help and know-how, thanks to Yvonne Hey (PICR microarray core facility) and Phyllis Ogston (LCM facility, Wellcome Functional Genomics Facility, University of Glasgow) and others who have helped in many different ways – Jo, Keith Vass, Margaret O'Prey, Robert Macfarlane (the list goes on and on!). To my fellow PhD students in R4, Alex, Aurnab, and Paul, thank you for help and support when things were difficult, and for sharing in the times when we made progress.

At Glasgow Dental Hospital, thanks to the other half of the supervisor/advisor team, Professors Gordon MacDonald and Jeremy Bagg for keeping me focused and protecting me from the aspects of an academic job that are more cumbersome and time consuming. Joyce Hope requires thanks for her help and masterful cutting of sections, often at very short notice. The list of people who have encouraged me and helped me to remain focussed is so long – you know who you are – thank you one and all!

Last but not least, thank you to my long-suffering wife Isabella, who has endured the months of writing up without once complaining about being a "thesis widow", and to Katie whose development over these months has made life so enjoyable, even at this time. I love you both very much.

The work described in this thesis has been supported by a staff fee scholarship from the University of Glasgow, and by a generous grant from the British Society for Oral and Maxillofacial Pathology (BSOMP).

Soli Deo Gloria.

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Abbreviations

ALT	Alternative lengthening of telomeres
ANOVA	Analysis of variance
ARF	CDKN2 Alternate Reading Frame (p14)
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia related
BCH	Basal cell hyperplasia
CDK	Cyclin dependent kinase
CDKN	Cyclin dependent kinase inhibitor
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl suphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetate
EMT	Epithelial Mesenchymal transition
FAD	flavine adenine dinucleotide
FAL	Frequency of allele loss
FDR	False discovery rate
G-CSF	Granulocyte colony stimulating factor
GAPDH	Glyceraldehyde phosphate dehydrogenase
GEF	guanine nucleotide exchange factor
GEP	Gene expression profile
GM-CSF	Granulocyte colony stimulating factor
GO	Gene Ontology
H&E	Haematoxylin and eosin
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
IHC	Immunohistochemistry
ISD	Information and statistics division

IVT	In-vitro transcription
LoH	Loss of heterozygozity
MIAME	Minimum Information About a Microarray Experiment
MDM2	Mouse double minute 2
MM	Mismatch
MOPS	3-(N-Morpholino)-propanesulfonic acid
NHOK	normal human oral keratinocyte
o/n	over night
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	PBS/EDTA
PM	Perfect match
PML	premalignant lesion
RAR-β	Retinoic acid receptor beta
Rb	retinoblastoma protein
RMA	Robust multi average
RNA	Ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SAM	Statistical analysis of microarrays
SCC	Squamous cell carcinoma
SEM	Standard error of the mean
TBST	tris-buffered saline with tween 20
TERT	telomerase reverse transcriptase
TGF-β	Transforming growth factor beta
TR	telomerase RNA template
TRAP	Telomerase Repeat Amplification Protocol
UTR	Un-translated region
WT	Wild type

The work described in this thesis is that of the author, unless otherwise stated.

Chapter One: Clinical and pathological aspects of head and neck squamous cell carcinoma and related premalignant lesions.

1.1 General introduction

Squamous cell carcinoma of tissues of the head and neck (HNSCC) continues to be a major cause of morbidity and mortality in many parts of the world. Indeed, head and neck cancer (90% of which is HNSCC) is the sixth most common cancer worldwide, with a much higher incidence in particular subpopulations, especially in areas of France, the Indian sub-continent and Far East. This disease can have devastating effects on the sufferer, as treatment often involves mutilating surgery that compromises the functions of the upper aero-digestive tract, in addition to the unfortunate social stigma of the facial disfigurement that treatment often involves. Patients, particularly if at advanced stage on presentation, often follow a protracted clinical course involving repeated recurrences with a requirement for long-term multi-disciplinary care. This is distressing for the patient and their family and places a significant financial and logistical burden on the treating healthcare institution.

1.2 Epidemiology

1.2.1 Incidence

HNSCC is a heterogeneous group comprising cancers of the lip, oral cavity, oropharynx, hypopharynx and larynx, each having clinically and biologically distinctive features, but clearly linked by common risk factors (Takes et al., 1998). In the United Kingdom, approximately 8000 new cases of HNSCC are diagnosed each year, with oral cancer comprising 1.6% of all new malignant disease. Historically, the sex ratio has shown a male predominance (5:1), but this has decreased as the female incidence rate has risen (now 1.9:1). Patients over 50 years of age comprise 85% of cases. (ISD at www.isdscotland.org).

The incidence of HNSCC in the UK as a whole has been stable over the last 25 years at around 16 per 100,000 population. However, this headline figure is misleading and conceals important trends. In Scotland, head and neck cancer incidence increased by 16.3% over the period 1990-2000 (Table 1.1 shows individual male and female rates). There was also an increase in the incidence

of cancers of the oral cavity, both in the UK as a whole, but particularly in Scotland, where data from the Scottish Cancer Registry (ISD at www.isdscotland.org) reveals an increase of 34% in males and 43% in females over a similar period (Table 1.2). This contrasts with the 20% reduction in male lung cancer incidence in Scotland over 1990-2000, an intriguing phenomenon as HNSCC and lung cancer share many common risk factors. Similar rises in HNSCC incidence have been seen in many areas of the world, particularly in Central and Eastern Europe, Japan and Australia (La Vecchia et al., 2004; La Vecchia et al., 1997). However, in the USA, the incidence of HNSCC amongst whites (especially males) is falling (Ries et al., 2004). A reduction has also been seen in some areas of high incidence such as India, Hong Kong and Brazil (Sankaranarayanan et al., 1998).

Another worrying feature hidden in the headline data is the rise in HNSCC incidence in young patients (younger than 45 years at diagnosis) in many high incidence and European countries (Boyle et al., 1995). Many of these patients fall into the traditional high risk factor groups, but an increasing proportion do not (Llewellyn et al., 2003; Llewellyn et al., 2004; Rodriguez et al., 2004). Such patients without the traditional HNSCC risk factors may constitute a distinct clinical and biological group (Koch et al., 1999). The notion that these young patients have a poor prognosis with a low five-year survival is not supported by studies in Scotland, as recent data revealed that the 15-44 year age group had a substantially higher five-year survival than any other age group (ISD at www.isdscotland.org). A recent study in Scandinavia also reported higher five year survival rates in young patients (Annertz et al., 2002).

Mortality

Headline figures for the overall five-year survival of patients with HNSCC are also misleading as they conceal trends in particular subgroups of patients. These summary statistics do not take into account the diversity of stage and anatomical subsites involved. Aggressive surgical treatment of early disease at some subsites, for example lip, can produce five-year survival figures of 80-90% (McCombe et al., 2000; Veness et al., 2001). However, in many parts of the world, including Scotland, despite progress in the understanding of the disease, the headline survival rates for head and neck cancer have not changed

markedly over the last few decades (ISD at www.isdscotland.org). The five-year survival rate is 54.3% for males and 56.6% for females (Table 1.1). Indeed, in Scottish males, the five-year survival from head and neck cancer has fallen slightly over the period 1977-2001. Many reasons have been proposed, including a less favourable case mix and an increase in the proportion of patients from deprived areas (Macfarlane et al., 1996). In contrast, over the same period, five-year survival from oral cavity cancer improved by 15%, in both males and females (ISD at www.isdscotland.org).

The recent overall worsening of HNSCC survival in Scottish males is disappointing. Whilst developments in primary surgical and oncological treatment have resulted in better apparent control of primary disease, there has been little improvement in the management of metastatic disease. This is becoming a problem, as an increasing proportion of patients now survive long enough to present with distant metastases (Taneja et al., 2002). Thus, it has become clear that the way forward in tackling this devastating disease must be on two fronts. Emphasis must be placed on prevention and early detection of HNSCC lesions and the careful clinical and pathological monitoring of any lesions that may harbour an increased risk of development of HNSCC. The Health Education Board for Scotland (HEBS) has highlighted this in recent media campaigns. Additionally, advancement in the knowledge of the biology and pathogenesis of HNSCC may result in the development of molecular based prognosis assessment tools and therapies, which may help to reduce the burden of continued disease, foremost for the patient, but also on the healthcare system.

Summary statistics	Period	Males	Females
Rank	2001	4	10
Relative frequency	2001	5.6%	2.4%
Registrations	2001	687	313
Deaths	2003	252	113
Change in incidence (%)	1992-2001	+11.6%	+21.80%
Relative survival (%)			
- 1 year	1997- 2001	78.7	77.3
- 5 years		54.3	56.6

Table 1.1 Summary statistics for cancer of the head and neck in Scotland

This Table comprises the most recent data from Scottish Health statistics, ISD Scotland, released on 26 November 2004 (ISD at www.isdscotland.org). This covers IARC classification codes ICD-9 140-149, 160-161; ICD-10 C00-C14, C30-C32.

Summary statistics	Period	Males	Females
Relative frequency	1999	1.9%	0.9%
Registrations	1999	222	113
Deaths	2001	81	35
Change in incidence (%)	1989-99	+35.4%	+43.5%
Relative survival (%)			
- 1 year	1995-99	72.8	71.1
- 5 years		43.1	51.3

Table 1.2 Summary statistics for oral cavity cancer in Scotland

This Table comprises data collected for IARC classification codes ICD-9 143-145; ICD-10 C03-C06. Data from Scottish Health statistics, ISD Scotland (ISD at www.isdscotland.org).

1.3 Risk factors

1.3.1 Tobacco and alcohol

The main risk factors for HNSCC are tobacco usage (in any form) and alcohol consumption, but this varies significantly between subsites. A recent report found a 20 fold increased risk of oral and pharyngeal cancer below age 46 for heavy smokers, with a five-fold increase for heavy drinkers. The combination of heavy smoking and drinking led to an increased risk of almost 50 fold (Rodriguez et al., 2004). In Western populations, the most common sites for small, asymptomatic SCC lesions within the oral cavity are the ventral aspect of the tongue and floor of the mouth, presumably because this is where such carcinogens collect. Oral cancer is also prevalent in particular communities, such as in the Indian subcontinent, where other forms of tobacco consumption, such as betel quid chewing, are prevalent: indeed, in some Indian subpopulations, HNSCC accounts for almost half of all cancers in males (Sanghvi, 1981; Saranath et al., 1993).

Carcinogens produced by tobacco, for example benz-(a)-pyrene and nitrosamines, are known to produce the precise types of guanine nucleotide transversions found in critical genes involved in HNSCC development, such as p53 (Burns et al., 1993; Chang et al., 1994). The role of alcohol is less clear, but it appears to act as a co-carcinogen by being metabolised to acetaldehyde. This can damage DNA and trap glutathione, an important peptide in detoxification of carcinogens (reviewed in Poschl & Seitz, 2004; Seitz et al., 2001). Alcohol can also induce CYP2E1, an enzyme involved in activation of various pro-carcinogens found in alcoholic beverages and tobacco smoke.

1.3.2 Human papilloma virus

Human papilloma virus (HPV) has also been implicated in HNSCC development (Gillison et al., 2000), but this has proved controversial (Ha et al., 2002a). One case control study found an increased risk of HNSCC with the presence of high risk HPV types (HPV16) in exfoliated oral cells, in concert with heavy alcohol consumption and tobacco use (Smith et al., 2004). However, the concordance between oral HPV status and HPV positivity of the tumour in this study was weak and thus the data is open to other interpretation. Much of the data on the

role of HPV in HNSCC carcinogenesis is semiquantitative and hampered by difficulties in interpretation. The recent study by Ha and co-workers utilised quantitative PCR in assessment of HPV DNA copy number in oral carcinogenesis. This study concluded that sufficient copy numbers of HPV to allow a causal association to be inferred were only present in 1 of 102 premalignant oral lesions and 1 of 34 oral SCCs (Ha et al., 2002a).

Recent evidence more convincingly implicates HPV in a less aggressive form of HNSCC, particularly in tonsillar and oropharyngeal cancers, but to a lesser extent in oral cancer (Herrero et al., 2003; Ringstrom et al., 2002). These HPV-positive oropharyngeal cancers are clinically and biologically distinct and such patients had an improved prognosis (Gillison et al., 2000). Braakhuis and co-workers have shown that HNSCC with transcriptionally active HPV DNA fail to show the pattern loss of heterozygosity (LoH) at chromosomes 3p, 9p and 17p normally associated with HNSCC (Braakhuis et al., 2004b). The implications of this will become apparent in later discussion of the importance of such chromosomal loci.

1.3.3 Other risk factors

A multitude of other factors has been implicated. The development of lip cancer is primarily related to exposure to UV irradiation (Ju, 1973). Marijuana has also been postulated to play a role by increasing mutagen sensitivity (Zhang et al., 1999), but the evidence from cohort studies does not support such a link (Hashibe et al., 2002; Rosenblatt et al., 2004). Poor diet, particularly a low consumption of fruit and vegetables has been implicated (Macfarlane et al., 1995). This has been pinpointed as a potential cause of the increase in HNSCC incidence in young people, although it is likely that such an effect is additive to that of high tobacco and alcohol consumption (Llewellyn et al., 2004; Mackenzie et al., 2000). These collective risk factors may account for the higher incidence of HNSCC in socially and economically deprived communities, notably in the West of Scotland (Macfarlane et al., 1996). Nutrients, such as folic acid, may be protective (Pelucchi et al., 2003). A small number of patients may have an increased susceptibility to oral cancer because of inherited genetic defects (Prime et al., 2001). In disorders of DNA repair mechanisms, such as

xeroderma pigmentosum, these is an increased incidence of secondary malignancies, including oral cancer (Patton & Valdez, 1991).

Genetic susceptibility and environmental risk factors often interact. About 40% of Japanese carry a mutant allele of the acetaldehyde dehydrogenase-2 (ALDH2) gene that encodes an inactive enzyme. This mutant ALDH2 allele is a strong risk factor for head and neck cancer and oesophageal cancer among heavy alcohol drinkers in Japan (Yokoyama et al., 2002). Polymorphisms in alcohol dehydrogenase-2 (ADH2) also affects risk of head and neck cancer in alcoholics (Yokoyama et al., 2001). Indeed, it has been calculated that the polymorphisms in the ALD2 and ALDH2 genes account for 82% of the population attributable risks for HNSCC in Japanese alcoholics (Yokoyama et al., 2001).

There is some variation in the risk factors associated with different subsites within the upper aero-digestive tract. Smoking and alcohol in combination is a higher risk factor for oral cavity and pharyngeal cancer than for larynx (Baron et al., 1993). The differences may lie in the presence of unspecified factors in the oral cavity and pharynx, but not in the other upper aero-digestive tract sites, which potentiates the effects of alcohol and smoking in the oral cavity and pharynx. Separate analysis of smoking and alcohol in relation to risk at the various subsites also reveals that the highest risk for either factor is in the oral cavity and lowest in the larynx (Franceschi et al., 1990; La Vecchia et al., 1990). Various dietary factors have been reported as having differential effects in terms of risk including red chilli and tea drinking (pharynx) (Notani & Jayant, 1987). Patients who present with none of the traditional risk factors predominantly have oral cavity cancers (Wiseman et al., 2003).

1.4 Prognostic factors in HNSCC

Many clinical prognostic factors in SCC are well established. These include anatomical site, tumour size, lymph node status, the presence of distant metastases and certain histological subtypes (Quon et al., 2001; Tralongo et al., 1999; Woolgar et al., 1999). Histological examination also adds tumour grade (a measure of differentiation), depth of invasion and the presence of invasion related to nerves or lympho-vascular channels. Much of this information is summarised in the TNM classification (Sobin & Wittekind, 2002). Whilst useful

at a population level, these prognostic factors may be limited in their predictive value for the identification of patients who have a high risk of recurrence, relapse or death (Fries et al., 1976). As a result, many other clinico-pathological staging and prognosis systems have been developed, with varying degrees of success, and with limited acceptance (Groome et al., 2001).

As is often the case for lung cancer patients, the risk of the development of continued or subsequent malignant disease in patients with HNSCC is high. Many patients will develop either synchronous or metachronous second (or subsequent) upper aero-digestive tract tumours. The probability of developing a second, metachronous HNSCC in the five years after initial treatment is approximately 22% (de Vries et al., 1986; Schwartz et al., 1994).

1.5 Field cancerization

There is evidence that primary HNSCC and subsequent disease (and premalignant lesions) are derived by additional mutations acquired within a 'field' of genetically altered cells within the mucosa (Bedi et al., 1996). The mucosa affected may be clinically and/or histologically normal. This has been termed 'field cancerization', (Slaughter et al., 1953). These alterations arise from exposure of large areas of the epithelium of the upper aero-digestive tract to carcinogens and/or growth promoters in tobacco or alcohol (Bedi et al., 1996). Thus, whilst these tumours are ultimately clonally divergent, they share certain common cytogenetic features with the rest of the field (Braakhuis et al., 2003). The abnormalities may be due to epigenetic or genetic changes induced in the field of exposed cells. Jang and co-workers have also suggested that, in a minority of cases, there may be mucosal spread of malignant or premalignant cells within the oral cavity, resulting in multiple, discrete tumours that are truly clonal in origin (Jang et al., 2001). The mechanism of this effect has not been elucidated.

Consequently, some of the genetic changes characteristic of a particular primary HNSCC can often be found in clinically and histological normal mucosa up to a distance of 7 cm away from the primary tumour (Braakhuis et al., 2003; Tabor et al., 2002a). This has obvious repercussions for decisions relating to the size of excision margins required at the time of primary surgical

management. Thus, second or subsequent HNSCCs may be of three types: they may be:

- "True" recurrence; derived from incomplete eradication of the primary SCC

- Second "field cancers"; these may have a different, but overlapping, spectrum of genetic changes having developed from an intervening field of abnormal oral mucosa (Braakhuis et al., 2002).

- Second primary tumours, with a clonally independent origin.

1.6 Premalignant lesions

The presence of extensive areas of genetically abnormal epithelium raises the possibility of precursor lesions, which may be amenable to preventative intervention. These lesions may be clinically evident, and are often referred to as premalignant lesions (PMLs). Clinically evident mucosal lesions that possess an increased risk of progression to HNSCC predominantly appear as white patches (leukoplakia) or, less commonly, as red patches (erythroplakia). These lesions are illustrated in Figure 1.1A and C. Other conditions, such as lichen planus, may have premalignant potential (Epstein et al., 2003).

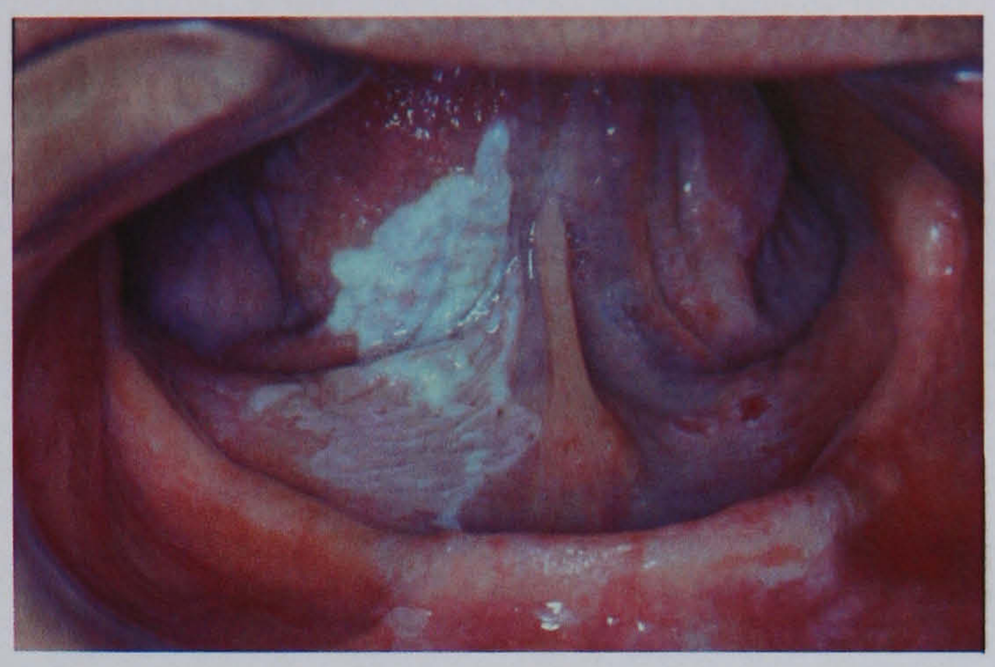
1.6.1 Malignant transformation in PMLs

The evidence that oral leukoplakia has premalignant potential is mainly based on cohort studies in hospital-based populations. This introduces an element of case selection bias into the assessment of the proportion of these patients who develop HNSCC within their PML. The consequence of this is a large range of malignant transformation rates from multiple studies, whose populations are not readily comparable (Table 1.3). Recent studies estimated the prevalence of homogeneous oral leukoplakia in the general population of the USA at 0.37% (Scheifele et al., 2003). Extrapolation of this, in conjunction with the incidence of HNSCC in European countries where there is reasonable integrity of cancer registry data, indicates that the true malignant transformation rate of oral leukoplakia is probably less than 1% per year (Scheifele & Reichart, 2003). Malignant transformation may be higher in the small proportion of leukoplakias that are dysplastic, but regression of dysplastic changes may also be seen,

particularly where there is cessation of the precipitating risk factor(s) (Pindborg et al., 1977).

Various clinical parameters alter the malignant transformation rate of leukoplakia. In Western populations, lesions located on the floor of the mouth and ventral surface of the tongue have been shown to have a higher malignant potential than other areas of the mouth (Kramer et al., 1978), although Schepman and co-workers could not substantiate this in a later study (Schepman et al., 1998). Thus, the concept of high-risk sites is not well supported in the literature. However, it is surely significant that the proposed "high-risk" PML sites are also those that are most commonly associated with early oral cancer lesions. Erythro-leukoplakia or "speckled" leukoplakia, a form of non-homogenous leukoplakia (Figure 1.1B) also has a higher malignant transformation rate (Mehta et al., 1981), presumably related to the presence of areas of erythroplakia within the lesion. The concurrent presence of infection with Candida species also correlates with the presence of oral epithelial dysplasia (McCullough et al., 2002).

In contrast, the malignant transformation rate of erythroplakia is much higher. Erythroplakia is much rarer than leukoplakia, but often reveals carcinoma-in-situ or frank carcinoma at the time of original diagnosis (Mashberg, 1977; Mashberg, 1978; Shafer & Waldron, 1975). Subsequently, a substantial proportion of patients will develop a SCC over the following five-year period. The reasons for the high prevalence of dysplasia in erythroplakias are not clear. It has been suggested this may due to a greater frequency of p53 mutations in erythroplakias (Qin et al., 1999), but once smoking status has been taken into account, this does not seem to be the case (Lazarus et al., 1995; Lazarus et al., 1996).



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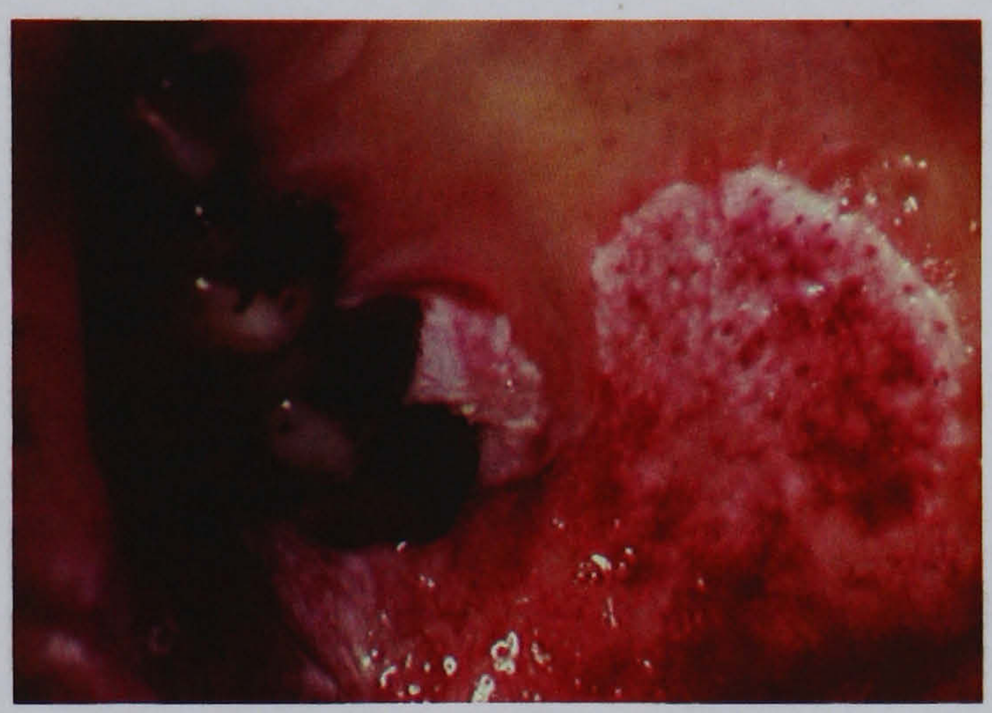

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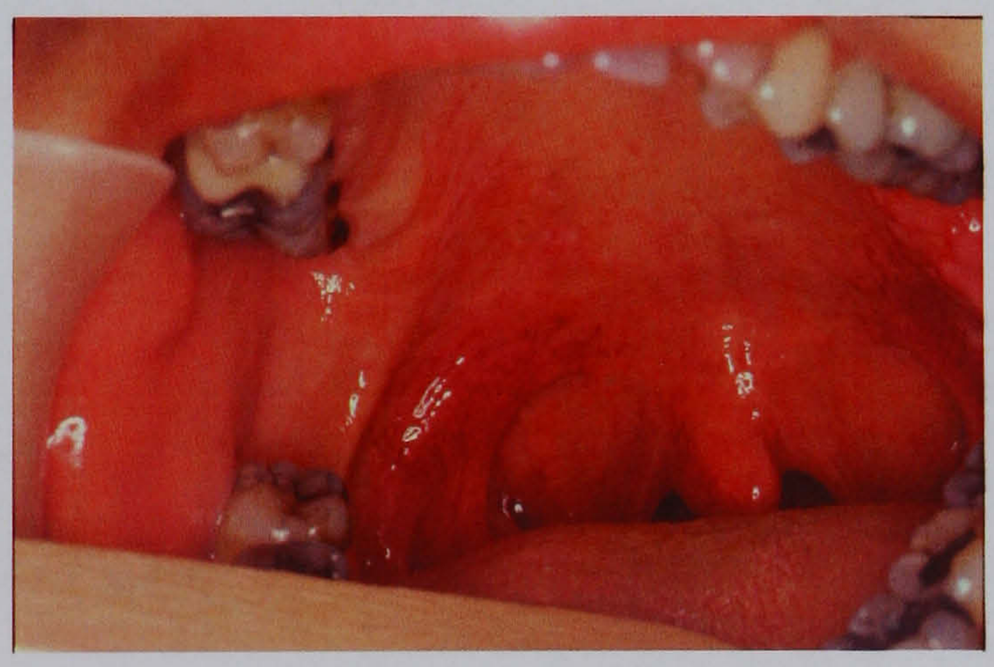


C

Figure 1.1 . Clinically premalignant lesions

A. leukoplakia of anterior floor of mouth/ventral tongue

B. erythro-leukoplakia of soft palate complex

C. erythroplakia of right anterior pillar of fauces

Photographs courtesy of Professor DG MacDonald (A and C) and Dr John Eveson, © Bristol Biomedical Image Archive, University of Bristol (B)

Study/year	Country	No of patients	follow-up (years)	% malignant transformation
(Pindborg et al., 1968)	Denmark	248	3.9	4.4
(Silverman & Rosen, 1968)	USA	117	1-11	6.0
(Kramer et al., 1970)	UK	187	unclear	4.8
(Mehta et al., 1972)	India	117	10	0.9
(Silverman et al., 1976)	India	4762	2	0.9
(Banoczy, 1977)	Hungary	670	9.8	6.0
(Silverman et al., 1984)	USA	257	7.2	17.5
(Lind, 1987)	Norway	157	9.3	8.9
(Lummerman et al., 1995)	USA	240	20	13.8
(Schepman et al., 1998)	Netherlands	166	2.5	12
(Shiu et al., 2000)	Taiwan	435	10	14
(Cowan et al., 2001)	N. Ireland	165	20	15
(Sudbo et al., 2001a)	Norway	150	9	24

Table 1.3 Selected studies on malignant transformation of dysplastic oral leukoplakia.

The table is adapted from a review by Reibel (Reibel, 2003), with some added information from more recent studies.

1.7 The relationship between PMLs and HNSCC

It is impossible to be precise as to the fraction of HNSCC that are preceded by PMLs. Indeed, in some patients, the precursor lesion may not be clinically evident. Certainly, many patients present initially with advanced HNSCC, but it is unclear to what extent previous PMLs may have been missed due to infrequent intraoral examinations. However, as the risk factors for the development of oral premalignant lesions are very similar to those for HNSCC itself (Hashibe et al., 2003), it seems reasonable that a proportion of patients will develop their SCC within a histopathologically evident precursor lesion, whether or not it is clinically visible. Histological studies indicate that, as a minimum estimate, in the region of 36% of SCCs have adjacent dysplastic areas (Bouquot et al., 1988). This clearly leaves a large proportion of SCCs that either have developed in epithelium that was not previously dysplastic, or have completely subsumed the original dysplastic lesion. Pindborg found that a proportion of carcinomas developed in lesions that had not shown dysplasia in previous biopsies (Pindborg et al., 1977). Another study concluded that leukoplakia was associated with 48% of oral SCCs (Hogewind et al., 1989). This indicates that whilst commonly associated with HNSCC, PMLs are not obligatory precursor lesions.

1.8 Epithelial dysplasia and PML prognosis

Assessment of epithelial dysplasia on histological sections has been the traditional method of judging the potential for malignant change. Dysplasia is the sum of atypical structural and cytological features within the epithelium and is traditionally graded as mild, moderate or severe (Figure 1.2 A-C). Once the histopathological features of dysplasia affect the full thickness of the epithelium, but with no evidence of invasion, the term carcinoma-in-situ is used (Figure 1.2 D). Despite WHO guidelines to standardise assessment (Pindborg et al 1997), evaluation of changes within the epithelium remains subjective (Pindborg et al., 1985) and poorly reproducible (Abbey et al., 1995; Onofre et al., 1997).

Some studies have demonstrated a relationship between increased histological grade and increased risk of malignant transformation (Schepman et al., 1998; van der Waal et al., 1997) but others have failed to corroborate this (Partridge et

al., ; Saito et al., 2001). The disparity most likely arises from a combination of the subjectivity of dysplasia assessment and a lack of knowledge of the relative importance of the individual histopathological features (Warnakulasuriya, 2001). Indeed, the morphological changes seen in a dysplastic epithelium may merely be surrogate features that are additional and superfluous to the important changes occurring in the genome and transcriptome of the progressing precancerous oral lesion i.e. epiphenomena (Partridge et al., 2000b). It is clear that although certain histological features of dysplasia are associated with risk of malignant transformation in oral leukoplakia in general, there is a poor correlation between the histological grade of dysplasia (as mild, moderate or severe) and the prognosis of an individual lesion. Such confusion clearly demonstrates the inadequacies of the present predictive methodologies for a patient.

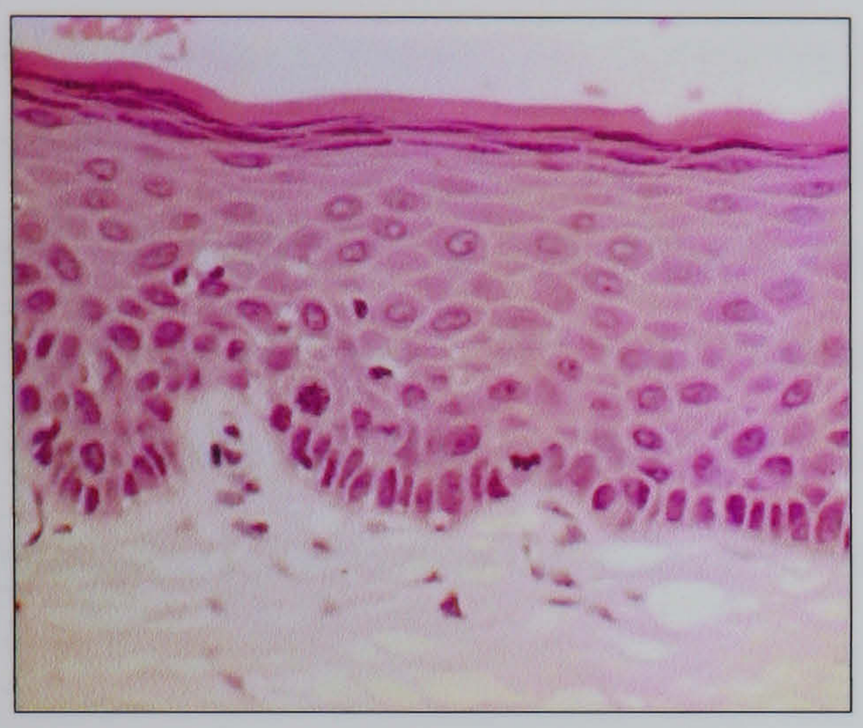
1.9 Treatment of PMLs

Despite initial promise, medical treatment of PMLs with the aim of reducing the malignant transformation rate has proven disappointing. Vitamin A (retinyl palmitate) or its synthetic retinoid derivatives have been used for treatment of oral leukoplakia and prevention of second primary cancers (Hong & Doos, 1985; Hong et al., 1986; Koch, 1978). Retinol/retinoids are known to play an important role in regulating keratinocyte differentiation and high dose retinoids can reverse epithelial dysplasia and precipitate resolution of oral leukoplakia (Hong et al., 1986). However, they cause significant toxicity and the beneficial effect is transient, as the clinical lesion often returns after cessation of treatment (Lodi et al., 2004). The initial promise that retinoids might prevent second cancers developing (Hong et al., 1986) has not been confirmed in recent trials (van Zandwijk et al., 2000). The biology of retinoids will be discussed in more detail later.

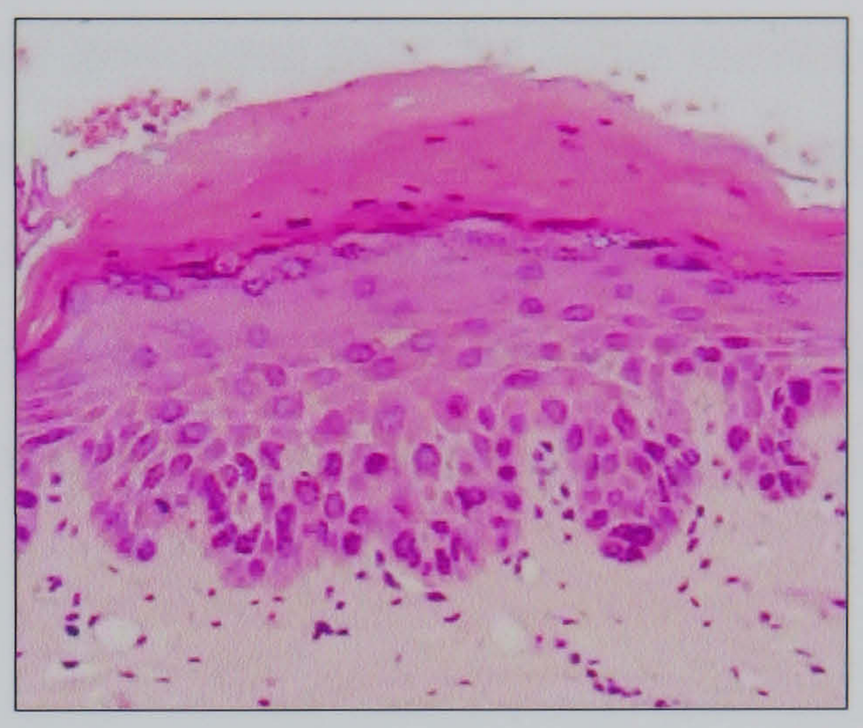
Based on promising preclinical data, inhibition of cyclooxygenase 2 (COX2) is one of the novel treatments currently being actively tested in various cancer models. This includes upper aero-digestive tract tumours, either for cancer prevention or treatment (reviewed in Altorki et al., 2004; Dannenberg & Subbaramaiah, 2003). Antioxidants are another class of agents that may offer promise for treatment of oral PMLs. For example, oral administration of N-

acetyl-L-cysteine reduced the frequency of DNA adducts and abnormal micronuclei in oral mucosa in healthy smokers (Van Schooten et al., 2002) and oral dysplasias showed a clinical and histological response to lycopene, a naturally occurring carotenoid (Singh et al., 2004).

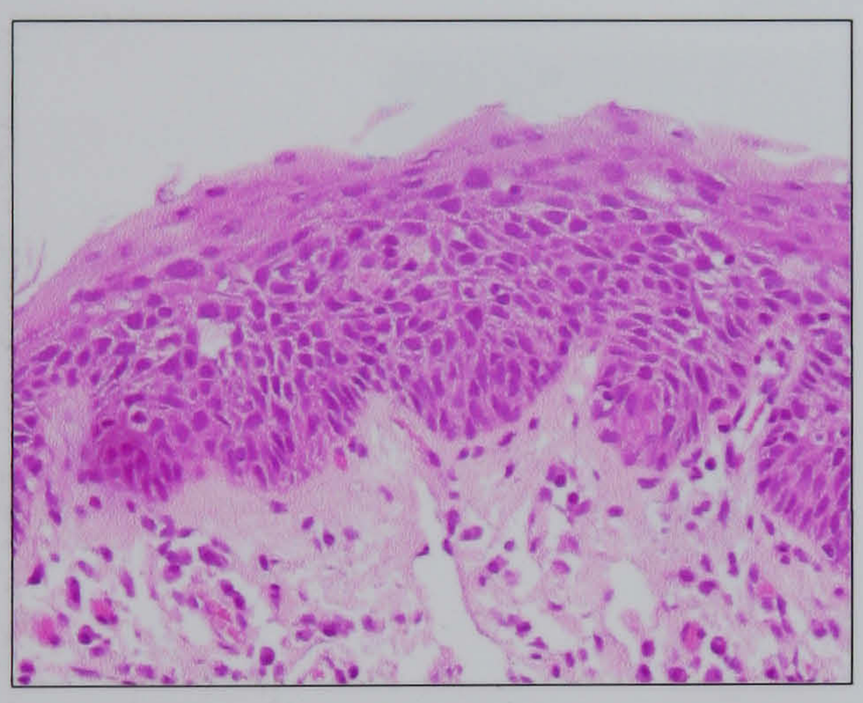
For many patients, surgery provides the only available method for control of their disease, but multifocal lesions are not amenable to curative surgical intervention. Indeed, recent studies have suggested that surgical removal of leukoplakias with chromosomal aneuploidy may be futile (Sudbo et al., 2004). Allied to this, the failures of medical treatment and the uncertainty in assessment of these lesions have led to the search for other predictors of prognosis in HNSCC and malignant transformation in PMLs. It is hoped that these markers may also reveal promising targets for therapy, both in malignant and premalignant disease.



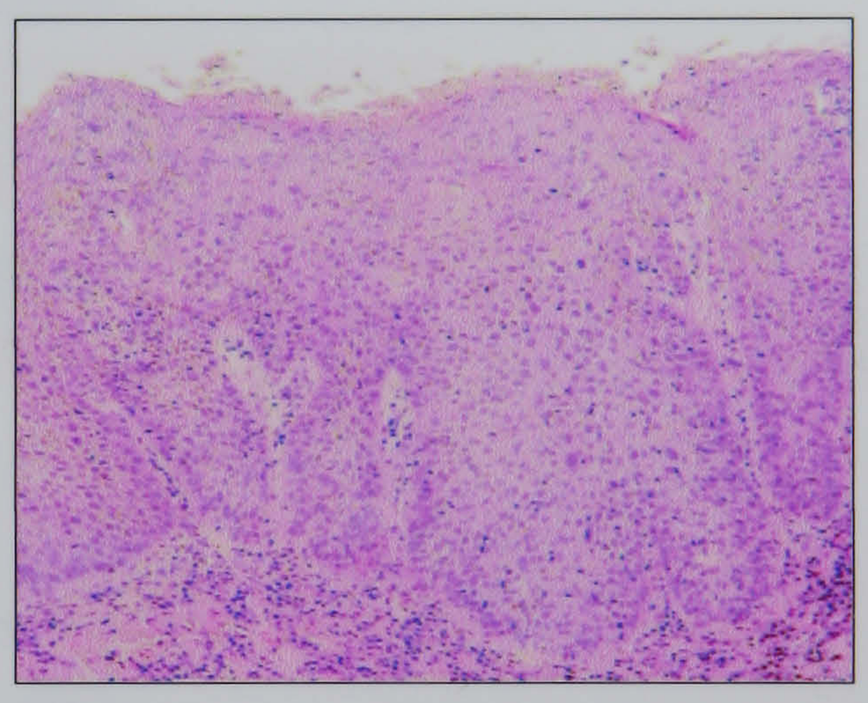
A mild dysplasia



B moderate dysplasia



C severe dysplasia



D Carcinoma in situ

Figure 1.2 Oral epithelial dysplasia

Features assessed after the method of Kramer et al (Kramer et al., 1978). Generally, in mild dysplasia (A) abnormal tissue and cytological features are confined to the deep third of the epithelium, moderate (B) to middle and deep thirds and severe dysplasia (C) extends into the superficial third of the epithelium. This can be modified up or down depending on cytological features. Carcinoma in situ (CiS) (D) refers to similar features to severe dysplasia affecting the whole thickness of the epithelium.

Chapter Two: Carcinogenesis

Over the past three decades, much evidence has accumulated that carcinogenesis is a multi-step process in which various genetic and epigenetic alterations in normal cells cooperate in the generation of highly malignant derivatives. Despite the complexity of cancer genomes and the large number of possible cellular pathways to target, Hanahan and Weinberg have suggested that alterations in six basic cellular functions are required (Hanahan & Weinberg, 2000). The six "hallmarks" of cancer are illustrated in Figure 2.1. These features involve not only the neoplastic cell population per se, but also interactions with the tumour stroma.

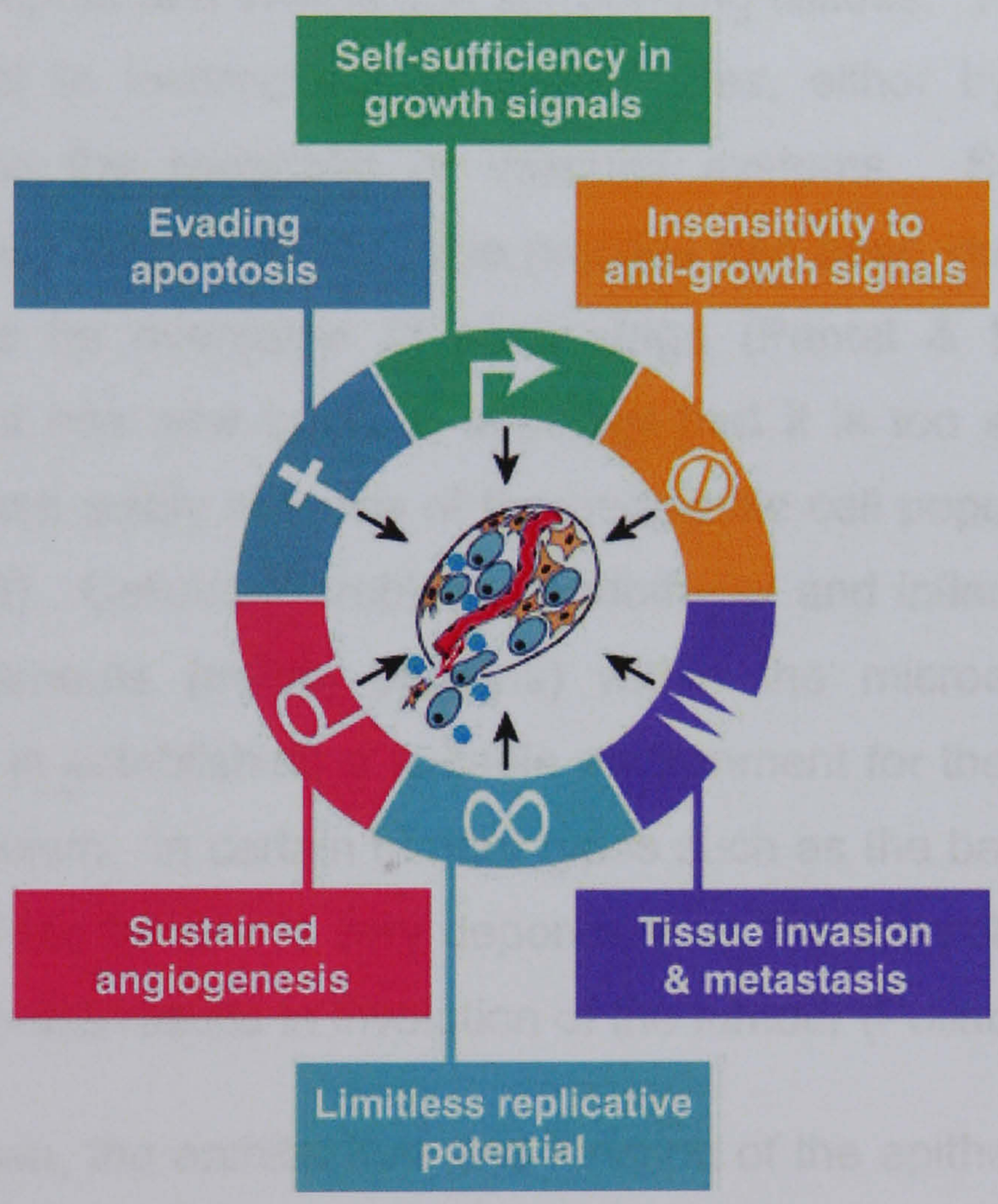


Figure 2.1 The "hallmarks" of cancer.

The Figure shows the six consistent features or "hallmarks" of most cancers, as described by Hanahan and Weinberg. Reproduced from the review by Hanahan and Weinberg (Hanahan & Weinberg, 2000)

The "hallmarks" comprise some of the most complex basic cellular control mechanisms and as such, there are multiple points of intervention that may lead

to similar effects. Conversely, one particular defect may have a different effect in different cell types and the effect may even differ within cells of the same type under different environmental contexts. Thus limiting such "capabilities" to six in number does not predicate against multiple pathways which cells may take on their way to becoming malignant. Thus, certain common alterations (e.g. p53 mutation) may only be present in a proportion of a particular cancer type, but defects in the p53 pathway may be more common (Save et al., 1998). Similarly, the timing and the order in which these capabilities are acquired may vary extensively.

2.1 Invasion and metastasis

The defining feature of malignant disease is its ability to escape normal biological constraints and invade into surrounding tissues. These invasive cells may then travel to locoregional or distant sites, either by direct spread or dissemination in the lymphatic or vascular systems. Secondary tumours (metastases) may then develop. The process has been likened to a cascade with barriers to be overcome at each stage (Pantel & Brakenhoff, 2004). Consequently, it has now become apparent that it is too simplistic to regard malignant disease solely in terms of the neoplastic cell population (Hanahan & Weinberg, 2000). Cellular (fibroblasts, endothelial and inflammatory cells) and non-cellular elements (matrix proteins) within the microenvironment play important roles in establishing a suitable environment for the development of a malignant neoplasm. In certain tumour types such as the basal cell carcinoma, development of the tumour is very dependent on the correct microenvironment as removal from this results in involution of the tumour (Pollack et al., 1982).

In normal mucosa, the architecture and function of the epithelium is maintained by a complex interaction between the epithelial cells and their microenvironment. The dynamic interplay with the extracellular matrix (ECM) controls various functions within the epithelial cell such as differentiation, proliferation and apoptosis. These functions are vital in the control of growth and development, healing and the tissue response to injury (Streuli, 1999). Multiple cellular products and components of the ECM, including adhesion molecules, such as the integrins, and the transforming growth factor-beta (TGF-β) signalling pathway, mediate this control. Alterations in the microenvironment

of a tumour may result from similar genetic damage to that which precipitated the neoplastic growth in the epithelial cells (Park et al., 2000). Alternatively, tumour cells can induce the formation of a "tumour stroma" via the aberrant secretion of growth factors or induction of growth factor receptors (Mueller & Fusenig, 2002). Certain haemopoietic growth factors, such as G-CSF and GM-CSF in addition to tumour derived TGF-β may be important in this regard (Obermueller et al., 2004). The cellular components of the tumour stroma may then reciprocally influence the tumour cells.

TGF-β plays a double-edged role in carcinogenesis (Akhurst & Derynck, 2001). In the early stages, TGF-β acts as a potent tumour suppressor, as it inhibits growth and induces apoptosis (Derynck et al., 2001; Tang et al., 1998). Further effects may include suppression of telomerase activity and the induction of senescence (Katakura et al., 1999; Zhao et al., 2003). However, later in the carcinogenic process, this growth inhibitory effect is lost and TGF-β acts to promote the malignant phenotype (Barrack, 1997). This results in increased angiogenesis and motility, promotion of epithelial-mesenchymal transition (EMT) and escape from apoptosis (Lu et al., 2004; Prime et al., 2004). The alteration in the effect of TGF-β has been related to defects in the receptors and their signalling pathways, particularly via the SMAD transcription factors (Maliekal et al., 2003). Defects in TGF-β signalling in HNSCC have been demonstrated, but many HNSCC cell lines maintain a full or partial inhibitory response to TGF-β (Malliri et al., 1996). However, even a partial defect in TGF-β signalling through the receptor TβR-II, has been shown to result in the emergence of the malignant phenotype, with increased proliferation and invasion *in vitro* (Huntley et al., 2004).

Further functions of TGF-β may include the acquisition of a motile phenotype that allows neoplastic cells to disseminate locally and to distant sites. This may be possible via a partial epithelial-mesenchymal transition (EMT). In normal embryogenesis, this fundamental process governs morphogenesis (Shook & Keller, 2003), but in tumours, it promotes loss of intercellular adhesion and induces cell motility (Mueller & Fusenig, 2004). The TGF-β pathway is one of many that have been implicated in induction of EMT. In breast carcinoma, the transcription factor TWIST is suppressed and this allows EMT to occur, due to loss of E-cadherin mediated effects (Kang & Massague, 2004). Loss of E-

cadherin expression by promoter methylation has been reported in HNSCC (Chang et al., 2002; Chow et al., 2001). The main consequence of loss of E-cadherin is nuclear accumulation of β-catenin, but whilst this has been described in gastric adenocarcinoma, there is no convincing evidence that this occurs to any significant degree in HNSCC in vivo (Chow et al., 2001; Patturajan et al., 2002). However, it is possible that other modulators of EMT, such as certain receptor tyrosine kinases, will play a role. However, HNSCC cell lines showing decreased E-Cadherin expression also have higher levels of vimentin and other markers of EMT (Taki et al., 2003).

Many tumours show changes in the spectrum of cell adhesion molecule (CAM) expression including alteration in E-Cadherin and various integrins. These CAMs mediate adhesion to the ECM and are involved in a variety of functions such as motility, proliferation and differentiation via various intracellular pathways, such as activation of focal adhesion kinase (FAK) (Akiyama, 1996). Numerous studies have demonstrated changes in the expression, distribution and functions of integrins in tumour cells. Aberrant expression and function promotes cell migration, invasion and angiogenesis (Brooks et al., 1994) in cancer and may suppress apoptosis (Thomas & Speight, 2001). Consistent upregulation of certain integrin subunits, such as $\alpha V\beta 6$, in oral cancer suggests that this may play a more active role in oral carcinogenesis (Thomas et al., 2001a; Thomas et al., 2001b).

Alterations in stromal remodelling can also contribute to the tumour-stroma interaction and may modulate the activity of the neoplastic cell population. Many enzymes, including the matrix metalloproteinases (MMPs), remodel the extracellular matrix. This family of zinc-dependent endopeptidases plays a crucial role in ECM turnover, but alterations in the expression of various MMPs and/or their inhibitors have been reported in many cancers. The levels of various MMPs may be increased by many signals including TGF-β, epithelial growth factor receptor (EGFR) and Vascular Endothelial Growth Factor (VEGF) (Hiratsuka et al., 2002; O-Charoenrat et al., 2000b; Rougier et al., 1997). MMPs promote carcinogenesis by multiple mechanisms, including cell growth, migration and angiogenesis (Pepper, 2001), due not solely to ECM turnover, but also to the release of matrix-bound growth factors (Belotti et al., 2003) and interaction with various classes of cell adhesion molecules (Coussens et al.,

2002). Alterations in many MMP family members and some of their inhibitors have been found in HNSCC including MMP2, MMP9 and TIMP2 (Katayama et al., 2004; O-Charoenrat et al., 2001; Ruokolainen et al., 2004). High levels of MMP9 in HNSCCs has been correlated with advanced T stage, an infiltrative pattern of growth and poor prognosis (O-Charoenrat et al., 2001; Ruokolainen et al., 2004). However, this has not been reproduced *in vitro*, perhaps due to difficulties in fully reconstituting the ECM in tissue culture (Robinson et al., 2003).

Analysis of tumour cell motility by a combination of methods has led to the conclusion that there are many different mechanisms of tumour cell motility. (Friedl & Wolf, 2003; Sahai, 2005) The two main types, with subgroups, are illustrated in Figure 2.2. Collective type motility involves the movement of clusters of cells and is dependent on the production of MMPs at the invasive front to clear a path for the tumour to move (Nabeshima et al., 2002). This method, although common in epithelial malignancy, is poorly understood, as it is difficult to model *in vitro*. There are also two types of individual motility. Mesenchymal motility is related to EMT, and requires the production of MMPs to degrade the ECM (reviewed in Friedl & Wolf, 2003). On the other hand, amoeboid movement, which is characteristically seen in haematological malignancy, demonstrates no such requirement, nor an EMT. It is very similar to the rounded Rho- and Rock-dependent form of motility that has been described (Sahai & Marshall, 2003). This allows the cell to squeeze through the ECM, rather than degrading it. It also is likely that cells are able to switch their mechanism of motility in response to different environments (Wolf et al., 2003).

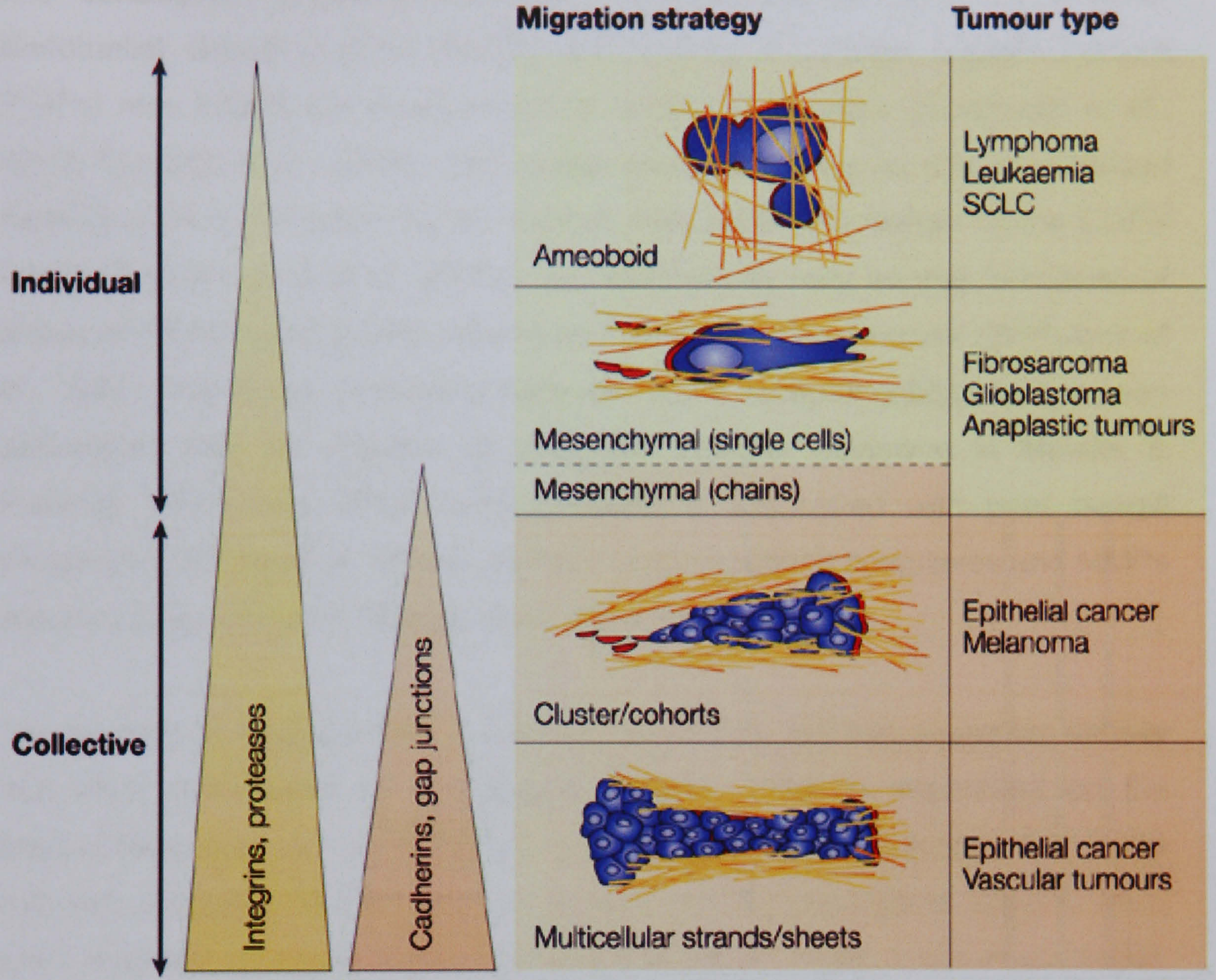


Figure 2.2 Mechanisms of invasion

The figure illustrates the present understanding of the different mechanisms of invasion of cancer cells and their varying dependence on different molecular programmes (triangles). From individual (top) to collective (bottom) movements integrins and proteases play an increasing role in cell-ECM interaction. The most common pattern of invasion in epithelial cancers is collective movement as clusters. Reproduced from (Friedl & Wolf, 2003).

2.2 Angiogenesis

Tumour angiogenesis is another vital component of "successful" malignant neoplasia. The ability to stimulate the development of new blood vessel growth allows a tumour to continue to grow without the potential hindrance of hypoxia and consequent ischaemic necrosis. Growth factors such as Vascular Endothelial Growth Factor (VEGF) and various Fibroblast Growth Factors (FGFs) may initiate the development of tumour vasculature (Compagni et al., 2000; Giavazzi et al., 2003). The increase in activity may be due to increased production of such factors by the tumour cells under stimulation by the EGFR family (O-Charoenrat et al., 2000c), but alternatively may be due to release of stores of ECM-bound growth factors by increased ECM turnover (Whitelock et al., 1996). Increased numbers of particular VEGF receptor subtypes have been associated with the initiation of malignant disease (reviewed in Mueller & Fusenig, 2002) and VEGF overexpression is associated with poor overall prognosis (Uehara et al., 2004). Various integrin subunit complexes and MMPs are also pro-angiogenic (Brooks et al., 1994; Pepper, 2001).

Assessment of angiogenesis in tumours is difficult, but microvascular density has often been used as a surrogate marker. This is dependent on the immunohistochemical identification of endothelial lined channels, but some tumours, including HNSCC may construct a pseudo-vasculature using tumour-lined channels for blood supply (Folberg & Maniotis, 2004; Shieh et al., 2004). In HNSCC carcinogenesis, some groups have demonstrated that the vascularity of oral tissues increased with disease progression from normal mucosa to through the grades of dysplasia to carcinoma (Macluskey et al., 2000; Pazouki et al., 1997), whilst others have found no such increase (Tae et al., 2000). An increase in VEGF has been noted in HNSCC and this is related to EGFR overexpression (O-charoenrat et al., 2002). In particular, increases in VEGF-A and VEGF-C have been correlated with the presence of lymph node metastases (Shintani et al., 2004).

2.3 Self sufficiency in growth signals

Normal cells require mitogenic stimuli to initiate and sustain proliferation. Many of these signals are mediated by autocrine or paracrine signals that are relayed to the nucleus from the cell surface by various classes of transmembrane receptor. Alterations in several of these critical pathways were originally identified as a consequence of research into oncogenic viruses (Harvey, 1964). Such viruses have sequences in their genome that are similar to genes found in normal human cells. These sequences were aberrantly expressed subsequent to integration into the host genome. These genes, termed oncogenes, have subsequently been shown to belong to several functional groups of genes, many of which are involved in the transmission of mitogenic stimuli from the cell surface to the nucleus.

2.3.1 Growth factors and their receptors

Many of the alterations in oncogenes involve growth factors and their receptors. Tumours may produce their own growth factors to act in an autocrine/paracrine manner on the tumour cell population (e.g. FGF-1, Myoken et al., 1994). This obviates the normal control exercised by the microenvironment. Overexpression of growth factor receptors will also increase the intensity of the signal. One example is overexpression of the epidermal growth factor receptor (EGFR), a member of the EGFR/c-erbB family of growth factor receptors. This family of receptors binds six major ligands, namely, epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), betacellulin, heparin-binding epidermal growth factor-like growth factor (HB-EGF), amphiregulin and heregulin (O-Charoenrat et al., 2000a; Prigent & Lemoine, 1992). Ligand binding promotes dimerisation of receptor subunits and activation of the intrinsic tyrosine kinase activity of the cytoplasmic domain of the receptor. The signal is then propagated to the nucleus via a cascade of intracellular kinases. These include the Stat family of signal transducers, which may also show altered expression in tumours (Grandis et al., 1998b).

EGF is required for proliferation of oral keratinocytes in culture, which illustrates the dependence of these cells upon this signalling pathway. Thus, it is not surprising that alterations of EGF signalling are seen in many epithelial cancers

(Cheng et al., 2002; Cowley et al., 1986; Hendler & Ozanne, 1984; Ito et al., 2001; Perry et al., 1998). Overexpression of EGFR may allow the neoplastic keratinocytes to respond inappropriately to low levels of ligand in the extracellular environment. EGFR upregulation has been demonstrated in many cancers including HNSCC (Ozanne et al., 1986), although it does not seem to be a ubiquitous alteration. Inhibition of EGFR or TGF-α expression using antisense oligonucleotides leads to a decrease in the proliferation of HNSCC cells lines, with limited effects on normal cells in culture (Grandis et al., 1998a; Rubin Grandis et al., 1997).

However, Stanton and co-workers noted that the relative overexpression of EGFR was higher in primary HNSCC cell cultures than in their matched tumour samples (Stanton et al., 1994). Indeed, the expression of EGFR in the tumours was higher than normal tissue in only two of ten samples. Whilst this suggests that there is potential for EGFR overexpression in at least a subset of cells within most HNSCC lesions, it does not explain why some tumours over-express EGFR and others do not. The same work also suggested that overexpression of EGFR is a late event in HNSCC carcinogenesis, since cells derived from two premalignant lesions that progressed to SCC over a short period did not have high levels of EGFR expression (Stanton et al., 1994). The small number of premalignant lesions analysed may be a limiting factor in this conclusion, as much larger studies have concluded that EGFR is upregulated in dysplastic oral tissues (Shin et al., 1994b). Even in these studies, the EGFR upregulation demonstrated in dysplastic lesions is somewhat modest (in some cases 2 fold, assessed by immunohistochemistry), compared to the much larger increase in EGFR expression which occurs in HNSCC (Shin et al., 1994b). Alterations in the other family members (c-erbB2, c-erbB3 and c-erbB4) have also been reported, but their role in HNSCC carcinogenesis is less clear (O-Charoenrat et al., 2002).

Nevertheless, clinicopathological studies found that high levels of EGFR and TGF-α in HNSCC were a statistically significant predictor of recurrent disease and poor overall survival (Grandis et al., 1998c). Similar data in other epithelial malignancies has lead to the development of novel anti-EGFR therapies for use in many cancers, including HNSCC. Initial preclinical and phase I/II trials appeared promising (Bonner et al., 2002), but the results of recent larger trials

of EGFR inhibition with chemotherapy and radiotherapy have shown limited improvements in disease free survival, with only a minority of patients responding (reviewed in Raben et al., 2004).

2.3.2 Signal transduction

Components of various intracellular signal transduction pathways have also been implicated as oncogenes. Such signalling networks include the Ras-Raf-MAPKinase pathway, which is downstream of many growth factor receptors. The Ras proteins were amongst the first to be assigned a function related to cell growth and proliferation (Yuspa et al., 1983). This family of proteins controls the activity of several key signalling pathways. Three members of the family HRAS, KRAS and NRAS are mutated in 20% of human tumours (Bos, 1989). Point mutations in RAS lead to constitutive activation by compromising the GTPase activity of RAS, leading to accumulation of RAS in an active form (Tabin et al., 1982). This increases the signalling traffic in several pathways, resulting in loss of control over cell growth and an increase in invasiveness and angiogenesis (reviewed by (Shields et al., 2000).

The reported frequency of RAS alterations in HNSCC varies in the literature (reviewed in Downward, 2003). Several studies have documented a very low frequency of RAS mutation in HNSCC in the UK, despite the high proportion of tobacco smokers (Chang et al., 1991; Yarbrough et al., 1994; Yeudall et al., 1993). A similar pattern has been found in premalignant lesions (Matsuda et al., 1996). However, in populations where tobacco is chewed, or used in association with betel quid (pan), the prevalence of activating RAS point mutations may be as high as 35% (Chang et al., 1991). Alterations in targets downstream of RAS, such as BRAF, are also rare in HNSCC (Weber et al., 2003).

2.3.3 Targets in the cell cycle

The cell cycle comprises a highly regulated series of steps that result in the duplication of the cellular DNA and subsequent cytokinesis (Figure 2.3). The machinery of the cell cycle is thus intimately involved in the control of cell proliferation. The regulatory elements of the cell cycle includes Cyclins, Cyclin

dependent kinases (CDKs) and various negative regulators, including inhibitors of Cyclin/CDK complexes.

One of the ultimate targets of the mitogenic cell-surface signals is Cyclin D1 (Loyer et al., 1996; Quelle et al., 1993). This is a member of the family of D-type Cyclins and is involved in control of the transition from G1 to S phase of the cell cycle (Figure 2.4). Upon induction by mitogeneic stimuli, Cyclin D1 expression increases forming transient functional complexes with cdk4 and cdk6. Interaction of this complex with retinoblastoma protein (Rb) results in Rb phosphorylation (Kato et al., 1993). This removes the inhibitory effect of Rb on certain transcription factors, such as E2F, which allows transcription of proteins required for G1/S phase transition (Figure 2.4).

Investigations in many different cancers, including breast carcinomas, lymphomas and SCCs have demonstrated frequent amplification of chromosome locus 11q13 (Schuuring, 1995). This locus contains several putative oncogenes, including Cyclin D1, also known as PRAD1 (Motokura et al., 1991). Loss of control of Cyclin D1 expression may be attained by chromosomal translocation, DNA amplification, abrogation of controls in mitogenic signalling pathways and retroviral integration. The resulting overexpression accelerates passage of the cell through G1 phase and reduces the requirement for mitogens (Quelle et al., 1993).

A number of workers have demonstrated that Cyclin D1 overexpression is a late change in HNSCC carcinogenesis (Schoelch et al., 1999) and have related such overexpression to poor prognosis (Bova et al., 1999). The proportion of patients with Cyclin D1 overexpression varies greatly, particularly in those studies based on immunohistochemistry (Lam et al., 2000). The subjectivity in assessment of Cyclin D1 by IHC may account in part for the great variation in proportion of tumours and subsequent conclusions. Detection of Cyclin D1 amplification may be a more sensitive measure of deregulation of Cyclin D1 expression, but it does not account for all possible routes by which deregulation may be achieved. Amplification has been described in 30-50% of primary tumours, but in virtually all HNSCC cell lines (Callender et al., 1994; Jares et al., 1994; Okami et al., 1999; Rousseau et al., 2001; Sartor et al., 1999). The presence of amplification of Cyclin D1 has been related to aneuploidy, which

has also been related to poor prognosis (Callender et al., 1994; Nimeus et al., 2004). However, work in our own group did not identify any consistent overexpression of Cyclin D1 expression in cell cultures from dysplastic lesions or HNSCC (McGregor et al., 2002). Thus, whilst it seems likely that deregulation of Cyclin D1 does play a role in the development of at least subset of HNSCC there are other points in the machinery controlling G1/S transition that may also be altered in the process of carcinogenesis.

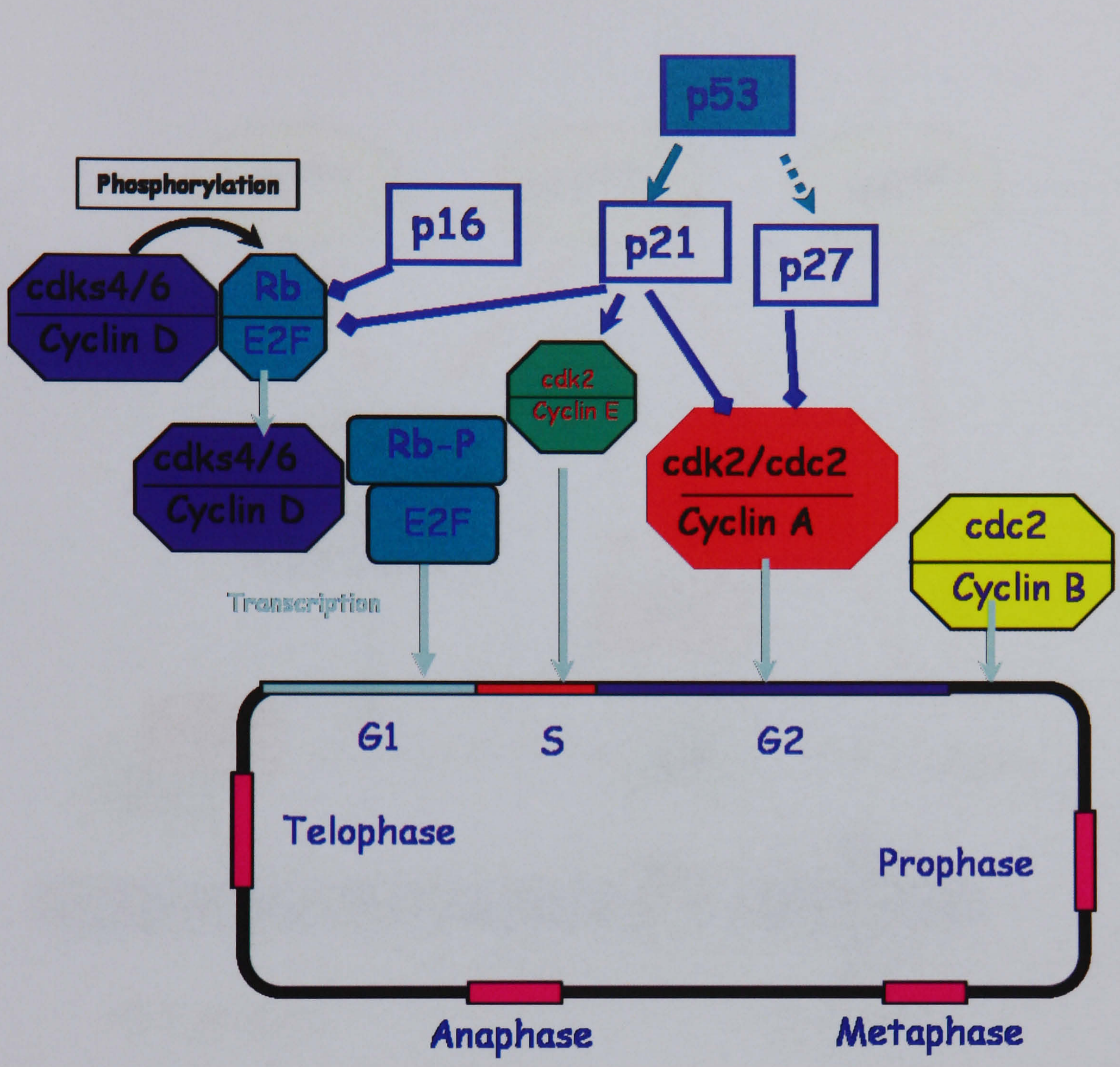


Figure 2.3 The cell cycle

Illustration of the phases of the cell cycle and its regulators. The major Cyclins, Cyclin dependent kinases and cdk inhibitors are shown (see text for discussion).

G1 = gap phase 1; S = DNA synthesis phase; G2 = gap phase 2; M = mitosis. Adapted from Goodger, 1997 (Goodger et al., 1997).

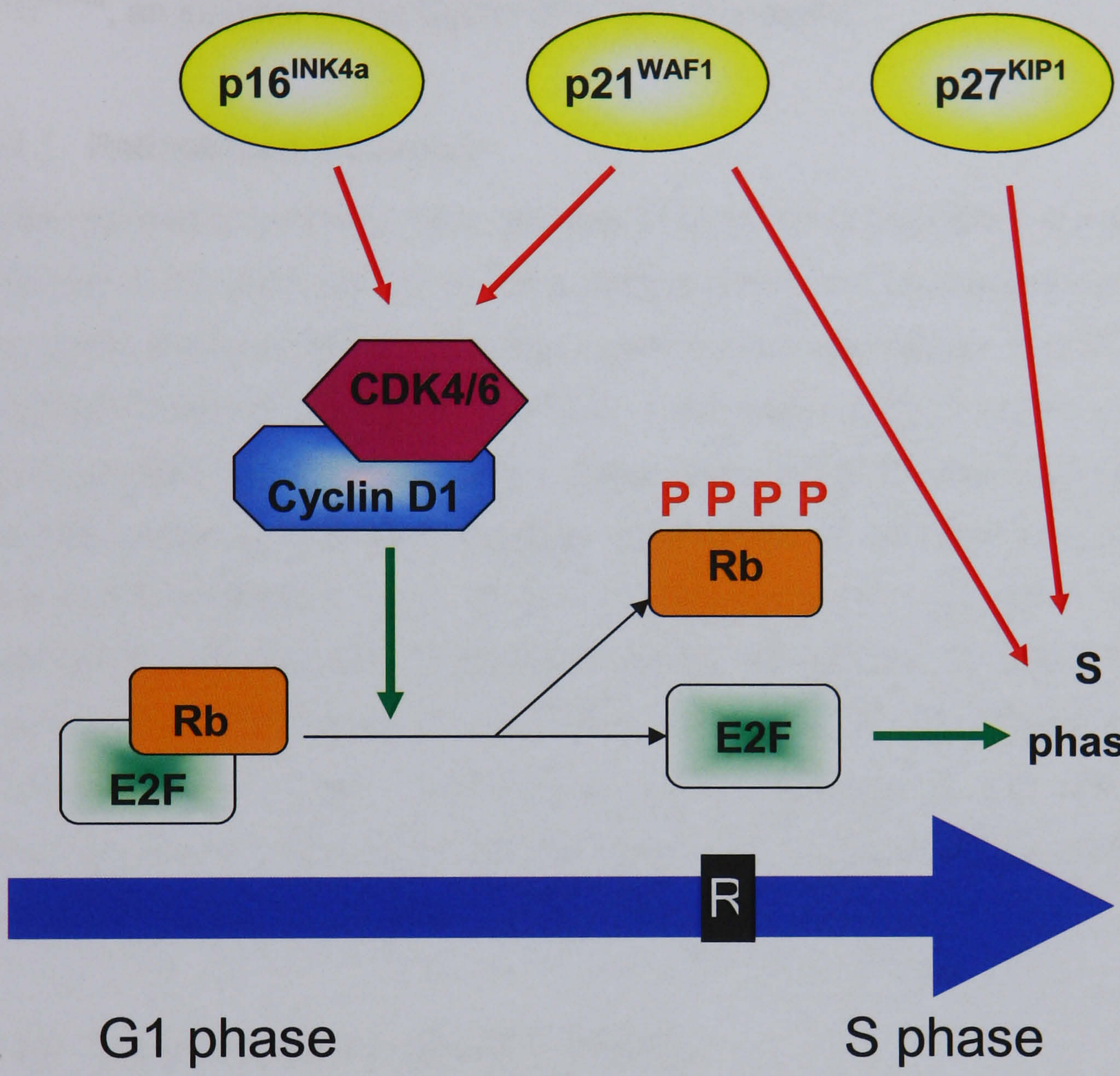


Figure 2.4 Control of G1 to S phase transition by the retinoblastoma protein pathway

Phosphorylation of Rb is accomplished by the CyclinD1/cdk4/6 complex. This complex is under the control of p16, and to a lesser extent, p21. p21 also acts on genes expressed in S-phase, together with p27, to inhibit the progress of S-phase

R= restriction point P=phosphorylation

2.4 Insensitivity to anti growth signals

The abrogation of inhibitors of the cell cycle may also contribute to the loss of tight control of cycle progression. Other possible targets, which may render Cyclin D1 overexpression redundant, include alterations in Rb itself, or in $p16^{INK4a}$, an inhibitor of the Cyclin D1/CDK4/6 complex.

2.4.1 Retinoblastoma protein

In its hypophosphorylated form, Rb acts to block G1/S transition. It may also be involved in the promotion of terminal differentiation and permanent exit from the cell cycle (Adams, 2001). Rb also regulates the expression of $p16^{INK4a}$ via a negative feedback loop (Li et al., 1994). Inactivation of Rb, by point mutation or by interaction with HPV protein E7, deregulates the G1/S transition and allows the cell a selective growth advantage. In HNSCC, Rb mutation appears to be a rare event (Ambrosch et al., 2001). Alterations in Rb expression have been reported in some cancers, including non-small cell lung cancer (Jin et al., 2001), oesophageal SCC (Xing et al, 1999), and to a variable extent in HNSCC (Ambrosch et al., 2001; Pande et al., 1998; Schoelch et al., 1999). When alteration in Rb expression occurs, this may be early in the carcinogenic process (Pande et al., 1998).

2.4.2 Cyclin dependent kinase inhibitors

It is more common for the alteration within the Rb pathway to involve inhibitors of the Cyclin/CDK complexes, such as $p16^{INK4a}$ (hereafter referred to as p16) or $p27^{KIP1}$ (hereafter referred to as p27). Inhibitors of the cell cycle belong to two main families – the ink4 family which includes p14, p15, p16 and p18 or the Cip/Kip family, which includes p21, p27 and p57.

2.4.2.1 The INK4 family of Cyclin dependent kinase inhibitors

The INK4 proteins are products of the complex INK4a locus on chromosome 9p21. p16 transcripts include exons 1α, 2 and 3 whilst an alternate reading frame (ARF) uses exon1β, which is located upstream from exon 1α (Quelle et al., 1995). This comprises the first exon of p14ARF, with exons 2 and 3 the

same as p16. These alternative products have separate promoters and perform very different actions within the cell.

2.4.2.2 p16

p16 was initially identified in co-immunoprecipitation experiments with CDK4 (Xiong et al., 1993). Subsequent investigation has demonstrated it to be a specific inhibitor of the Cyclin D kinases, CDK4 and CDK6 (Serrano et al., 1993) and to be able to destabilise cdk4/Cyclin D1 complexes *in vitro* (Sandhu et al., 2000). Thus, Rb phosphorylation is inhibited and the G1/S transition of the cell cycle does not proceed. Regulation of p16 expression is primarily at the transcriptional level and increased p16 levels have been related to a number of stimuli including replicative senescence (discussed later), oncogenic RAS (Serrano et al., 1997) and inactivation of Rb, presumably due to the loss of negative feedback (Parry et al., 1995).

Alterations in p16 were initially described in many cancer cell lines, including those derived from bladder, lung, pancreas and head and neck cancer (Cairns et al., 1995; Liu et al., 1995). Abnormalities reported include homozygous deletion (often of small segments) or point mutation. The linkage with cancer was further strengthened by the identification of germline mutations of p16 in patients with a familial form of malignant melanoma (Ranade et al., 1995). Epigenetic silencing of p16 expression by promoter methylation has been identified as another method of transcriptional loss (Merlo et al., 1995). In cell lines demonstrating this phenomenon, re-establishment of p16 expression was possible by treatment with a demethylating agent, such as 5-aza-2-deoxycytidine (McGregor et al., 2002; Merlo et al., 1995).

In HNSCC, the predominant mechanisms of p16 inactivation are homozygous deletion and promoter methylation. The prevalence of p16 mutations is low (Jefferies et al., 2001; Tsai et al., 2001). Promoter methylation may be concurrent with homozygous deletion indicating that initial epigenetic inactivation may be made permanent by subsequent deletion (Shintani et al., 2001). From many different studies, it has been estimated that around 60-80% of all primary HNSCCs have either a genetic or epigenetic alteration in p16 (Reed et al., 1996; Shintani et al., 2001). There is however, much variation as studies based solely on immunohistochemical detection of p16 expression have

found a lower prevalence of alteration (Tsai et al., 2001). Discrepancies in the frequency of deletions and mutations detected in primary tumours when compared to cell lines has also been reported (Sartor et al., 1999; Zhang et al., 1994). These studies concluded that the difference in p16 alterations might be the result of adaptation to tissue culture. However, the presence of normal cells within the mixed tumour samples, which may have masked small deletions, was not investigated in either study.

Loss of p16 expression in various SCCs has been linked with poor prognosis. For example, p16-negative lung SCC patients having a significantly lower five year survival than those with p16-positive tumours (Huang et al., 2000; Jin et al., 2001). In HNSCC, loss of p16 has been related to a poorer prognosis in patients with SCC of anterior tongue (Bova et al., 1999). Yuen and coworkers found a link with more locally advanced disease, but not with overall survival (Yuen et al., 2002), a finding confirmed by other studies (Geisler et al., 2002; Ogi et al., 2002).

Many clinical studies have also found p16 alterations in dysplastic oral epithelium (Ambrosch et al., 2001; Papadimitrakopoulou et al., 1997; Shahnavaz et al., 2001). Papadimitrakopoulou and coworkers described loss of p16 protein expression in 38% of oral leukoplakias and a good correlation was found with LOH at 9p21 (Papadimitrakopoulou et al., 2001). This work, along with similar studies in Barrett's oesophagus, indicates that alterations in p16 are common, early abnormalities in SCC (Wong et al., 2001). Interestingly, hypermethylation has been reported as the predominant mechanism of loss of p16 expression in severely dysplastic lesions, especially in lesions of the floor of the mouth (Kresty et al., 2002). In terms of assessment of the risk of malignant transformation, loss of p16 expression appears to have predictive value only in combination with other markers, such as p53 overexpression (Gallo et al., 1997).

Work previously conducted in the Beatson Institute for Cancer Research has focused on p16 alterations in HNSCC and oral dysplastic lesions *in vitro*. Loss of p16 expression was found in all 19 immortal carcinoma and six of seven immortal dysplasia cultures, but only in one of six mortal carcinoma and one of eight mortal dysplasia cultures (Loughran et al., 1996; McGregor et al., 2002;

McGregor et al., 1997). In the carcinoma cultures, p16 was inactivated by homozygous deletion in 8 of 18 cultures and by transcriptional silencing in the remaining 10 (Loughran et al., 1996). In two of these cell lines, p16 transcripts were readily detectable after treatment with demethylating agents. In addition to this, we have previously demonstrated p16 re-expression after treatment with 5-aza-2- deoxycytidine in 2 of 5 immortal dysplasia cultures, but in none of the immortal carcinomas (McGregor et al., 2002). These results confirm the importance of homozygous deletion and DNA methylation as mechanisms of p16 inactivation, consistent with that described elsewhere in the literature. Additionally, they suggest a relationship between p16, replicative senescence and the acquisition of immortality, rather than with the malignant phenotype per se. This issue will be discussed in detail later.

2.4.2.3 p14ARF

p14 has a role in the p53 signalling pathway, as it binds to MDM2, targeting it for degradation (Zhang et al., 1998b). This results in blockage of MDM2-mediated p53 degradation and stabilisation of p53 (see later). The consequence of this is p53 dependent cell cycle arrest or apoptosis. p14ARF has also been implicated in non-p53 mediated events, such as an ability to inhibit rRNA processing and other effects on gene expression (Ayrault et al., 2004). Expression of p14ARF is under the control of E2F (Dimri et al., 2000), thus the Rb and p53 pathways are linked (Bates et al., 1998; Robertson & Jones, 1998). Increased expression of ARF has also been related to sustained oncogenic signals, such as those resulting from Myc overexpression or Ras mutation (Zindy et al., 1998). Such effects may be unrelated to p53 function.

Loss of p19ARF (the mouse homologue of human p14ARF) expression has been shown to predispose to cancer early in life (Kamijo et al., 1997). However, despite this and the high frequency of alterations reported in the INK4 locus, specific p14ARF abnormalities have been found with much less regularity than those in p16. As all of the cell cycle inhibitory functions of p14ARF are contained within the sequence of the unique 1β exon, the significance of mutations in exon 2, which is shared with p16, is disputed (Quelle et al., 1997). The frequency of either mutations in, or deletion of, exon 1β in oral carcinogenesis has been reported to be very low (Kresty et al., 2002; Munro et

al., 1999; Shahnavaz et al., 2001). However, epigenetic abnormalities in the p14ARF promoter have been reported in oesophageal cancer (Smeds et al., 2002) and oral SCC (Shintani et al., 2001). Interestingly, the study by Smeds and coworkers found no relation between p14ARF promoter methylation with p53 status, indicating that the non-p53 mediated effects of p14ARF may be of greater importance (Smeds et al., 2002). Despite this, at present, there is no convincing evidence that abnormalities in p14ARF play an important role in the development of oral cancer.

2.4.3 The cip/kip family of Cyclin dependent kinase inhibitors

This family of CDKNs includes p27, $p21^{WAF1}$ and p57. These inhibit a wider spectrum of Cyclin/CDK complexes including Cyclin E/cdk2, Cyclin A/cdk2 and Cyclin D/cdk4. This results in cell cycle arrest by intervention at various points in the cell cycle (see Figure 2.3).

2.4.3.1 $p27^{KIP1}$

The cellular level of p27 is responsive to a wide variety of growth inhibitory and stimulatory signals, including TGF-β and growth factors (Polyak et al., 1994). In various cell types, including fibroblasts, epithelial cells and T lymphocytes, downregulation of p27 in response to mitogenic stimulation allows the cell to progress from a quiescent to a dividing state (Eblen et al., 1995; Malek et al., 2001). The main regulation of p27 expression is post-transcriptional, as mitogen-stimulated cells degrade p27 in an ubiquitin-dependent manner via the proteosome (Kawada et al., 1997). This complex regulation of quiescent and dividing cells demonstrates the importance of p27 in cell differentiation and development (Zhang et al., 1998a), but functions in the control of cellular senescence (Munro et al., 2001), susceptibility to apoptosis and cell adhesion have also been demonstrated (reviewed in Sgambato et al., 2000).

Many cancer studies have demonstrated poor prognosis related to loss of p27 expression (summarised in Slingerland & Pagano, 2000). The mechanism of such an effect is probably enhanced degradation of p27, although other mechanisms, such as sequestration by Cyclins D1 and D2 cannot be discounted (Bouchard et al., 1999). In HNSCC, low p27 expression in tongue tumours is associated with an increased risk of lymph node metastases and

poor overall survival (Mineta et al., 1999). This loss of p27 immunoreactivity has also been demonstrated in dysplastic oral lesions (Jordan et al., 1998).

2.4.3.2 $p21^{WAF1}$

Inhibition of several Cyclin/CDK complexes by $p21^{WAF1}$ (p21) results in cell cycle arrest. p21 is one of the main downstream effectors of p53 mediated cell cycle arrest in either G1 or G2 (Waldman et al., 1995). p21 also binds to PCNA, which may interrupt DNA replication (McCormick & Hall, 1992).

Loss of expression or mutation of p21 are infrequent in HNSCC (Ibrahim et al., 2003). Indeed, Van Oijen and co-workers found increased p21 expression in HNSCC and dysplastic tissue and this can occur in HNSCCs with no functional p53 (van Oijen et al., 1998). Others have substantiated this, by showing that p53 mutation did not necessarily lead to deregulation of downstream targets such as p21, Bax and Bcl-2, (Lavieille et al., 1998 2272). Thus, p53 independent functions of p21 may be advantageous to the neoplastic cell, including resistance to apoptosis (Javelaud & Besancon, 2002; Wang & Walsh, 1996). In dysplastic oral lesions, p21 was increased in 60% of samples, the intensity and amount of parabasal staining increasing with a more advanced histological grade (van Oijen et al., 1998). However, others have found no differences in p21 level when comparing lesions which progressed to SCC and those which did not (Hogmo et al., 1998). These conflicting findings indicate that loss of p21 is not useful as a marker of progression in HNSCC.

2.4.4 p53

The ability of the cell to delay DNA synthesis and mitosis in order to allow repair of DNA damage due to various injurious stimuli is an important mechanism in the protection of the integrity of the cell and its genome. Signals arising from DNA damage, mitotic spindle damage, hypoxia, nucleotide deprivation and oncogene activation, amongst others, represent stresses to which the cell must respond. The p53 tumour suppressor protein sits at this pivotal point in control of the cell cycle and other cellular responses to such stresses. The two main responses of the cell, namely cell cycle arrest and apoptosis, are mediated, at least in part, by the activity of p53.

p53 is a transcription factor, whose activity is regulated primarily at the post-translational level. The half-life of wild type p53 is very short under normal conditions. This is due to its interaction with the mouse double minute 2 protein (MDM2). This interaction interferes with the p53 transactivation domain, thus rendering p53 unable to assemble fully the machinery required for transcriptional activation (Oliner et al., 1993). The interaction with MDM2 also leads to the degradation of p53 by the ubiquitin-proteasome pathway, as MDM2 has been demonstrated to act as a p53-specific E3 ubiquitin protein ligase (Honda et al., 1997). One of the genes targeted by activated p53 is MDM2, thus forming the basis of a negative feedback loop (Wu et al., 1993).

Stimulation of p53 stabilisation and activation can be achieved via several signalling pathways that form the primary responses to cellular stresses (Figure 2.5). Injurious stimuli, such as ionising or ultraviolet radiation and certain chemotherapeutic agents, such as adriamycin, elicit a rapid induction of p53 activity via non-transcriptional means. The DNA damage caused by such agents is relayed via signalling pathways such as ataxia telangiectasia-mutated (ATM) and ataxia telangiectasia-related (ATR) proteins (Enoch & Norbury, 1995; Tibbetts et al., 1999). These signal via the protein kinases Chk2 and Chk1 (Liu et al., 2000) respectively, with the result being phosphorylation of p53 (Hirao et al., 2000; Liu et al., 2000). A schematic representation of these pathways is seen in Figure 2.5. Other cellular stresses are mediated via other intracellular signalling pathways (reviewed in Giaccia & Kastan, 1998). For example, deregulated expression of various oncogenic signals, such as E1A, Ras, Myc and β catenin leads to a p53 response via the induction of p14ARF (de Stanchina et al., 1998; Zindy et al., 1998). ARF can bind to MDM2, reducing binding to p53 and thus inducing p53 stabilisation (Honda & Yasuda, 1999) Thus, the response of p53 to oncogenic stimuli may be an important direct tumour suppressive role.

The phosphorylation of p53 is one of the major mechanisms for regulation of its activity. The protein can be phosphorylated on many amino acid residues, including serine 15 and 20, which are the main targets for the ATM/ATR pathways (Dumaz & Meek, 1999; Fiscella et al., 1993; Tibbetts et al., 1999). These residues lie in the transactivation domain that regulates binding to MDM2. Phosphorylation within this domain reduces affinity for MDM2 and thus

reduces p53 degradation. However, p53 activity can also be modified by acetylation (Li et al., 2002) or glycosylation of various amino acid residues (reviewed by Brooks & Gu, 2003). This may lead to increased DNA binding.

Activation of p53 induces many genes including p21 (Figure 2.5). This, at least in part, is responsible for cell cycle arrest in G1 (Waldman et al., 1995). Participation of p53 in other cell cycle checkpoints, such as at G2/M and G0/G1 has also been described, but the molecular mechanisms have not been established (Itahana et al., 2002; Taylor & Stark, 2001). The functions of p53 in replicative senescence may also be mediated via these pathways (Shay et al., 1991). This will be discussed in a subsequent section.

The other cellular response that may be elicited is apoptosis, mediated in some cells by Bax, a member of the bcl2 family of apoptosis promoters (Miyashita & Reed, 1995). Induction of other genes, such as PUMA, is also pro-apoptotic (Nakano & Vousden, 2001). The appropriate cellular response depends on the context in which p53 stimulation is achieved. If p53 signalling co-exists with stimulation of factors related to cell survival, cell cycle arrest may predominate.

2.4.4.1 p53 in cancer

Loss of p53 function is well documented in many cancers, including HNSCC. In rodents, homozygous disruption of p53 results in predisposition to malignancy, with an increased cancer risk also present in p53 heterozygous animals (Harvey et al., 1993; Jacks et al., 1994). Disruption of p53 function is often achieved by point mutation, but p53 function can also be inactivated by interaction with viral proteins such as HPV16 E6 (Shay et al., 1993). Mutation of p53 most frequently occurs in the DNA binding domain (Olivier et al., 2002), a consequence of which is reduced DNA binding and transcriptional activation capacity, in addition to inhibition of MDM2 binding. This results in stabilisation of p53, explaining the increased levels of mutated protein often reported by immunohistochemical studies. Often the remaining wild type allele also becomes deleted. Patients with mutant p53 show defects in cell cycle control and apoptosis, but also have an increased tendency to genomic instability (Shao et al., 2000; Shin et al., 2001a).

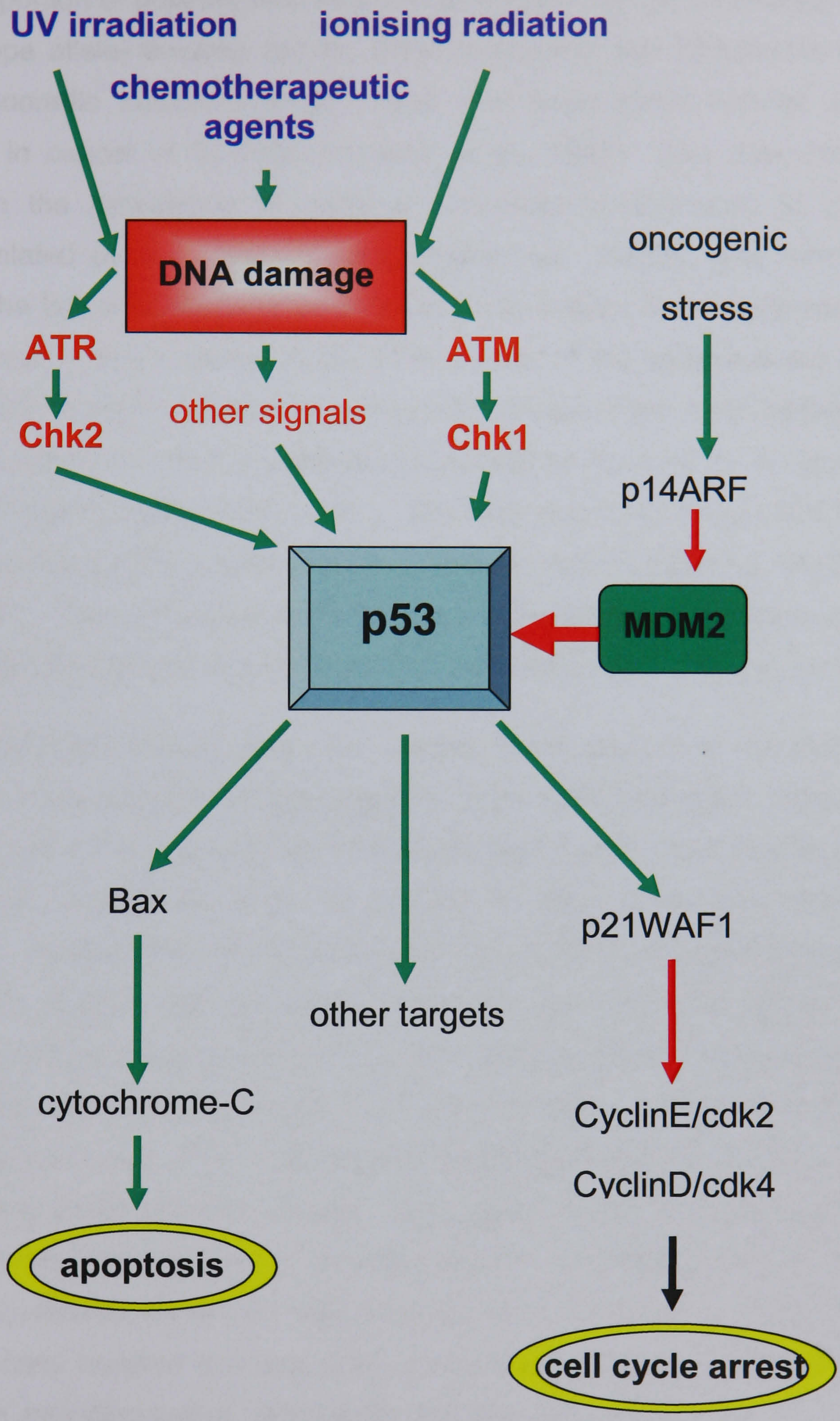


Figure 2.5 Upstream and downstream events in the p53 pathway

DNA damage signals to p53, resulting in the activation of various intracellular responses. p53 exerts its control on cell cycle progression mainly through p21 and its inhibitory effect on CyclinA/cdk4. The activity of p53 is regulated primarily by its interaction with MDM2.

Green arrows – stimulation Red arrows - inhibition

A high proportion of patients who inherit mutated p53 (i.e. are heterozygous for the wild type allele) develop cancer, often at an early age (Srivastava et al., 1990). Sporadic mutations in p53 have also been noted with an overall frequency in cancer of 50-55% (Hollstein et al., 1991). The importance of tobacco in the prevalence of guanine nucleotide transversions in p53 in smoking related cancers, such as lung, bladder and HNSCC, was mentioned earlier. The low prevalence of p53 mutations in lesions from never-smokers bears witness to this (Lazarus et al., 1995). Most of the mutations are in the DNA binding domain encoded by exons 5-8. These have been termed the "hotspot", regions in which mutations are claimed to account for in excess of 90% of the total (Hollstein et al., 1991). We have previously shown that two of seven immortal dysplasia cultures have mutations outwith this area (McGregor et al., 2002). Thus, emphasis solely on sequencing exons 5-8 in many studies may have resulted in under-reporting of the prevalence of p53 mutation *in vivo*.

The link between p53 mutation and protein stabilisation has resulted in a plethora of immunohistochemically based studies of p53 malfunction. However, the complexity of the regulation of p53 protein level makes interpretation of this data difficult, particularly when no attempt is made to analyse the DNA sequence. Additionally, the large number of variables in methodology and assessment of p53 staining, together with the use of many different p53 antibodies, makes drawing clear conclusions problematic. A poor correlation of p53 mutation with increased immunoreactivity has been demonstrated in some studies (Burns et al., 1993). The effect of particular mutations on p53 protein structure may explain this, for example, the creation of a premature stop codon, or a frameshift, which creates a truncated protein. In addition, some tumours express elevated levels of p53 without mutation (Bradley et al., 2001). Such difficulties have resulted in many, often contradictory studies relating p53 level to outcome, including overall survival and freedom from recurrence.

The reported frequency of p53 mutation in HNSCC varies from study to study and is dependent on the methodology utilised. Most of these studies confine sequencing to exons 5-8/9 (Kashiwazaki et al., 1997; Koch et al., 1996; Matsuda et al., 1996; Rowley et al., 1998). However, in a study by Kropveld and co-workers, p53 was mutated in 91% of HNSCC, with 33% of these outwith

the core domain of exons 5-8/9 (Kropveld et al., 1999). This is considerably higher than in most other studies. Overall, p53 sequencing consistently demonstrates that 50-55% of HNSCC have mutations in exons 5-8/9, with approximately 20% present in the other exons (2-4 and 10-11). This gives an approximate overall prevalence of p53 mutation in HNSCC of 75%. The mutations found in the primary tumours have also been found in metastatic lesions (Burns et al., 1994; Tabor et al., 2002b), indicating that p53 mutated clones may be positively selected during HNSCC progression. The difficulties outlined above with immunohistochemical prognosis have resulted in contradictory conclusions about p53 (reviewed in (Nylander et al., 2000), but mutations, particularly in the DNA binding domain, have been associated with poor prognosis (Mineta et al., 1998; Yamazaki et al., 2003).

The prevalence of p53 mutations in some precancerous lesions of the head and neck indicates that this may be an early step in HNSCC carcinogenesis (Boyle et al., 1993). The reported rate of mutation is less than that for invasive disease, with the highest rate recorded in severe dysplasia within erythroplakia (50%, Qin et al., 1999). Other workers have even suggested that p53 mutation is the initiating event in HN carcinogenesis (Braakhuis et al., 2004a). However, in a study of sequential biopsies from patients who eventually developed HNSCC within a dysplastic lesion, p53 mutations (in exons 5-8) were only found in pre-invasive lesions in two of ten patients (Shahnavaz et al., 2000). The conclusion drawn from this was that p53 mutations occur late in disease progression, but this assumes that any p53-mutated clone present in the tissue would be present in the portion of the premalignant lesion actually analysed. Tissue heterogeneity within the epithelium, as demonstrated later in this thesis, probably dictates that this is not necessarily so.

Increased p53 immunoreactivity has been described in dysplastic oral lesions, often variably related to the grade of dysplasia (Lippman et al., 1995; Sauter et al., 1994; Shin et al., 1994a). This may be a genuinely early change, as increased immunoreactivity is seen in all grades of dysplasia (Regezi et al., 1995). Alterations in the spatial distribution of p53 staining have been suggested as of more value than staining intensity. The presence of suprabasal p53 staining in high-grade dysplasia is associated with an increased risk of malignant transformation (Cruz et al., 2002). However, some groups have found

that the link between p53 expression and HNSCC carcinogenesis is less robust (Murti et al., 1998) and that the link may reduce with long term follow-up (Lee et al., 2000). However, the prognostic value of the combined p53 and p16 status is more powerful than for either alone (Gallo et al., 1997).

2.5 Evasion of apoptosis

As seen earlier in the discussion of p53 function, one of the possible responses of the cell to damage and stress, is the activation of programmed cell death or apoptosis. Apoptosis is an energy dependent process of "cell suicide", and can be induced by activation of receptors on the cell surface, such as FAS and TNF-α receptor (the "extrinsic" pathway, Wajant, 2002). Additionally the DNA damage signalling pathways via p53 result in the upregulation of pro-apoptotic mediators, such as Bax (an example of the "intrinsic" pathways Wajant, 2002). Bax in turn stimulates mitochondria to release cytochrome C (Jurgensmeier et al., 1998). Signalling via FAS, or release of cytochrome C, results in the activation of the caspase cascade, via the initiation caspases, such as caspase 9 (Figure 2.6). The product of this is activation of several effector caspases, such as caspases 3, 6 and 7 (Slee et al., 2001). These effect the cell death programme, by destruction of the organelles and dissolution of the genome (reviewed in Okada & Mak, 2004).

The abrogation of apoptosis in cancer generally was confirmed by the finding that the oncogene bcl 2, which is overexpressed by translocation in follicular lymphoma, has profound anti-apoptotic effects (Tsujimoto et al., 1984a; Tsujimoto et al., 1984b). It is also a transcriptional target of p53, with activated p53 reducing bcl 2 expression (Haldar et al., 1994). Overexpression of bcl 2 has been described in the early stages of various epithelial malignancies, including lung, breast and bladder, but this is often followed by loss of expression in advanced disease (Ghosh et al., 1999; Mauri et al., 1999; Nakopoulou et al., 1998). However, whilst this has not been found to be a consistent effect in HNSCC (Piattelli et al., 2002), a general trend towards a reduced bax/bcl 2 ratio has been found in tumour samples, but not in pre-invasive lesions (Lavieille et al., 1998).

The most common defect in pro-apoptotic signalling reported in HNSCC is loss of p53 function by mutation. Mice with p53 deficiency show defects in apoptosis *in vivo* (Lotem & Sachs, 1993). In terms of human tumour biology, this may manifest itself as resistance to chemotherapy. There have been clear links made between specific p53 mutations and resistance to doxorubicin in breast cancer (Aas et al., 1996), and response to chemotherapy in HNSCC (Cabelguenne et al., 2000).

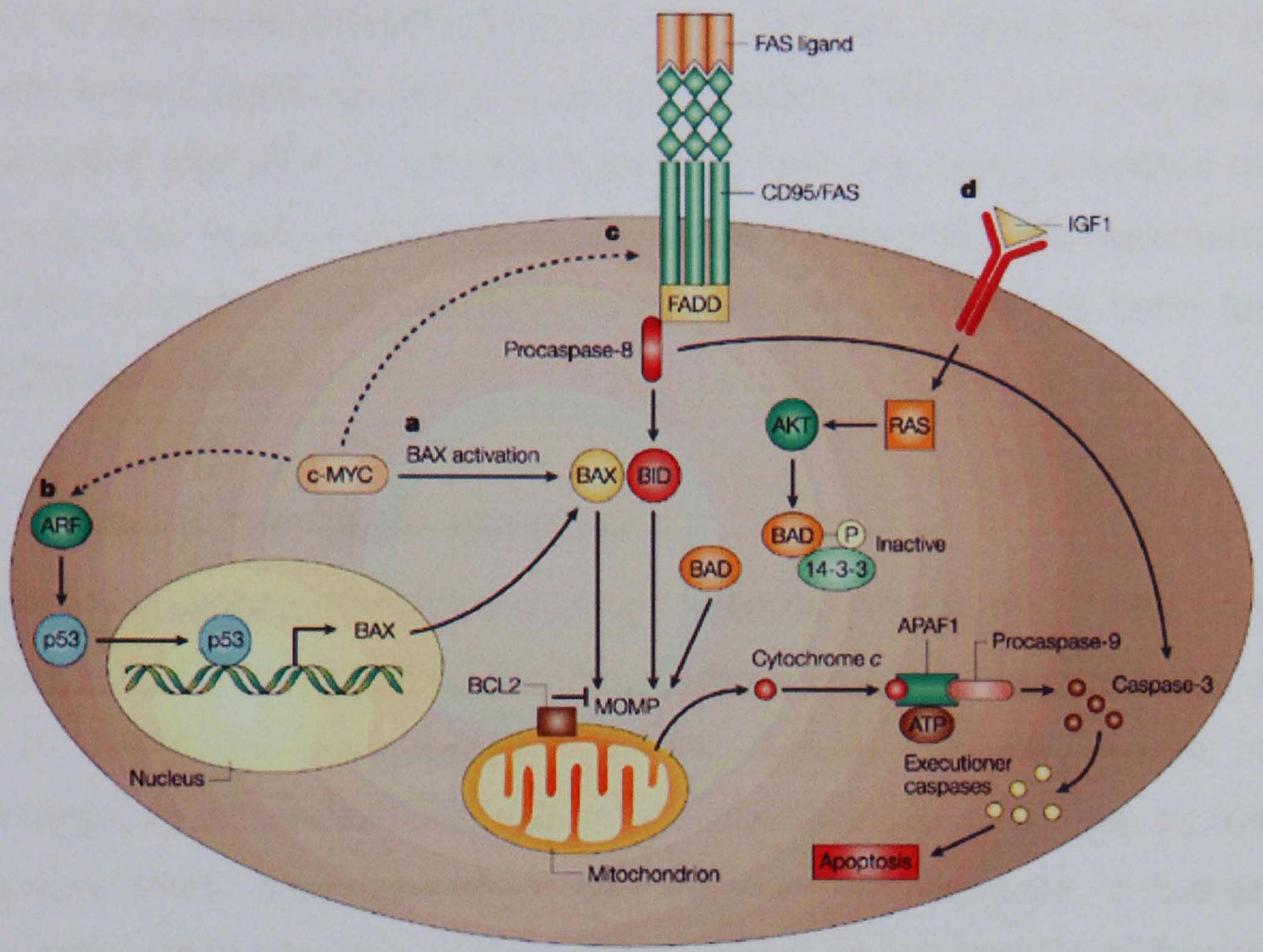


Figure 2.6 Apoptosis – an overview

The graphic shows some of the various pathways to apoptosis including signalling via p53 (DNA damage response), FAS receptor and c-myc. The pathways converge on the release of cytochrome c from mitochondria. Once released, cytochrome c interacts with apoptotic protease activating factor 1 (APAF1) and procaspase 9. In the presence of ATP, caspase 9 is activated, leading to the activation of downstream effector caspases. Survival signals, such as those mediated by IGF1 may reduce the pro-apoptotic signalling in some pathways.

This figure has been reproduced from (Pelengaris et al., 2002).

2.6 Limitless replicative potential

The genetic changes described above allow proliferation of cancer cells outwith the strict controls placed on normal cells. Such unrestricted growth should allow tumours to develop unhindered. However, there are further multiple barriers to the limitless proliferation of cells. Hayflick originally demonstrated that cells have a finite replicative potential (Hayflick, 1965). The concept of the "replicometer" was then introduced (Hayflick, 1997), this being a method for the cell to count the number of divisions and cease dividing after the maximum limit had been reached. This cessation of growth and division has been termed replicative senescence.

2.6.1 Barrier 1: Replicative senescence

Normal diploid embryonic cells, including fibroblasts and epithelial cells undergo approximately 40-70 population doublings before entering the stable, but viable state of replicative senescence (Hayflick, 1965). There is now much accumulated evidence that senescence is controlled, at least in part, by attrition of telomeric DNA. The observation that telomeres shorten both in vivo and in vitro suggests that telomeric attrition is the most likely "counting" mechanism for cell divisions (Allsopp et al., 1995). Other cellular clocks have been proposed, including a progressive increase in DNA methylation as senescence approaches (Machwe et al., 2000). The cellular signals that elicit senescence include activation of the DNA damage response (d'Adda di Fagagna et al., 2003).

Telomeres are repetitive DNA sequences (TTAGGG) on the ends of chromosomes. A complex of proteins binds the telomeric DNA, which is important in the maintenance of the complex structure of the telomere (Figure 2.7 A). The "end replication problem" dictates that some of these repeats are lost when DNA is copied (Levy et al., 1992), although larger portions may also be lost through homologous recombination at the telomere (Wang et al., 2004). Telomerase was first described in yeast (Greider & Blackburn, 1985). It is a holoenzyme complex, consisting of a reverse transcriptase component (TERT, on chromosome 5p15.33) and a RNA template (TERC/TR) and is responsible

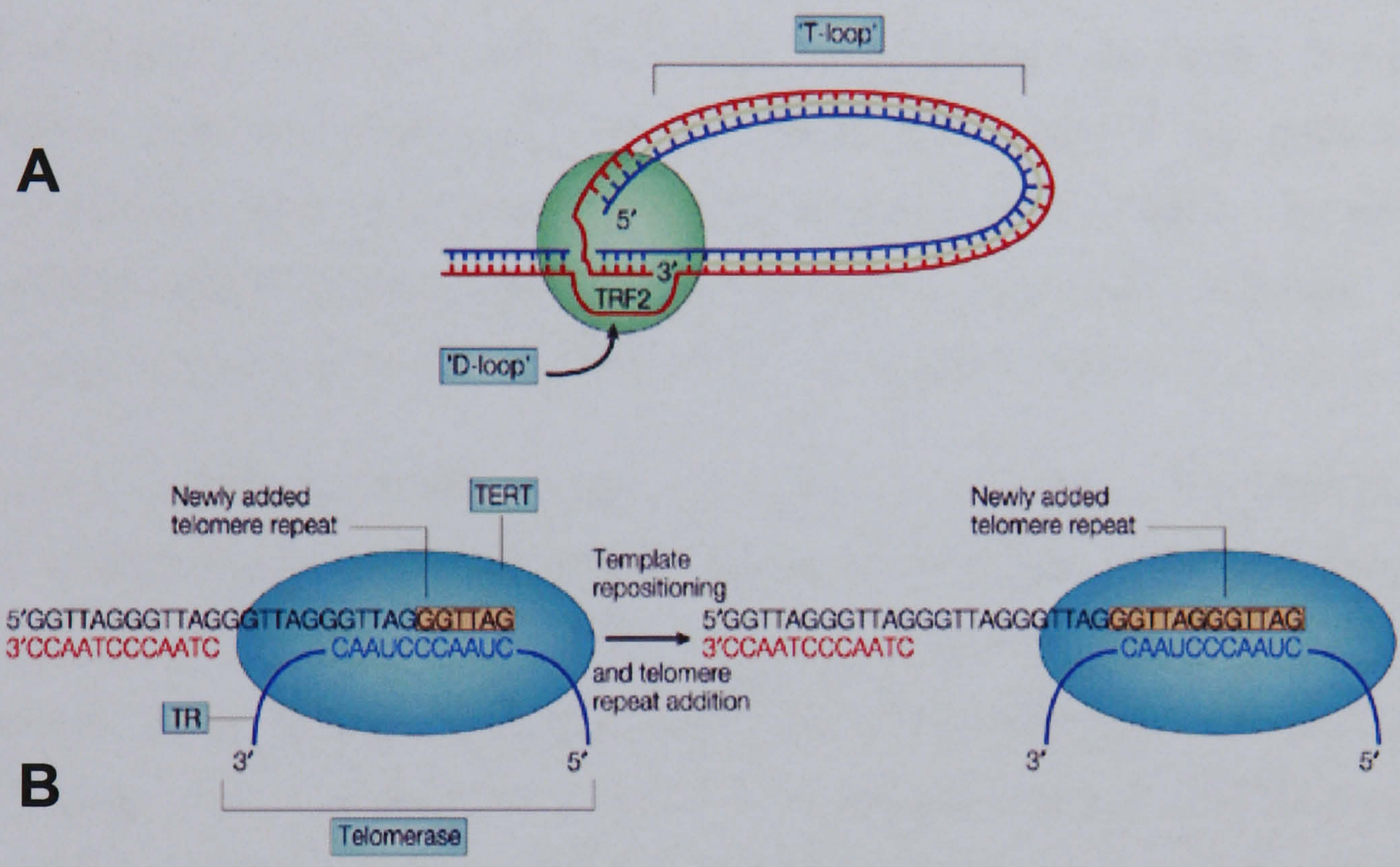


Figure 2.7 Telomere structure and the role of telomerase

A: Schematic diagram of the structure of the telomere, showing the formation of D and T loops, in conjunction with various telomere associated proteins. These act to protect the telomeric end of the chromosome.

B: The action of telomerase. hTERT catalyses the addition of new telomere repeats, with hTR as the template.

Reproduced from Mathon and Lloyd, 2001 (Mathon & Lloyd, 2001)

for synthesis of the TTAGGG repeats on the end of chromosomes in germline cells (Figure 2.7 B).

In normal somatic tissues, telomerase activity is generally absent, but is present at low levels in the proliferative (stem) cells in continually renewing tissues, such as epithelia (Harle-Bachor & Boukamp, 1996; Kolquist et al., 1998). In these cells, telomerase expression may depend on the proliferative state of the tissue.

Expression of hTERT and hTR have also been described in the basal cells of normal oral mucosa (Fujimoto et al., 2001). Such activity may retard, but does not abolish telomere attrition (Oh et al., 2004) and is lost if the cells enter terminal differentiation or a quiescent state (Sharma et al., 1995). As normal oral keratinocytes approach senescence, telomerase expression reduces. This may be due to hypermethylation of the hTERT promoter (Shin et al., 2003).

The normal regulation of telomerase expression is complex. Transcription of hTERT is modulated by multiple signals including members of the Myc family of transcription factors, Sp1 (Park et al., 2001), and human papilloma virus E6 (Klingelhutz et al., 1996). It is also postulated that alternative splicing of the hTERT gene may contribute to control of telomerase activity (Ulaner et al., 2000), but this has not been confirmed under physiological conditions (Kotoula et al., 2002; Liu et al., 1999). Repression of hTERT expression may be under the control of various, as yet unidentified transcriptional repressors, such a gene from a possible candidate region on chromosome 3p (Cuthbert et al., 1999). Rb and E2F may also have hTERT repressive activity, either directly or indirectly (Crowe & Nguyen, 2001).

In cell culture, it has been demonstrated that telomerase activity is closely related to hTERT expression and that in alterations of telomerase activity, such as those mentioned above in relation to senescence, hTERT reduces, whilst hTR remains constant (Shin et al., 2003). However, in a mouse model, haploinsufficiency of hTR disrupted telomere maintenance (Chiang et al., 2004). *In vivo* studies have also shown hTR to be upregulated in some solid tumours, including cervical carcinoma and non-small cell lung carcinoma (Soder et al., 1998; Soder et al., 1997). Additionally, the coordinate upregulation of hTR and hTERT has been demonstrated in many tumours *in vivo* (Stanta et al., 1999), as

has hTR downregulation upon proliferation in the small number of telomerase-positive cells in normal tissue (Sugihara et al., 1999). This clearly supports a role for hTR expression in the regulation of telomerase activity *in vivo*. The mechanisms of regulation of hTR expression have not been elucidated.

Ectopic expression of TERT in human diploid fibroblasts (HDFs) is sufficient for immortalisation. This indicates the importance of telomere dependent pathways in the establishment of barriers to growth in this type of cell. However, this does not apply to mammary epithelial cells or keratinocytes, both of which require additional alterations to allow immortalisation to occur (Muntoni et al., 2003). The additional alterations that are necessary in human oral keratinocytes most likely include loss of p16 expression. In one mortal cell culture, which had lost expression of p16, ectopic expression of hTERT was sufficient to result in immortalisation, without pressure toward p53 mutation. Mortal cultures which had intact p16 expression, were not immortalised (Muntoni et al., 2003).

As mentioned earlier, p16 has been suggested as playing a central role in the establishment of replicative senescence. Recent elegant investigations in fibroblasts have indicated that p16 does not have an essential role in fibroblast senescence (Brookes et al., 2004; Herbig et al., 2004), although this is still disputed (Bond et al., 2004). More importantly, the experiments demonstrated that the mechanism by which senescence is achieved could vary even between cells within the same culture (Herbig et al., 2004). Thus in terms of senescence, it seems likely that individual cells may differ in their response to multiple signals, resulting in the development of mosaic cultures. Senescence triggered by coordinate upregulation of p14 and p16 has been reported in murine fibroblasts, but a role for p14 in keratinocyte senescence has not been established (Munro et al., 1999).

In human keratinocytes, senescence is a two-stage phenomenon, with initial replicative arrest mediated by p16, and its effect on the Rb pathway (Kiyono et al., 1998; Loughran et al., 1994). This has been termed mortality stage 0 (M0). The upstream pathways are unclear, but roles for loss of expression of the transcription factor Id1 (Alani et al., 2001) and increase in the expression of 14-3-3σ have been suggested (Dellambra et al., 2000; Rheinwald et al., 2002). The rise in p16 expression may also be mediated by telomeric attrition (Jacobs

& de Lange, 2004), but the effector pathways are yet to be fully elicited (Zheng et al., 2004). Some groups have suggested that the accumulation of p16 in keratinocyte cell culture is related to inadequate culture environment and that only co-culture with feeders prevents the accumulation of p16 (Ramirez et al., 2001). This has not been borne out by experimentation in mortal cells in our laboratory as, despite co-culture with feeders, the accumulation of p16 still occurs (McGregor et al., 2002).

Once the growth arrest mediated by p16 has been overcome, the continued telomeric attrition results in the activation of p53, as described earlier. This is termed mortality stage 1 (M1) (Figure 2.8). Shortened telomeres activate p53 via ATM, as the uncapped telomere is recognised in a similar manner to a double strand DNA break. A single short telomere may be sufficient for this pathway to be activated (Hemann et al., 2001), and the effect may be mediated by alterations in the binding of telomere-related proteins to shortened telomeric DNA (Karlseder et al., 2002). It is also possible that senescence can occur in the absence of telomere shortening. Some types of cell, including certain keratinocytes, may be telomerase positive and yet these cells still undergo senescence (Kang et al., 1998). The main effector in this case was reported to be p16 (Kang et al., 2004). However, in many instances these results may merely reflect whether M0 or M1 is being investigated.

2.6.2 Overcoming senescence

Escape from senescence can occur in many ways, largely dependent on the cell type, but most require alterations at some point in the p53 and Rb pathways (Shay et al., 1991). Fibroblasts require loss of signalling via Rb, but not the loss of p16 function per se (Wei et al., 2003). This may be because fibroblast senescence is maintained primarily by p53 (Beausejour et al., 2003). Other cell types, such as normal human mammary epithelia cells, may spontaneously escape senescence (Romanov et al., 2001). In human oral keratinocytes, inactivation of p16 is invariably associated with the immortal phenotype in HNSCC cell cultures (Munro et al., 1999). Inactivation of the DNA damage signal is also required and this is most commonly acquired by mutation of p53. Cells that have escaped senescence proliferate until a second major replicative barrier is reached, namely “crisis”.

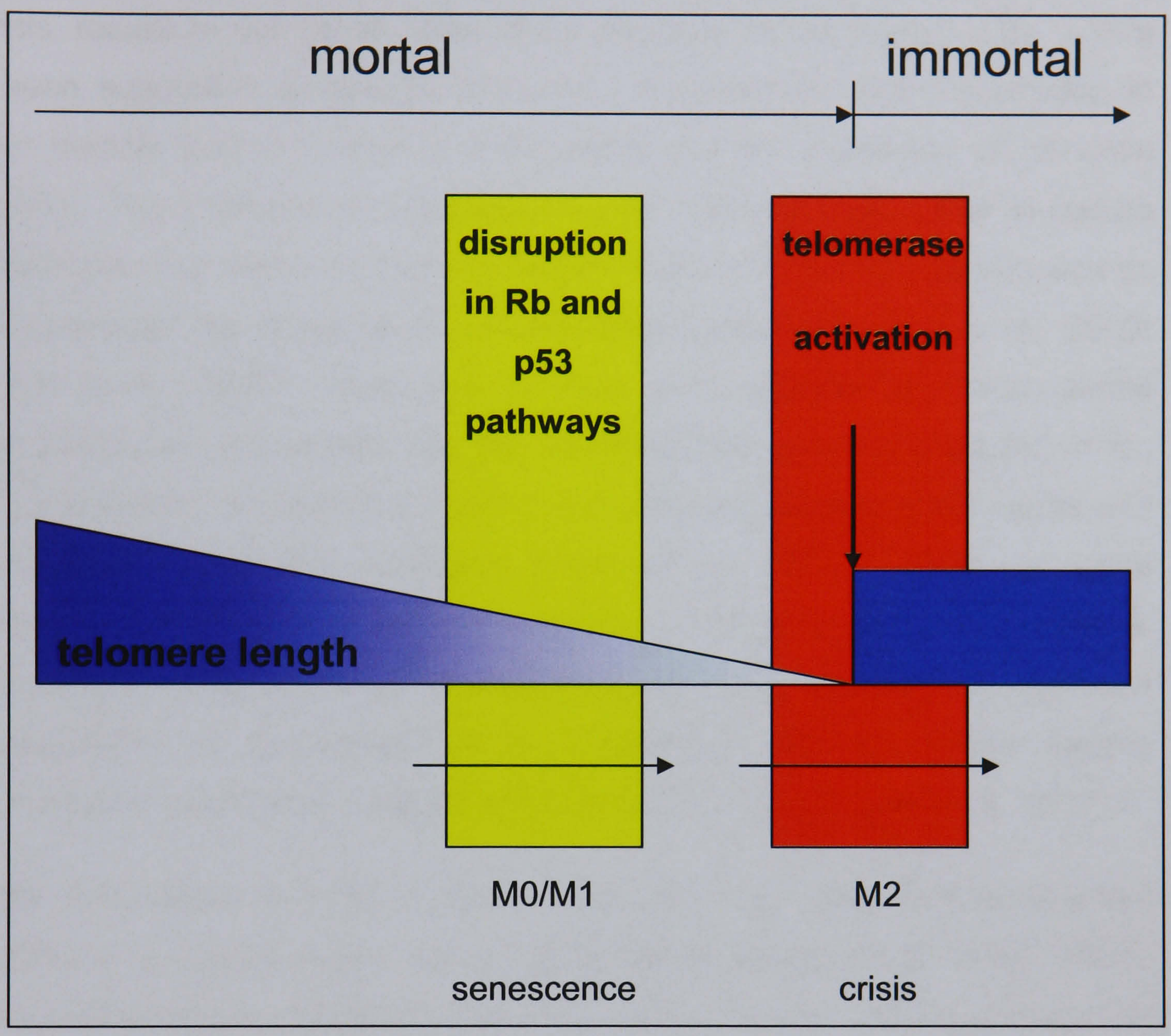


Figure 2.8 Barriers to immortalisation

Schematic representation of the relationship of telomere length and passage through senescence and crisis. Human cells must surmount both replicative senescence and crisis to become immortal. Replicative senescence is controlled by the actions of the pRb, p53 and telomere maintenance pathways. Telomere stabilisation permits cells to avoid crisis and become immortal

Figure adapted from Hahn, 2003 (Hahn, 2003)

2.6.3 Barrier 2: Crisis and immortalisation

Following further cell division, multiple telomeres in each cell become critically short and uncapped. This generates dicentric chromosomes and genomic instability (Ducray et al., 1999). This barrier to indefinite growth, known as M2 or crisis, results in cell death rather than cell cycle arrest (Figure 2.8). Crisis has been suggested to precipitate tumour development and progression in animal models and in human breast cancer via the promotion of genomic instability. The presence of anaphase bridges in mitotic cells, either in culture or in tumours, has been used as a surrogate marker of telomere dysfunction as it demonstrates the presence of chromosome fusions (Gordon et al., 2003; Rudolph et al., 2001). Moreover, in mice, such imbalanced chromosomal translocations are associated with the immortalisation process (Rudolph et al., 1999) and cancer (Artandi et al., 2000), including the erosion of telomeres and the development of p53 mutations (Shao et al., 2000). Thus, in some circumstances, variants emerge from crisis to become immortal. This is accomplished by the correction of telomere dysfunction, by either up-regulation or reactivation of telomerase or by alternative methods, which involve recombination (alternative lengthening of telomeres, ALT) (Bryan et al., 1997).

Despite telomerase activity in many tumours, it is clear that telomerase activation is a double-edged sword (reviewed in Masutomi & Hahn, 2003). Absence of telomerase activity results in a limited lifespan, via the activation of senescence, but if senescence is bypassed, this can result in genomic instability. However, telomerase activation reduces chromosomal instability by stabilising the telomere, but allows cells to become immortal. Once telomerase has been activated, the telomeres appear to be maintained at a very short setting in some tumours. This allows for a measure of continued genomic instability (Chin et al., 2004; Gisselsson et al., 2001; Gordon et al., 2003).

Expression of telomerase is increased in HNSCC, both in cells derived from dysplastic oral lesions and from tumours (McGregor et al., 2002; Parkinson et al., 1997). The level of activity is related to tumour grade, as the highest levels have been demonstrated in poorly differentiated HNSCC (Kannan et al., 1997). Increases in both hTERT and hTR expression have been described (Downey et al., 2001 ; Kim et al., 2001; Zhang & Zhang, 1999). Whilst increased

telomerase activity has been noted in dysplastic oral lesions (Mao et al., 1996a), reports linking the grade of dysplasia with telomerase activity have been variable (Miyoshi et al., 1999; Mutirangura et al., 1996; Zhang & Zhang, 1999). In most studies, a proportion of the tumours are telomerase-negative. These findings suggest that the immortal and invasive phenotypes may be acquired independently in HNSCC development. This concept will be explored more fully below and later in this thesis

2.6.4 ALT

Whilst many tumour types have high levels of telomerase activity, some do not. There may be several reasons for this. However, the presence of immortal, telomerase negative cell lines is indicative of the existence of other telomere lengthening mechanisms (Bryan et al, 1995). These cells have very long and heterogeneous telomeres, and there is evidence of elongation of these telomeres by the alternative lengthening of telomeres (ALT) mechanism (Bryan et al., 1997), which involves intratelomeric recombination and copy templating for the synthesis of telomeric DNA (Dunham et al., 2000). Whilst ALT has been shown to occur in oral keratinocytes under certain circumstances *in vitro* (Opitz et al., 2001), there is presently no evidence that ALT occurs in HNSCC carcinogenesis (Henson et al., 2002; Loughran et al., 1997).

2.6.5 Evidence for senescence and immortalisation in vivo

In studies from the Beatson Institute for Cancer Research, about 60% of primary HNSCCs are immortal, as are all the HNSCC recurrences (although the latter are more limited in number) (Burns et al., 1993; Edington et al., 1995). Numerous studies link the process of immortality with genetic instability. p53 mutations, p16 loss, LOH and aneuploidy are all characteristics of cells that have escaped senescence and become immortal (Edington et al., 1995; Loughran et al., 1997). It is very unlikely that the acquisition of the immortal phenotype is merely a culture phenomenon since the genetic signature of immortal HNSCC cell lines is also found in a large subset of HNSCC tumours and dysplasias *in vivo* (Loughran et al., 1997; Mao et al., 1996b; Rosin et al., 2000; Weber et al., 1998). In addition, the presence of unbalanced chromosome translocations and anaphase bridges in over 80% of HNSCC

tumours *in vivo* is suggestive of their escape from senescence (Gordon et al., 2003).

Despite, the importance of immortality in the generation of the majority of HNSCC, it is becoming clear from work conducted in the Beatson Institute and others that a subset of tumours may develop into invasive carcinomas without acquiring immortality. Furthermore, other published data on uncultured tumour samples reveal minor subsets of oral carcinomas that have no detectable LOH (Field et al., 1995) or unbalanced translocations (Weber et al., 1998), or are still diploid (Sudbo et al., 2003). The conclusion that the ability of SCCs to establish as cell lines in culture (i.e. become immortal) correlates with poorer survival is supported in the literature (Shimada et al., 2003). This suggests that some primary SCCs can still be mortal in vivo; whereas in others an immortal population has developed by further mutation and this is responsible for local recurrence and metastasis. Offner et al found that disseminated tumour cells in bone marrow from a variety of primary tumour types (presumed to be micro-metastases) rarely have p53 mutations and are therefore unlikely to be immortal, unlike the primary tumours; in contrast, p53 mutations in the primary tumour appear to favour lymphatic dissemination of lung carcinoma cells (Offner et al., 1999).

The existence of mortal SCCs is supported by *in vivo* data that some HNSCC are telomerase negative (Mao et al., 1996a) and, as mentioned above, there is currently no evidence for ALT mechanisms in head and neck cancer (Henson et al., 2002). Additionally, subsets of HNSCC have a low level of fractional allele loss and develop without the characteristic genomic changes (LoH at 3p, 9p and 17p) that may underpin the development of immortality (Nunn et al., 1999). Others have extended this observation to bladder cancer, showing that all superficial transitional cell carcinomas (TCCs) were mortal whereas invasive TCCs spontaneously bypassed senescence by acquiring genetic alterations involving p16/pRb loss and an additional alteration (p53 mutation, changes at chromosome 20q, 3p or 8p) (Yeager et al., 1998). Another study on glioblastoma divided the tumours into three groups: telomerase positive, telomerase-negative-ALT positive and telomerase negative ALT negative; they concluded that the third group of tumours do not have a telomere maintenance mechanism of any kind and that these tumours appeared to have a better

prognosis (Hakin-Smith et al., 2003). Given that all data available shows that telomere erosion denies mammalian cells an indefinite lifespan (Bodnar et al., 1998; Niida et al., 1998), this data seems persuasive evidence for the existence of mortal tumours *in vivo*.

Like HNSCCs, both mortal and immortal dysplasias exist, though the balance is in favour of the former (60:40) (McGregor et al., 2002; McGregor et al., 1997). Of the 19 dysplasia biopsies for which we have derived primary cultures, 6 of 7 of the immortal cultures showed four molecular changes: loss of $p16^{INK4A}$ and retinoic acid receptor-beta (RAR-β) expression, p53 mutations and high telomerase activity; whereas 11 of 12 mortal cultures lacked these changes (McGregor et al., 2002; McGregor et al., 1997; Muntoni et al., 2003). The two exceptions have an intermediate phenotype (McGregor et al., 2002; McGregor et al., 1997). No evidence of HPV infection was found in any of the cultures. An important conclusion from this evidence is that acquisition of immortality and invasiveness may be independent events in cancer progression, since some dysplasias are immortal and, conversely, some carcinomas are mortal.

2.7 Genomic instability

One further overarching feature may hold all these alterations together and act as an enabling backdrop upon which the other abilities may appear. This is genomic or chromosomal instability. Genomic instability is characterised by the occurrence of losses and gains of chromosomal segments or even whole chromosomes. A large proportion of human tumours demonstrate abnormalities in both the number and structure of their chromosomes (Hittelman, 2001).

2.7.1 How does genomic instability arise?

Chromosomal instability may arise by multiple mechanisms. The basis of instability consequent to telomeric attrition has been discussed above. Abrogation of the DNA damage signal, by loss of signalling via p53 or intermediates within the p53 DNA damage pathway (Figure 2.5), results in unrepaired damage and this may lead to chromosomal aberrations (Shao et al., 2000). Loss of the DNA damage recognition signal after the loss of p53, together with telomeric attrition, results in the cell entering crisis (Feldser et al., 2003). The fusion of chromosome ends, seen in tumours as anaphase bridges, drives chromosomal instability via breakage-fusion-bridge cycles.

Amongst the other mechanisms, which may operate in certain tumours, are chromosomal segregation defects (Minhas et al., 2003). The cellular machinery active in mitosis is complex and alterations detected in tumour cells include spindle abnormalities, centrosome amplification (Mayer et al., 2003) and abnormal cytokinesis (reviewed by Gollin, 2004). Amplification of certain genes which control mitotic spindle activity has been described. One such important gene is Aurora A (serine threonine kinase 15). Amplification of this gene has been associated with centrosome amplification and the formation of multipolar spindles (Jeng et al., 2004; Zhou et al., 1998). In mammary carcinogenesis, amplification of Aurora A has been associated with the transition from in situ disease to invasive carcinoma (Hoque et al., 2003).

Investigations in colorectal cancer have demonstrated that defects in single genes can result in genomic instability (Rajagopalan et al., 2003). This in turn

leads to further loss of genomic material, as assessed by loss of heterozygozity (LoH).

2.7.2 Karyotypic abnormalities

The largest scale abnormality seen in genetically unstable cells is karyotypic alteration, such as tetraploidy and aneuploidy. These may arise from non- or partial disjunction of cells in mitosis. Aneuploidy has been reported in many cancers, reflecting the complex karyotypes that may result from genomic instability. The presence of aneuploid cells in HNSCC carcinogenesis has been known for some time (Munck-Wikland et al., 1994; Munck-Wikland et al., 1992). The presence of aneuploidy in oral tissues may even precede the development of a clinically or histopathologically evident lesion (Ai et al., 2001; Sudbo et al., 2001b). Analysis of modal chromosome number in a panel of HNSCC cultures indicated that almost all, regardless of whether mortal or immortal, showed some karyotypic abnormality (Edington et al., 1995). This demonstrates the likelihood that multiple pathways to alterations in the karyotype exist, not merely those that involve passage through crisis.

In recent work using DNA image cytometry to assess the DNA content of nuclei from HNSCC and various dysplastic lesions, Sudbo and colleagues have shown that leukoplakias from the oral cavity containing aneuploid cells are more likely to develop into tumours (Sudbo et al., 2001a). However, the risk of progression to SCC did not increase further as the proportion of aneuploid cells in the sample increased. Such lesions are also more likely to be aggressive and spread to other sites, compared to diploid or tetraploid leukoplakias (Sudbo et al., 2001a; Sudbo et al., 2004). However, the malignant transformation rate in these studies is high, compared to other reports of leukoplakia progression the HNSCC. Thus, it is likely that the study population is biased towards lesions that were clinically or histologically more advanced. However, these finding are clearly important and merit careful confirmation in independent patient groups with a prospective study design.

2.7.3 Comparative genomic hybridisation and loss of heterozygozity

Comparative genomic hybridisation (CGH) is a powerful tool for the analysis of gains and losses across the whole genome and has therefore been widely used

in the analysis of tumours. It appears that particular types of cancer have characteristic (although not ubiquitous) patterns of genetic alteration. This may now be conducted on a high throughput, genome-wide scale using array CGH, which combines microarray technology with the ability to explore chromosomal changes. This technique allows detection of single copy number changes. Loss of heterozygozity (LoH) indicates consistent loss at specific, predefined loci. This tool has been widely used but is less powerful as known markers must be used to investigate particular areas of interest in the genome.

The areas which are consistently altered in HNSCC are gains on 3q, 5p 8q and 11q13, and losses on 3p, 9p and 17p (Bockmuhl et al., 1996; Brzoska et al., 1995; Hermsen et al., 1997; Roz et al., 1996; Soder et al., 1995; van der Riet et al., 1994), although this varies by subsite to some extent (Huang et al., 2002). Late-stage and high grade tumours have large numbers of gains and losses (Hermesen et al., 2001 Bockmuhl, 1996). In general, SCCs have more alterations than dysplastic, non-invasive lesions (Weber et al., 1998). LoH has also been associated with poor prognosis, particularly at distinct regions within 3p (Partridge et al., 1996) or within 22q (Ashman et al., 2003), but little evidence of specific patterns of abnormality related to risk of metastasis *per se* (Patmore et al., 2004).

Loss of heterozygozity at 3p, 9p and selected other loci, including 17p has been associated with development of SCC, even within lesions exhibiting only mild dysplasia (Lee et al., 2000; Mao et al., 1996b; Rosin et al., 2000). These areas have been mentioned previously in relation to certain markers related to the development of HNSCC, namely p53 (17p), p16ink4a (9p21) and RAR-β (see next section) or a putative telomerase inhibitor on 3p. Other, yet unidentified tumour suppressor genes may reside in other areas that show high prevalence of LoH (Partridge et al., 1999).

Much of the evidence for field cancerization in HNSCC development has come from LoH analysis of multiple premalignant and malignant lesions (Tabor et al., 2002a). These lesions often have a common pattern of LoH, indicating a common origin, with divergence at other loci later within the field (Jang et al., 2001). Others have found that other "early" markers, particularly at 9p21 can be maintained over a large area of the mucosa (Guo et al., 2001). This analysis

can be used to detect abnormal cells in clinically normal mucosa and in the resection margins of SCC and dysplastic lesions to assess the subsequent risk of development of HNSCC (Partridge et al., 2000a; Rosin et al., 2002; van Houten et al., 2004; Zhang et al., 2001).

The large number of such publications on patterns of LoH has resulted in the development of a model of HNSCC development, much along the lines of the Fearon & Vogelstein model of colorectal carcinogenesis (Fearon & Vogelstein, 1990). This model, proposed by Califano and coworkers, is based on a progressive accumulation of genetic alterations (assessed by LoH) on comparison of normal, dysplastic oral mucosa (of varying grade) and HNSCC (Califano et al., 1996). The model is illustrated in schematic form in Figure 2.9. However, this model has certain weaknesses, including the apparently false assumption that lesions must progress through the various grades of dysplasia before development of SCC. Additionally, it is possible that the "early" alterations detected (3p, 9p, 17p) reflect the escape of the cells from the growth barrier of senescence (RAR-β, p16 and p53 loss respectively). The subsequent massive increase in LoH detected may be the result of subsequent genomic instability during "crisis", an idea supported by the much greater frequency of allele loss reported in immortal HNSCC cultures compared to mortal cultures (Edington et al., 1995). This would give rise to LOH at multiple sites, some of which may not be associated directly with alterations in any particular gene in relation to cancer progression. As described above, HNSCCs and premalignant lesions often exhibit aneuploidy, which is another feature in cells that have passed through crisis. The ongoing increase in LoH in late stage tumours may merely reflect continuing genomic instability, and be a function of the amount of time the cell has been immortal, rather than related to the disease process *per se*. In this way, immortality may be related to disease progression and poor prognosis in HNSCC.

2.7.4 Microsatellite instability

Microsatellites are areas of repetitive DNA scattered randomly throughout the genome. Alterations can occur if DNA replication is not conducted carefully. If controls on normal DNA replication are lost, as may be the case in mismatch repair defects, areas of microsatellite DNA can be added or lost. This is termed

microsatellite instability (MI). In HNSCC, MI has been demonstrated with increasing frequency as lesions progress from normal to dysplastic and finally to carcinoma (Ha et al., 2002b). The technique has also been used to detect circulating tumour cells in patients with SCC of the oesophagus (Eisenberger et al., 2003).

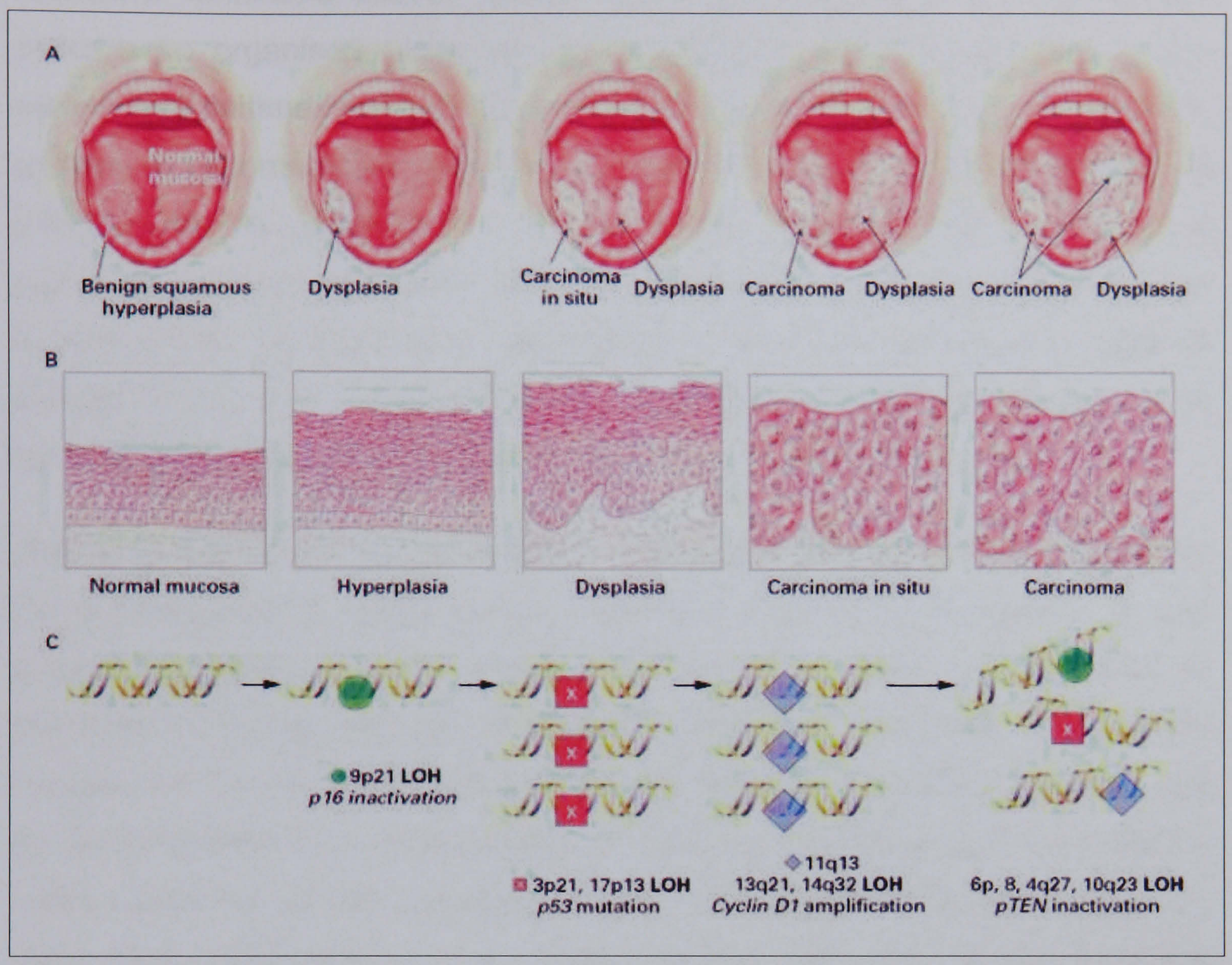


Figure 2.9 A model of the clinical, pathological, and molecular progression of oral cancer

This model was proposed by Califano et al (Califano et al., 1996).

Panel A shows the possible clinical presentation of oral cancer. Molecular approaches have yielded considerable understanding of the field cancerization hypothesis originally proposed by Slaughter (Slaughter et al., 1953). Although these lesions may have histopathological differences, as shown in B, they are often clonally related, arising from the same field of genetic alterations.

The possible histological features seen in progression from normal-appearing mucosa to invasive cancer are depicted in Panel B. Normal-appearing mucosa may harbor early genetic changes (Panel C), often with loss of 9p21 (possible inactivation of p16). Further clinical progression to dysplasia is associated with further genetic changes. Carcinoma-in-situ often harbours most of the genetic changes described in invasive carcinoma.

2.8 Retinoids and differentiation

Alterations in differentiation may also be linked with many of the genetic alterations described above. Within a stratified squamous epithelium, cells undergo an organised series of morphological changes, culminating in the presence of flattened cells on the surface of the tissue, which may, or may not, proceed to become fully keratinised. Control of the process of differentiation is complex and is accompanied by sequential expression of a number of cytoskeletal protein families, including the cytokeratins, and involucrin (Presland & Dale, 2000). Alterations in differentiation have been described in HNSCC (Kannan et al., 1994; Rheinwald, 1982), and the level of differentiation is one of the important clinico-pathological assessments of tumours in general.

Vitamin A and its analogues are known to play an important role in regulating the differentiation of epithelial cells (Kautsky et al., 1995), by binding to and activating members of the retinoic acid/retinoid receptors, two families of transcription factors that are members of the steroid hormone superfamily (reviewed in Sun & Lotan, 2002). Vitamin A is normally obtained from the diet and is transported and metabolised in the body by a family of transport proteins, cellular retinol or retinoic acid binding proteins (reviewed in Harrison & Hussain, 2001; Noy, 2000; Vieira et al., 1995). *In vivo*, there is a strong feedback mechanism tightly regulating and limiting the serum level of vitamin A at high dietary levels. However, synthetic vitamin A analogues ("retinoids") can escape this dose limiting mechanism and therefore their administration can result in much higher levels of retinoid acid metabolites *in vivo*.

Retinoids regulate gene expression and thus modulate differentiation and growth in cells (Lotan, 1996). Thus, there has been much interest in the effects that deregulation of this system may have in carcinogenesis. There are two main classes of retinoid receptor - RAR and RXR, both with several subtypes. The RAR family has three main subtypes α, β and γ plus alternatively spliced variants. Studies have shown that RARs are present in normal tissue, some showing RAR-α and RAR-γ present at all sites. Lotan and co-workers found RARβ was only prominent in normal tissue from non-keratinised intra-oral sites, despite the same group earlier finding no site difference in distribution (Lotan, 1996; Xu et al., 1994). Four isoforms of RAR-β have been described. RARβ1

and RARβ3 differ from RARβ2 in the 5'UTR and A coding region (see Figure 4.11). RARβ4 is generated by alternative splicing in exon 1 (Zelent et al., 1991). Isoform RAR-β2 has been specifically linked with mediation of retinoic acid inhibition of growth in certain cells (Toulouse et al., 2000).

In a variety of premalignant lesions the level of RARα was found to be slightly lower than in normal epithelium, but the most dramatic change consistently found during oral cancer development is the increasingly large reduction in RARβ levels on progression from hyperplasia to dysplasia to SCC (Chakravarti et al., 2001; Lotan et al., 1995; Xu et al., 1994). Loss of RARβ expression has been reported in a variety of other epithelial malignancies, including lung, prostate, and breast (Kikugawa et al., 2000; Xu et al., 1997).

In SCC and dysplastic tissue, the close correlation of keratin 19 expression and RAR-β expression is lost, representing aberrations in differentiation (Hu et al., 1991). It is also associated with acquisition of the immortal phenotype, as is loss of p16 (McGregor et al., 1997). The loss of RARβ mirrors the change from senescence to immortality in culture and work in our laboratory has shown that this is an early event in the development of dysplastic lesions (McGregor et al., 1997). However, Lee and coworkers found that loss of RARβ gave a SCC risk ratio of 0.9, suggesting that, loss of RAR-β alone was not necessarily associated with poor prognosis. This may be due to the modulatory effect of other receptors including RAR-γ (Lee et al., 2000).

The mechanism of the loss of RARβ expression does not seem necessarily to be deletion, since expression of RARβ receptors can often be re-induced by retinoids (Kurie et al., 2003; Lotan et al., 1995). However, many studies have implicated epigenetic silencing by promoter methylation in the abolition of RARβ expression (Virmani et al., 2000), and it has also been linked with aberrant histone deacetylation (Sirchia et al., 2000; Suh et al., 2002). Reduction in RAR-β expression in some immortal dysplasia cell cultures can be reversed by the use of de-methylating agents such as 5'aza2'deoycytidine (McGregor et al., 2002). The inducibility of RARβ correlates with clinical response in leukoplakia (Lotan, 1996).

Recently, retinoids have been implicated in the regulation of telomerase. Not only does constitutive expression of telomerase inhibit differentiation (Cerezo et al., 2003), but Pendino and coworkers also found that during retinoid treatment, hTERT is down-regulated and that this occurs by two pathways: an immediate RAR-mediated pathway and another indirect pathway (Pendino et al., 2001). In normal cells, expression of telomerase components is reduced in response to differentiation (Yashima et al., 1998). A link to p53 has also been suggested with stabilised p53 marking increased retinoid resistance (Lippman et al., 1995).

Historically, vitamin A or synthetic retinoid derivatives have been extensively tested in terms of reversing leukoplakias and prevention of second primary cancers. The rationale for this was initially based on evidence that cancer risk is greater in animals or humans with vitamin A deficiency. High dose retinoids can reverse histologically evident dysplasia, but have significant skin toxicity (reviewed in Lodi et al., 2004). Lower doses of retinoids in combination with interferon α and vitamin E are also effective, but genetic abnormalities persist (Hong et al., 1986; Papadimitrakopoulou et al., 1999; Shin et al., 2001b). Disappointingly, the initial promise that retinoids might prevent second cancers developing has not been confirmed in more recent trials (Hong et al., 1990; Khuri et al., 2003; van Zandwijk et al., 2000).

2.9 Other markers

A large number of studies have identified a variety of other gene expression changes in HNSCCs compared to normal oral mucosa by analysis of RNA extracted from biopsy tissue or by immunohistochemistry. In several studies, the expression of a large number of individual markers has been correlated with outcome in univariate analysis (Table 2.1). Not surprisingly, these changes affect a wide range of cellular processes known to be involved in cancer progression in many cancers, such as kinase signalling pathways; cell cycle/apoptosis; cell adhesion, motility and invasion; angiogenesis; immortalisation; chemokines and inflammation.

2.9.1 Cyclooxygenase 2

Alterations in cyclooxygenase 2 (COX2) have been described in many cancers. COX2 involvement was suspected from observations that patients treated with long-term non-steroidal anti-inflammatory drugs (NSAIDs) showed a reduced incidence of colorectal adenomas and adenocarcinomas (Thun et al., 1991; Waddell & Loughry, 1983). Subsequent investigation demonstrated overexpression of COX2 in colorectal adenoma and adenocarcinomas (Eberhart et al., 1994).

COXs are members of a family of dual function enzymes, prostaglandin endoperoxide synthases, involved in the generation of prostaglandins, an important class of biological regulators: COXs catalyse the first stage, the formation of prostaglandin H2 from the fatty acid, arachidonic acid. COX1 is widely expressed in cells, whereas COX2 is inducible by mitogens and is also highly expressed in many cancer types (reviewed by Goodin & Shiff, 2004). COX2 expression is increased in high risk oral dysplasias (Sudbo et al., 2003), probably as a consequence of EGF receptor overexpression and activation (Matsuura et al., 1999). Moreover, normal human mammary epithelia with silenced p16 overexpress COX2 and show features of premalignancy (Crawford et al., 2004).

Various mechanisms have been proposed to explain how COX2 may contribute to cancer progression: by producing reactive oxygen species as a by-product of its catalytic activity, stimulating proliferation, inhibiting apoptosis, modulating

Pathways affected	Changes in expression	References
Signalling pathways	EGF receptor & cerbB2	(Ang et al., 2002; Chen et al., 2003; Ulanovski et al., 2004)
	IL13 receptor α2	(Kawakami et al., 2003)
	phosphatidylinositol-3 kinase catalytic α peptide	(Estilo et al., 2003)
	RASSF1A	(Kuroki et al., 2003)
	Smad ubiquitin ligase (Smurf2)	(Fukuchi et al., 2002)
Cell cycle/apoptosis	cdc2	(Wada et al., 2004)
	p53 mutation	(Geisler et al., 2002; Mineta et al., 1998; Nathan et al., 2000; Nogueira et al., 1998)
	$p16^{INK4A}$	(Bova et al., 1999; Geisler et al., 2002)
	$p14^{ARF}$	(Ogi et al., 2002)
	Cyclin D1	(Bova et al., 1999; Nogueira et al., 1998)
	polo-like kinase 1	(Knecht et al., 1999)
	surviving	(Lo Muzio et al., 2003)
	DAP-kinase	(Brock et al., 2003)
Adhesion/motility	E-cadherin	(Chang et al., 2002; Chow et al., 2001; Lim et al., 2004)
	S100 Ca^{++} regulated protein A4	(Moriyama-Kita et al., 2004)
	CD44	(Gonzalez-Moles et al., 2003)
	hyaluronan	(Kosunen et al., 2004)
	metalloproteinase (MMP)-9	(Katayama et al., 2004)
	tissue inhibitor of MMPs (TIMPs)1/2/3	
	moesin	(Kobayashi et al., 2004)
	DCC	(Ogi et al., 2002)
	periostin	(Bao et al., 2004)
Angiogenesis	VEGF	(Shintani et al., 2004; Uehara et al., 2004)
	HIF-1α	(Beasley et al., 2002)
Transcription	HMGA2	(Miyazawa et al., 2004)
	BRG1/BRM	(Reisman et al., 2003)
Chemokines	CXCR4 receptor	(Delilbasi et al., 2004)
	CCR7 receptor	(Ding et al., 2003)
Immortalisation	telomerase	(Liao et al., 2004)
Others	COX2	(Chang et al., 2004)
	MINT1 & MINT 31	(Ogi et al., 2002)
	haem oxygenase	(Yanagawa et al., 2004)
	SCC-related oncogene	(Estilo et al., 2003)

Table 2.1 Gene expression changes in HNSCC that have been associated with prognosis compared with normal mucosa

The table includes some genes that are referred to elsewhere in the text.

immune surveillance or stimulating angiogenesis (Lin et al., 2002). Moreover, inhibition of COX2 overexpression may be one of the mechanisms whereby retinoids inhibit dysplasia progression (Subbaramaiah et al., 2002). Such data has led to great interest in the utility of specific Cox-2 inhibitors in chemoprevention (Koki et al., 2002). However, it is not certain that inhibition of growth of oral cancer cells by 'COX-2-selective' inhibitors is entirely mediated by reduced prostaglandin E2 synthesis as the magnitude of growth inhibition seen can be unrelated to the COX2 expression level (Minter et al., 2003; Schroeder et al., 2004). Disappointingly, despite this strong preclinical data, a recent randomised, double-blind, placebo-controlled trial of the COX inhibitor, ketorolac, as an oral rinse found no effect on response rates of oropharyngeal leukoplakia (Mulshine et al., 2004). However, this conclusion should be interpreted cautiously since the study lacked any investigation of biological endpoints in the target tissue to establish the effectiveness of the treatment on COX activity.

2.9.2 Gene expression profiling

More recently, several groups have compared the overall gene expression profiles of HNSCCs with normal oral mucosa by microarray analysis. In most cases, this has been performed using fresh biopsy material (El-Naggar et al., 2002; Gonzalez et al., 2003; Mendez et al., 2002; Sok et al., 2003) which obviously contains stromal and immune cells in addition to epithelial cells. In a few studies, pure epithelial cell material was obtained by laser capture microdissection (Alevizos et al., 2001; Leethanakul et al., 2003; Leethanakul et al., 2000) or pure epithelial cell cultures were isolated (Al Moustafa et al., 2002). Both approaches have their value, given recent work in other models indicating that gene expression changes occur in both tumour stromal and epithelial compartments of epithelial cancers and together facilitate cancer development (reviewed in Mueller & Fusenig, 2002; Park et al., 2000; TIsty, 2001).

Such studies have identified subgroups of HNSCCs with gene expression profiles that correlate with prognosis. One study of 25 primary HNSCCs, 16 locally recurrent HNSCCs and 13 normal oral mucosa samples found a gene expression signature associated with recurrence that included markers of proliferation, extracellular matrix proteins, cytokines and chemokines, and

immune response (Ginos et al., 2004). No attempt was made to correct for smoking status in this study and so this could account for some of the differences in gene expression profiles between normal subjects and HNSCC patients; however, the prognosis related differences in gene expression profiles amongst HNSCCs are likely to be unaffected since 90% of the patients were smokers. Another study of 60 HNSCCs (58 of which were from smokers) categorized the tumours into four groups associated with statistically significant differences in recurrence-free survival. These included a subtype with a possible epidermal growth factor (EGF) receptor pathway signature, a mesenchymal-enriched subtype, a normal epithelium-like subtype and a subtype with high levels of antioxidant enzymes (Chung et al., 2004).

Although not directly relevant to HNSCC, one study of 24 oesophageal SCCs and normal tissues, found a high rate of relapse to correlate with expression of molecules associated with cell cycle regulation, gene repair, apoptosis and chemoradiotherapy resistance (Ishibashi et al., 2003). Smoking-related changes in this study were controlled by comparing paired samples of normal and malignant tissues from the same patient. In another oesophageal SCC study, the gene expression signature profile associated with nodal metastasis in a training set of 36 cases was found to be predictive in an independent validation set of 18 cases (Tamoto et al., 2004).

Gene expression profiling offers one potential approach for better diagnostic methods for analysis of dysplasias in relation to prognosis. Ha et al found that there were many more differences in gene expression between normal and premalignant oral leukoplakias (mainly moderate to severe dysplastic lesions) than between these lesions and HNSCCs (Ha et al., 2003). However, no information regarding correlation with progression was available in this study. Although not directly relevant to squamous cancer, in another premalignant model, Barrett's oesophagus, screening of the expression profiles of a panel of 23 genes revealed that expression of three genes (PITX1, tetraspanin and thymidine phosphorylase) could distinguish Barrett's oesophagus from adenocarcinoma with an error rate of 28% in a cross validation test (Brabender et al., 2004).

2.10 Aims and rationale of approach

The work described in this thesis builds on the basis laid by the earlier publications from the group (McGregor et al., 2002; McGregor et al., 1997; Muntoni et al., 2003). The existence of mortal and immortal dysplastic and carcinoma cells, together with the characterisation of the immortal phenotype *in vitro*, has allowed us to assess the importance of some of the various components using functional studies.

However, sole use of cell culture technology to investigate HNSCC carcinogenesis has significant problems and is often subject to criticism (Ramirez et al., 2001). Thus, it is vital that the expression pattern of these surrogate immortality markers is established *in vivo*. This is vitally important as potential molecular markers, be it for prediction of prognosis or as potential for therapeutic intervention, must also show differential expression in the oral tissues.

Thus, this project aims to address the expression of the markers closely associated with the immortal phenotype (McGregor et al., 2002) *in vivo*. The further investigation of these cell cultures is also mandated, as it is apparent that, at least in terms of dysplasia progression, immortality does not entirely explain the presence of a subset of lesions that are at increased risk of progression to SCC. Thus, gene expression profiling has a large part to play in identification of other markers of immortality and of other important events in HNSCC carcinogenesis, such as early/initiating events, markers of high risk of dysplasia progression to carcinoma and gene expression profiles related to poor prognosis and recurrence/metastasis in HNSCC.

Studies with a relatively small group of cell cultures, all derived from different patients, are only likely to suggest hypotheses for future investigation. However, the aim is to identify novel markers potentially important in these important events and determine their expression *in vivo*, especially for mortal HNSCC and high risk dysplastic cell cultures. These markers may then be assessed in larger retrospective and prospective studies, which will ultimately determine the usefulness of these genes and particular patterns of gene expression in improving patient care.

Chapter Three: Materials and methods

3.1 Cell culture

All cell culture work was performed using strict aseptic techniques inside a laminar flow hood (Class II Microbiological Safety Cabinets, Medical Air technology Ltd., Manchester, UK). Epithelial cells were incubated at 37°C in 90 mm Falcon culture dishes in a dry atmosphere containing 5% (v/v) CO_2 (Heraeus, Essex, UK).

The derivation, characterisation and maintenance of the primary human keratinocyte cultures have been described previously (Edington et al., 1995; McGregor et al., 1997). All cells were maintained on irradiated Swiss 3T3 feeders (60Gy from a Co60 radiation source). The growth media used were flavine adenine dinucleotide (FAD) growth medium, composition 1:3 Ham's F12/Dulbecco's modified Eagles medium (DMEM) (both from Gibco BRL Europe Life Technologies, Paisley, UK) supplemented with 10% foetal bovine serum (FBS), 0.4 μg/ml hydrocortisone (Sigma-Aldrich, Poole, Dorset, UK), 5 μg/ml insulin (Gibco BRL Europe Life Technologies, Paisley, UK), 5 μg/ml transferrin (Sigma-Aldrich, Poole, Dorset, UK), 10^{-10} M cholera toxin (Sigma-Aldrich, Poole, Dorset, UK), 1.8×10^{-4} M adenine (Sigma-Aldrich, Poole, Dorset, UK). For FAD^{+} medium, 10 ng/ml epidermal growth factor (Gibco BRL Europe Life Technologies, Paisley, UK) was added.

The irradiated 3T3 feeders were removed by treatment with phosphate buffered saline (PBS)/0.02% EDTA (PE) for 15-20 seconds and vigorous pipetting before analysis of the keratinocyte cultures. Swiss 3T3 cells were maintained in 10C medium (DMEM plus 10% donor calf serum (Sigma-Aldrich, Poole, Dorset, UK). A summary of the clinical and biological features of the cell cultures used in the thesis is found in Table 3.1.

Oral fibroblast cultures were derived from the lamina propria related to one of the dysplastic cell cultures (D19). These were maintained in 10C medium. HeLa cells were maintained in modified Dulbecco's medium (Beatson formulation) (Gibco BRL Europe Life Technologies, Paisley, UK) supplemented with 10% foetal bovine serum (Harlan Sera-Lab, Crawley Down, Sussex, UK)

culture	age	sex	smoker	site	histology/ stage	PDL
NT	NK	M	N	tongue	normal	25
NB9	53	M	Y	tongue	normal	17
FNB3	43	M	N	BM	normal	17
FNB5	38	F	N	BM	normal	20
FNB6	33	F	N	BM	normal	17
D6	55	M	Y	tongue	moderate	25
D8	71	M	Y	tongue	mild	9
D17	61	M	Y	BM	mild/moderate	45
D25	58	M	Y	FoM	severe	28
D30	52	M	Y	FoM	mild	30
D36	35	M	Y	RM	keratosis only	
D41	55	F	N	Lat tongue	mild	3.5
D47	82	F	Y	FoM	moderate	20
D48	62	F	Y	FoM/VT	mod/severe	25.5
E1	NK	M	NK	tongue	CiS	24
E2	NK	M	NK	alveolus	CiS	17
E4	55	F	NK	tongue	CiS	41
E5	NK	M	NK	tongue	severe	31
D4	51	M	Y	tongue/FoM	CiS	immortal
D9	84	M	N	tongue	mild/moderate	immortal
D19	53	M	N	lat tongue	moderate	immortal
D20	50	M	N	lat tongue	mild	immortal
D34	54	F	Y	tongue	severe	immortal
D35	68	M	N	lat tongue	mild/moderate	immortal
D38	55	F	Y	FoM	CiS	immortal
BICR30	NK	M	Y	larynx	T4N1M0	17
BICR37	NK	M	NK	node	metastasis (T4 tongue SCC)	32
BICR66	55	M	Y	tongue	T2N0M0	37
BICR80	NK	M	Y	larynx	T4N2M0	62
BICR3	57	F	Y	alveolus	T2N0M0	immortal
BICR7	43	M	Y	tongue	T4N2cM0	crisis
BICR22	88	M	Y	tongue	metastasis (Tongue SCC)	immortal
BICR31		M	NK	tongue	T4N2bM0	immortal
BICR56	58	F	Y	tongue	T4N1M0	immortal
BICR68	74	M	Y	tongue	T4N0M0	immortal

Table 3.1 Basic clinical and biological features of the primary keratinocyte cultures used in this thesis

Site: BM = Buccal mucosa, FoM = Floor of mouth, VT = ventral tongue, lat=lateral, RM = retromolar

Histology: Mild/moderate/severe refers to the grading of dysplasia in the original biopsy. CiS = carcinoma in situ. Carcinoma stage refers to TNM classification at diagnosis.

PDL = population doublings in culture before senescence.

In every case, NK = not known

and 1mM glutamine (Gibco BRL Europe Life Technologies, Paisley, UK). WI38 cells (source) were maintained in modified Dulbecco's medium, supplemented with 5% fetal bovine serum and 1mM glutamine.

To freeze cell stocks for storage, semiconfluent cultures were trypsinized, pelleted and re-suspended at a concentration of approximately 10^6 cells/ml in chilled medium containing 50% serum and 10% (v/v) dimethylsulfoxide (DMSO) (Fisher Scientific, USA). Suspensions were aliquoted into 1ml cryotubes (Nunc, Wiesbaden, Denmark), placed in a polystyrene box and frozen, well insulated, at -70ºC overnight to ensure a slow rate of cooling (1ºC/min). The ampoules were then transferred to a liquid nitrogen tank until required.

Frozen stocks were recovered by removing the ampoules from liquid nitrogen and placing them into a small, covered bucket of water at 37°C. Once thawed, the cells were added to 10 ml of the appropriate pre-warmed growth medium, centrifuged, resuspended in fresh growth medium and transferred to 90 mm Falcon culture dishes (Becton Dickinson, Franklin Lakes, USA).

All cultures were tested for the presence of mycoplasma infection. This was assessed by Hoechst 33258 staining (Chen, 1977). Briefly, NRK cells were incubated for 3-4d with medium that had been in contact with the keratinocyte culture for at least 2d. These cells were fixed with methanol (3parts) and acetic acid (1 part). The fixative was then removed and 5ml of Hoescht 33258 (Sigma-Aldrich, Poole, Dorset, UK) added and left for 10 mins. The stain was then removed and the cells washed with distilled water. The cells were them mounted in distilled water and visualised under a fluorescence microscope. Extranuclear DNA staining indicated mycoplasma infection. No infected cells were identified during the period of this study.

3.1.1 Organotypic culture

After initial growth in a 90mm Falcon culture dish, the keratinocytes were grown on a collagen/fibroblast gel and raised to the air/medium interface. This allowed the development of a stratified squamous epithelium. The gel was mixed (on ice) using 8 volumes of 2mg/ml rat tail collagen solution (kindly provided by Dr M Edward, Department of Dermatology, University of Glasgow), with 1 volume of DMEM (pH adjusted to 7.2 with sterile 1M NaOH) and 1 volume of FBS. To

this gel mixture was added $2.5x10^5$ oral fibroblasts per ml of gel. The gel was mixed well and 1.5ml added to each Millicell-CM 0.4μm cell culture insert (Millpore Corporation, Bedford, MA, USA) in a six-well cell culture plate. This was incubated at 37°C for 30 min to set the gel. Each gel was then covered with 1ml of FAD^+ medium and incubated overnight at 37°C. The next day the medium was removed and the gels in the inserts plated with $8x10^5$ oral keratinocytes in 250μl of FAD^+. FAD^+ was also added each of the wells in the cell culture plate, adjusted to touch the underside of the semipermeable membrane on which the collagen/fibroblast gel rested. The medium was changed every 2d for 11d.

At the end of this period, the stratified epithelium was either scraped into RNA lysis buffer, for subsequent extraction of RNA, or placed, with the gel intact, into neutral buffered formalin for fixation prior to histological examination.

3.2 Detection of protein expression

3.2.1 SDS-PAGE and Western blotting

To prepare whole cell protein extracts for immunoblotting, cells in the proliferative phase of growth were washed twice with ice-cold PBS and then scraped off in 0.2ml of lysis buffer (20mM Hepes, pH 6.8, 5mM EDTA, 10mM EGTA, 5mM NaF, 0.1μg/ml okadaic acid, 1mM DTT, 0.4M KCl, 0.4% Triton X-100, 10% glycerol, 5μg/ml leupeptin, 50μg/ml PMSF, 1mM benzamidine, 5μg/ml aprotinin, 1mM sodium orthovanadate), incubated on ice for 20 min, followed by centrifugation at 13,000 rpm in a microfuge for 20 min and the lysate (supernatant) recovered. The supernatant was stored at -70^{0}C. Up to 50μg of protein sample (quantitated using the bicinchonic acid–copper (II) sulphate method, Smith et al., 1985) was mixed with an equal volume of 2x loading buffer (187.5 mM Tris-HCl, pH 6.8, 30% glycerol, 6.9% SDS, 2.1M β-mercaptoethanol, 0.1% bromophenol blue), boiled for 10 min before SDS-polyacrylamide gel electorphoresis (SDS-PAGE).

Gels containing different concentrations of polyacrylamide (i.e. 10%, 12%, and 15%) were used to resolve proteins according to their molecular weights. The resolving gel was made by adding the appropriate volume of 30% (w/v) acrylamide: 0.8% (w/v) bis-acrylamide to a solution of 0.45M Tris, 0.1% SDS, 0.08% TEMED and 0.1% (w/v) APS. The stacking gel comprised of 0.125M Tris, pH6.8, 0.1% SDS, 1.7ml 30% (w/v) acrylamide: 0.8% (w/v) bys-acrylamide, 0.2% APS and 0.3% TEMED. Proteins were analysed in parallel with BenchMark^{TM} prestained protein ladder (GibcoBRL,UK) and electrophoresis was performed o/n prior to transfer onto nitrocellulose using a Camlab semi-dry blotter (Camlab, UK), following the manufacturer's protocol.

The protein electrophoresis for validation of the microarray GEPs used 4-12% Bis-Tris Criterion XT precast gels (Bio-Rad Laboratories, Hercules, CA, USA). The 18 well format of the gel allowed for analysis of a large number of cultures on one gel. A Bio-Rad Criterion wet blotter (Bio-Rad laboratories, Hercules, CA, USA) was used for the transfer to nitrocellulose membrane, according to the manufacturer's instructions.

Western blots were blocked overnight at 4ºC in the presence of TBST (50mM Tris pH 7.5, 50mM NaCl, 1mM EDTA, 0.1% Tween-20) containing 5% non fat dried milk; washed 4 x 10min with TBST and then incubated with antibodies diluted in TBST containing 5% non fat dried milk or 0.1-1% bovine serum albumin (according to antibody manufacturer's instruction) and 0.1% sodium azide. The antibodies and their dilutions are shown in Table 3.2. Following incubation with the primary antibody the membrane was washed 4 x 10 minutes with TBST. This was followed by incubation in 20ml of a 1: 3000 dilution of the appropriate IgG HRP-linked antibody in blocking buffer for 1.5h. The secondary antibody solution was removed and the membrane was washed 3x 10 minutes with TBST. After removal of excess surface liquid, bound primary antibody was detected using ECL chemiluminescent reagent (Amersham, UK) source according to manufacturer's instructions.

For loading controls, the membranes were reprobed with a polyclonal antibody against total p38 MAP kinase to ensure even loading and transfer since it was found to be uniformly expressed in all keratinocyte cultures regardless of proliferative fate. The blots were stripped by incubation for 30min with 1x Stripping buffer (100mM 2-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH6.7) at 55^{0}C. The membrane was washed 3 times with TBST prior to proceeding to blocking and subsequent antibody incubation.

Antigen	Clone	Source	Dilution
p16	F12	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:250
p53	DO1	Santa Cruz Biotechnology	1:200
p38 MAPK		Cell Signalling Technology, USA	1:1000
UBE2C		Abcam Limited, Cambridge, UK	1:500
Cyclin B1	GNS1	Neomarkers, Fremont, CA, USA	1:500
SMC4Li		Upstate, Lake Placid, NY, USA	1:250
Involucrin		Cancer Research UK monoclonal antibody service	1:5000
S100A9	C-19	Santa Cruz Biotechnology	1:100
IGFBP2	H-75	Santa Cruz Biotechnology	1:200

Table 3.2 Primary antibodies used for Western blotting

3.2.2 Preparation of routine histopathological sections.

Tissue specimens 5μm in thickness were cut using a Leica 2035 Microtome (Leica, Heidelberg, Germany) and then mounted on plain glass sides. These were then oven dried for 15 minutes. The sections were deparaffinized for 30 minutes using Histoclear (Fisher Scientific, USA) followed by xylene for 10 minutes. Rehydration was carried out sequentially in 100% and 20% ethyl alcohols (5 minutes, each step) and finally water. The sections were then stained using haematoxylin and eosin (H&E, Surgipath, Peterborough, UK), dehydrated, mounted with a coverslip using Histomount (Hughes & Hughes, Wellington, UK).

3.2.3 Immunohistochemistry (IHC)

For immunohistochemistry, fresh 5μm sections were cut from the relevant diagnostic paraffin blocks and mounted on silane-coated slides. The sections were rehydrated as described above and then incubated in 0.3% H_2O_2 for 20 minutes followed by a 5 minute wash in distilled water. The sections were microwaved in 1.5l of citrate buffer for 4.5 minutes at full power (once full pressure had been reached). Immunohistochemistry was carried out using the Vectastain ABC (avidin-biotin-peroxidase, Vector Laboratories, Burlingame, CA, USA) kit for mouse or rabbit IgG, as appropriate, according to manufacturer's instructions. The washing buffer used at all stages was PBS pH7.6 with 0.1% BSA fraction V, as this reduces non-specific antibody binding.

The p53 IHC was performed on a DakoCytomation Autostainer (DakoCytomation Denmark). For all other IHC, the sections were incubated with primary antibody overnight at 4°C in a moist chamber. The antibodies used are shown in Table 3.3. Peroxidase substrate DAB (3,3'-diaminobenzidine) kit (Vector Laboratories, Burlingame, CA, USA) was used to visualize the primary antibody according to manufacturer's instructions. Counterstaining was carried out using haematoxylin and the section dehydrated with sequential treatment with water, 70% and 100% ethanol, and xylene. Finally the section was mounted in Histomount and covered with a coverslip.

Antigen	Clone	Source	Dilution
p16	F12	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:200
p53	DO1	Santa Cruz Biotechnology	1:300
Involucrin		Cancer Research UK monoclonal antibody service	1:10000
Cyclin B1	GNS1	Neomarkers, Fremont, CA, USA	1:800
S100A9	C-19	Santa Cruz Biotechnology	1:700

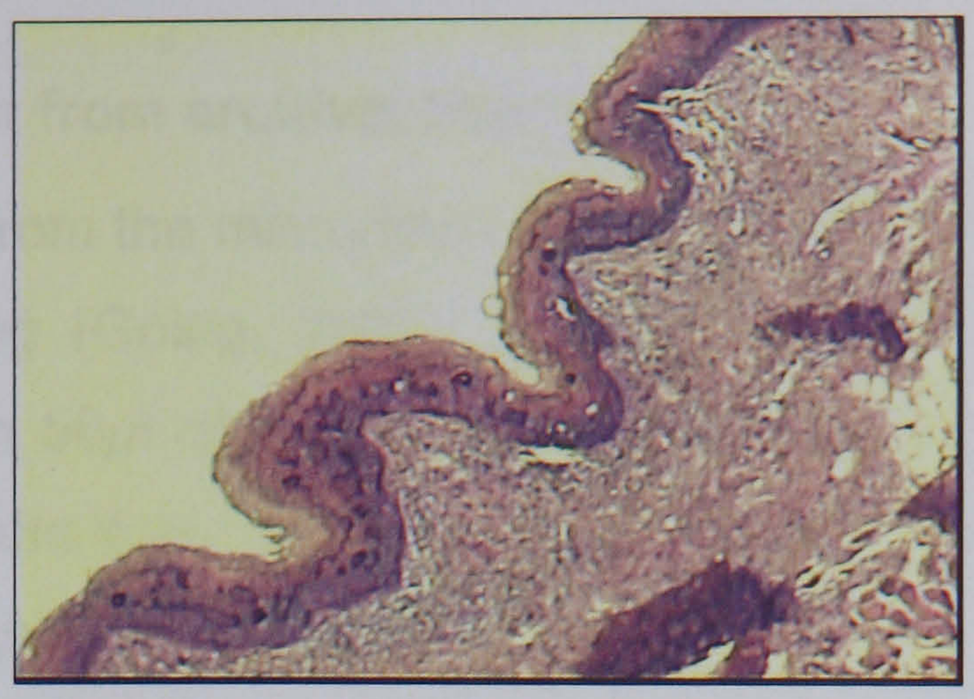
Table 3.3. Primary antibodies used for immunohistochemistry

3.2.4 Histopathological image capture

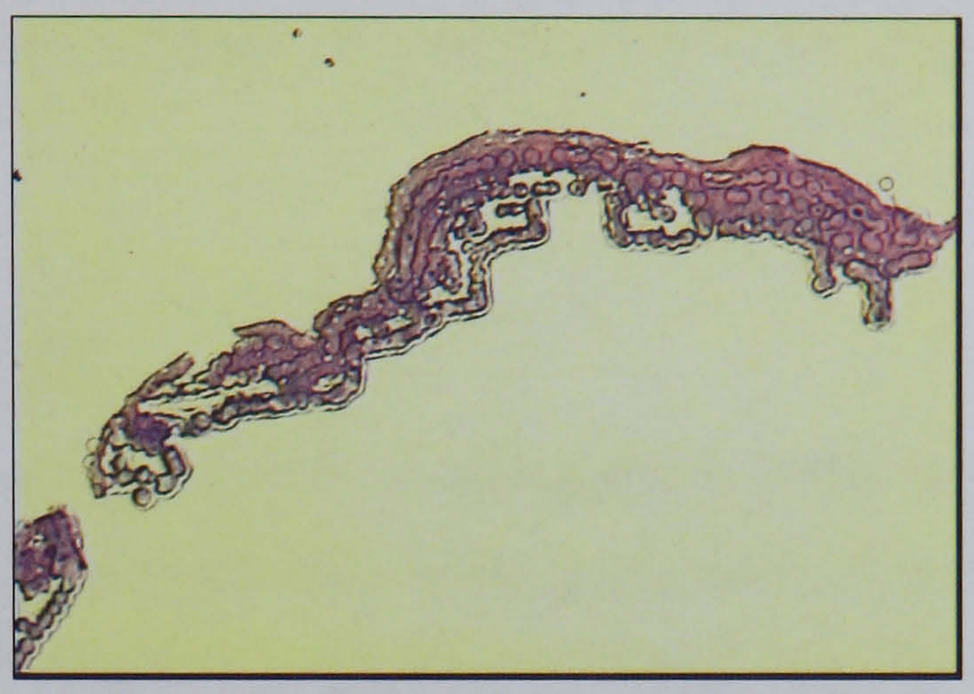
Routine H&E sections and immunohistochemistry were visualised using a Zeiss Axioskop 20 microscope (Zeiss, Germany) with an appropriate light source and photomicrographs recorded using the AxioVision ver. 3.0.6 software (Zeiss, Germany). The images were cropped in Adobe Photoshop Elements (Adobe Systems Incorporated, San Jose, CA, USA.

3.3 Laser capture microdissection (LCM)

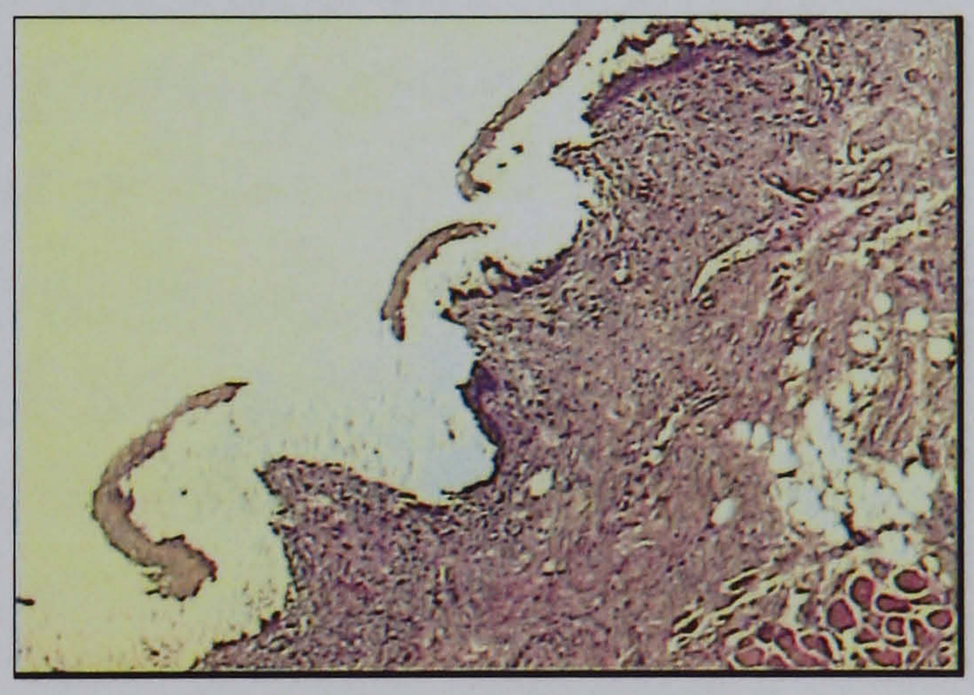
Fresh 6μm sections were cut from the relevant diagnostic paraffin blocks and mounted on plain glass slides, a maximum of 24 hours before use. The sections were rehydrated and briefly stained with haematoxylin and eosin. The slides were then maintained in a dry environment under a slight vacuum. The LCM was conducted using the Arcturus Pixcell® IIe, according to the manufacturer's instructions (Arcturus Bioscience, Mountain View, CA, USA). The Arcturus LCM cap was located on the apparatus and then placed on the tissue section. The cells required were identified. A pulse laser was then used to target these cells and the cap removed with the target cells adherent to the cap (see Figure 3.1).



D4 pre-capture



D4 captured epithelium



D4 lamina propria and surface keratin left in place

Figure 3.1 Laser capture microdissection for sampling of epithelium from tissue sections.

Panels show formalin fixed tissue stained with Haematoxylin and eosin and demonstrate the process of laser capture microdissection using the Arcturus Pixcell® IIe. The captured epithelium was subsequently be digested to release the DNA for PCR.

3.4 DNA extraction from archival tissue

DNA was extracted from the microdissected material by Proteinase K digestion, as outlined by Going (Going, 2003). Briefly, the microdissected tissue was digested overnight in 50μl of proteinase K digestion buffer at 37ºC. The tube was inverted to ensure that the entire surface of the LCM cap was covered. Proteinase K was then inactivated by heating at 95ºC for 10 min. 5μl of this digest was used in a standard PCR reaction. The DNA in the digest was amplified using GAPDH nested primers to confirm the isolation of DNA from the tissue (Table 3.4).

3.4.1 p53 sequencing.

This was performed using the proofreading DNA polymerase, Proofstart (Qiagen, Crawley, UK) and primers outlined in Table 3.4. These nested primers were designed using the Prime program (GCG Wisconsin package, Accelrys Ltd, Cambridge, UK). The primers used amplified a short product that covered the mutation previously demonstrated in the relevant culture (McGregor et al., 2002). A small aliquot of cDNA product was assessed by 5% acrylamide gel electrophoresis in Tris-borate-EDTA buffer (TBE), to check for the presence of product of correct size and the absence of non-specific products. The gel was subsequently stained with ethidium bromide (Sigma-Aldrich, Poole, Dorset, UK). The UVIdoc gel documentation system (Uvitech, Cambridge, UK) was used to visualise the gel.

Sequencing of fragments in both directions was performed according to the manufacturer's protocols (ABI prism big dye terminator reaction kit, Applied Biosystems, Foster City, CA, USA). Where a mutation was found by reading the traces by eye, the result was confirmed using an independent PCR amplification product.

As the standard sequencing protocol was unsuccessful for DNA from D9 and D20, the PCR products were cloned using the TOPO TA cloning kit (Invitrogen Ltd, Paisley, UK). A non proofreading Taq polymerase was used for the PCR reaction to provide the necessary TA overhang for cloning into the pCR4-TOPO constructs. The constructs were transformed into TOP10 competent E-coli. Once colonies had formed, they were picked and DNA minipreps prepared.

The DNA was then sequenced using T3 and T7 primers, as described above. This work was completed by Janis Fleming.

Sample	Sequence	Product size (bp)	Annealing temp (°C)
D4 & D19	5'-ACTTTCAACTCTGTCTCCTTCC-3' 5'CCATCGCTATCTGAGCAGC-3'	218	57
	5'-CCCTGCCCTCAACAAGATG-3' 5'-ACAACCTCCGTCATGTGC-3'	137	56
D9	5'-GCTTTTGATCCGTCATAAAGTC-3' 5'-TGGGCATCCTTGAGTTCC-3'	144	53
	5'-ACTTACTTCTCCCCCTCCTC-3' 5'-CCTCATTCAGCTCTCGGAAC-3'	77	53
D20	5'-AGGTTGGCTCTGACTGTACC-3' 5'-TGACCTGGAGTCTTCCAGTG-3'	116	54
	5'-GTGTAACAGTTCCTGCATGG-3' 5'-GAGTCTTCCAGTGTGATGATG-3'	69	52
GAPDH	5'-ATGCCTTCTTGCCTCTTGTC-3' 5'-CTCACCATGTAGCACTCACC-3'	145	52
	5'-GATTTGGTCGTATTGGGCG-3' 5'-TGAGGTCAATGAAGGGGTC-3'	93	51

Table 3.4 Nested oligonucleotide primers used for p53 sequencing

These primers were used for sequencing of culture and LCM tissue derived DNA. Sample refers to the tissue related to the culture indicated.

3.5 Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) assay.

RNA was extracted from the cultured cells using Qiagen RNeasy minikit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. After thawing, the tissue samples were disrupted and homogenised in the presence of the RNEasy lysis buffer using Omni-tip disposable generator probes in an OMNI TH220 tissue homogeniser (Omni international Inc Marietta, GA, USA). On column DNase digestion was performed during the RNA purification, using the RNase-Free DNase Set (Qiagen GmbH, Hilden, Germany). RT-PCR was performed using the GeneAmp RNA PCR core reagent kit (Perkin Elmer, Branchberg, NJ, USA) according to the manufacturer's protocols.

The p16 primers used were p16FP and p16RP (Table 3.5). These primers were designed to span the intron between exons 1α and 2, thus giving specificity for p16 and avoiding amplification of any contaminating DNA. The hTERT primers used were MS113 and MS114, as described by Dome and co-workers (Dome et al., 1999). Primers used in the detection of hTERT deletion splice variants (TERT2109 and TERT2531R) were those previously published by Yi and coworkers (Yi et al., 2001). The hTR primers were those published by Harada and co-workers (Harada et al., 2001). The RARβ2 primers used were RARβ2FP and RARβ2RP as described by Sirchia and coworkers (Sirchia et al., 2000). In each experiment, the levels of GAPDH mRNA were also measured as an internal control. The sequences, product size and optimal annealing temperature for each of these oligonucleotide primer sets are set out in Table 3.5. The reactions were carried out on a PCT200 DNA engine (MJ Research, Massachusetts, USA), according to the manufacturer's instructions.

The cDNA product was assessed by 5% acrylamide gel electrophoresis in TBE and subsequent staining of the gel by addition of 50μl of 5mg/ml ethidium bromide (Sigma-Aldrich, Poole, Dorset, UK), to 500ml water (final concentration 0.5μg/ml). The UVIdoc gel documentation system (Uvitech, Cambridge, UK) was used to visualise the gel. The gel images were captured as .tiff files.

Gene	Sequence (forward/reverse)	product (bp)	annealing temp (ºC)
p16	5'-TGCCCAACGCACCGAATAG-3' 5'-CACCAGCGTGTCCAGGAAG-3'	176	60
hTERT	5'-AGAGTGTCTGGAGCAAGTTGC-3' 5'-CGTAGTCCATGTTCACAATCG-3'	150	60
hTERT (DV)	5'-GCCTGAGCTGTACTTTGTCAA-3' 5'-AGGCTGCAGAGCAGCGTGGAGAGG-3'	various*	64
hTR	5'-TTTGTCTAACCCTAACTGAGAAG-3 ' 5'-TTGCTCTAGAATGAACGGTGGA-3 '	128	62
RARβ2	5'GACTGTATGGATGTTCTGTCAG-3' 5'-ATTTGTCCTGGCAGACGAAGCA-3'	256	62
GAPDH	5'-AAGGCTGAGAACGGGAAGCTTGTCATCAAT-3' 5'-AGCCCCAGCCTTCTCCATGGTGGTGAAGAC-3'	146	60

Table 3.5 Oligonucleotide primers used for RT-PCR.

DV = deletion variants. * The size of the products of the hTERT deletion variant RT-PCR are seen in Figure 4.7

The hTERT deletion splice variants were visualised using the Agilent 2100 Bioanalyzer (Agilent technologies, Germany). RNA was loaded into the RNA 6000 Nano-chip in a loading gel and with dye concentrate (Agilent technologies, Germany). The Bioanalyzer separates the different sizes of RNA in the sample by microcapillary electrophoresis. The different components are detected by laser induced fluorescence of the dye that intercalates directly with the RNA. The output may be viewed as either gel-like images or electropherograms (Figure 3.2).

3.6 Microarray RNA preparation and quality control

Total cellular RNA was isolated from the cell cultures using the Qiagen RNeasy minikit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The RNA was homogenized at the appropriate point in the RNeasy kit instructions using the QIAshredder homogenizer (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Initially, the quality of the RNA was assessed by spectrophotometry. Only RNAs with a 260/280nm ratio of >1.9 were of sufficient quality to proceed to the labelling stage. At the end of the labelling and fragmentation process, all the products were assessed using an RNA denaturing 1.1% formaldehyde/agarose gel electrophoresis in MOPS buffer. Later samples were analysed using the Agilent 2100 Bioanalyzer, (see earlier). Samples with 28S/18S ratio <2.0 were rejected (Figure 3.2). In either analysis, the integrity of the initial RNA was demonstrated and the expected size distributions for the cRNA and fragmented cRNA were confirmed.

3.6.1 Sample labelling and microarray analysis

Amplification and labelling of the RNA in the pilot and main studies were performed using the Affymetrix standard labelling protocol. An outline of this protocol is illustrated in Figure 3.3. Briefly, 15μg total RNA was reverse transcribed using the Superscript double stranded cDNA synthesis kit, according to manufacturer's instructions (Invitrogen Ltd, Paisley, UK) and T7(dT)24 primer (Helena Biosciences, Sunderland, UK). The cDNA was purified using buffer saturated phenol/chloroform/isoamyl alcohol (Ambion Inc, Austin TX, USA) in Phase lock gel (light) tubes (Helena Biosciences, Sunderland, UK). In vitro transcription (IVT) was performed using the Enzo Bioarray high-yield transcript-labelling kit (Affymetrix, Santa Clara, CA, USA), according to the manufacturer's instructions. Subsequent purification of the cRNA was carried out by the RNEasy minikit RNA clean-up protocol (Qiagen, GmbH, Hilden, Germany). If the yield from IVT was <40μg, the reaction was repeated, as it was assumed that there had been a problem with the IVT reaction.

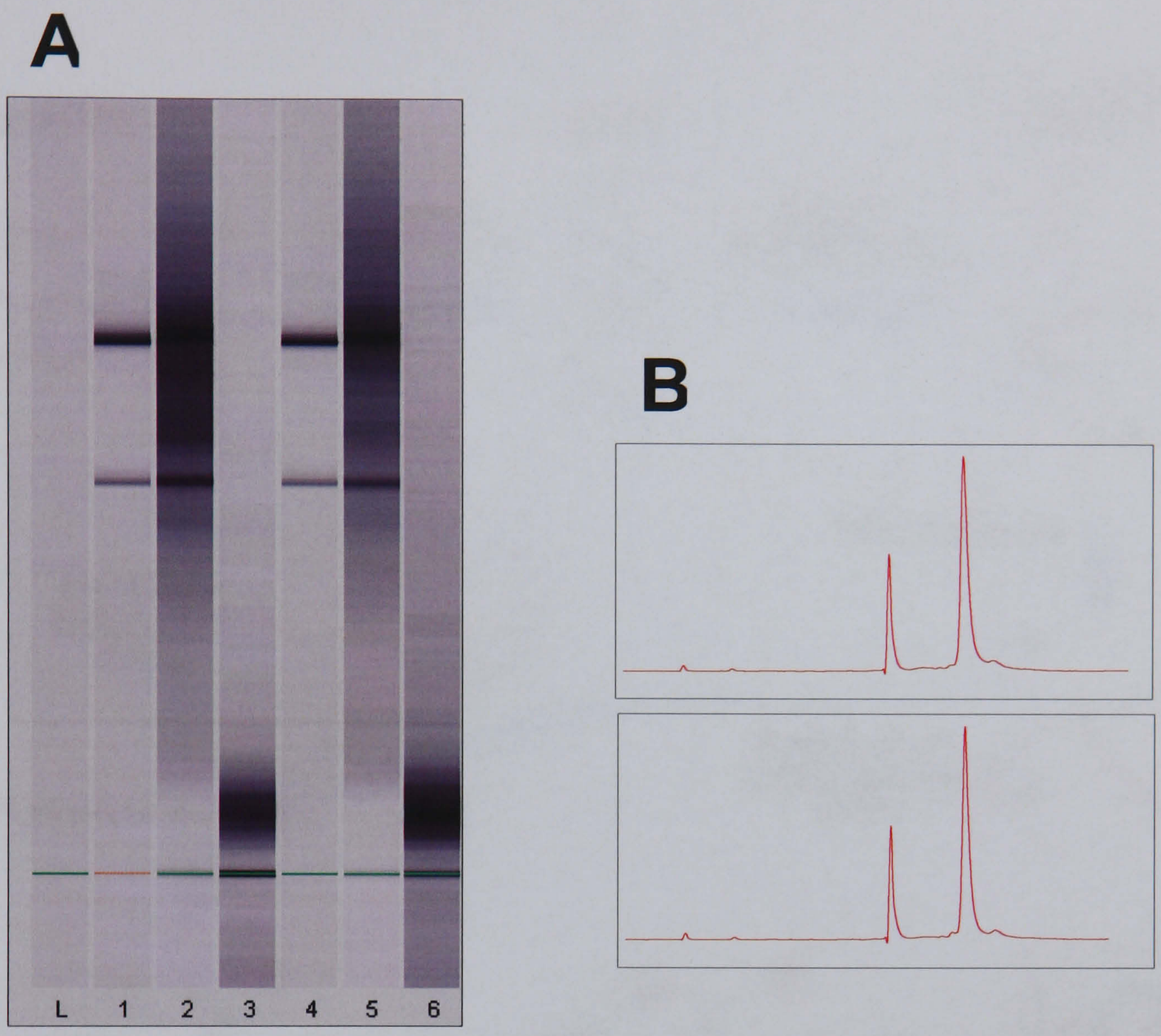


Figure 3.2 Quality control measures for RNA for microarray analysis

Total RNA was analysed on Agilent 2100 Bioanalyser using RNA nanochips.

Panel A shows a "virtual gel" trace of for QC of array samples
L=ladder
Lanes 1 - 3 BICR30a total RNA, labelled cRNA and fragmented cRNA
Lanes 4 - 6 BICR30b total RNA, labelled cRNA and fragmented cRNA

Panel B shows traces for lanes 1 and 3, showing 18S and 28S peaks. Samples with 28S/18S ratio <2.0 were rejected. Further QC was conducted at the PICR with test chips used for certain samples.

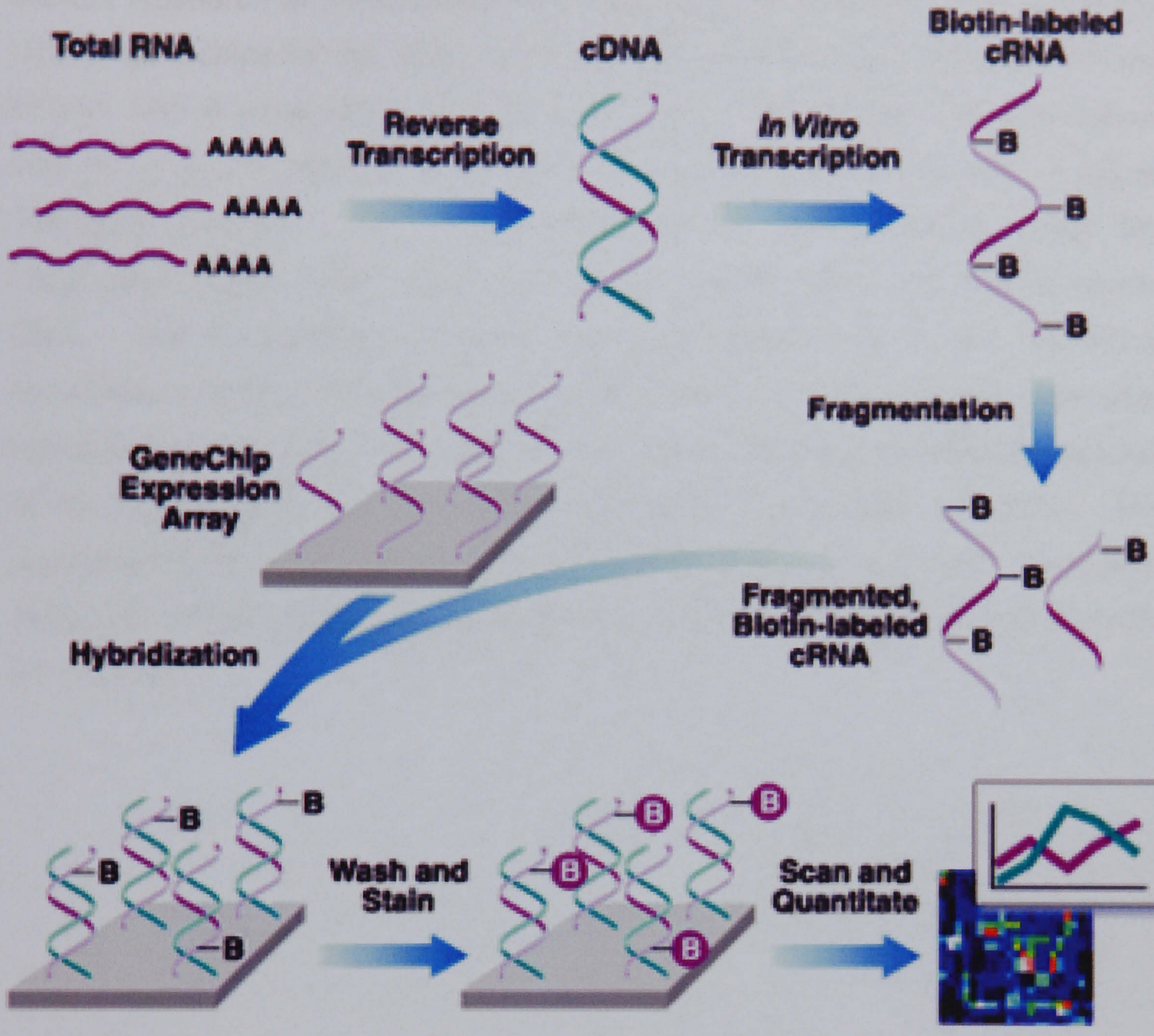


Figure 3.3 Standard eukaryotic gene expression assay.

Labelled cRNA targets derived from the mRNA (via cDNA) of each experimental sample are hybridized to nucleic acid probes attached to the solid support. By monitoring the amount of label associated with each oligonucleotide location, it is possible to infer the abundance of each mRNA species represented. Figure courtesy of Affymetrix.

The cRNA was fragmented in buffer containing 200mM Tris-acetate, pH8.1, 500nM KOAc and 150mM MgOAc for 35mins at 94ºC. After visualisation of the products and quality control as described above, the fragmented cRNA was sent on dry ice to the CRUK microarray core facility at the Paterson Institute for Cancer Research in Manchester, UK. The cRNA was hybridised to Affymetrix U133A genechips for the pilot and tissue/culture comparison experiments and U133A and B gene chips for the main study. The cRNA was processed according to the method outlined in the GeneChip® Expression Analysis Technical Manual. The hybridisation cocktail was prepared using the fragmented target cRNA, with probe array controls, BSA and herring sperm DNA. The hybridisation process was then carried out in the GeneChip hybridisation oven 640 (Affymetrix, Santa Clara, CA, USA). Immediately after hybridisation, the array underwent an automated washing and staining protocol on the GeneChip fluidics station 450 (Affymetrix, Santa Clara, CA, USA). The genechips were scanned using the GeneChip scanner 3000 with autoloader (Affymetrix, Santa Clara, CA, USA) and the resultant raw data returned to BICR for analysis.

3.7 Microarray data analysis

The first step in data analysis was quality assessment of the data. A number of elements were assessed, two of which are vitally important. Firstly, the number of probe sets detected should be similar for the arrays. The second assessment concerned the housekeeping genes. The Signal 3'/5' ratio of the GAPDH and β-actin probe controls should ideally be 1, but values up to 3 were considered acceptable. Higher values indicate either poor quality starting RNA or poor 1st strand cDNA synthesis. This occurred with only one sample, and this preparation was repeated.

The data was analysed in a standard fashion as outlined in Figure 5.1.1. The raw data was log2 transformed and normalized using the Robust Multi Average (RMA) method of Irizzary and coworkers (Irizarry et al., 2003), as implemented in RMAExpress. This is available for download from http://stat-www.berkeley.edu/users/bolstad/RMAExpress/ RMAExpress.html . The data was also loaded to the Affymetrix microarray analysis suite (MAS) 6.0. The perfect match (PM) and mismatch (MM) oligonucleotides were analyzed and the "Absent", "Marginal" or "Present" tags recorded. Only probesets which had 6 or more "Present" flags for that particular gene across the whole dataset of 80 arrays were forwarded to further analysis. Finally, after ascertaining that the reproducibility of the two culture replicates was good, the mean of the replicates was calculated and this figure was used for further analysis of the expression of a particular gene in a sample.

The expression data for the normalized, filtered genes were loaded in to Bioconductor in R (Gentleman et al., 2004), TMeV (http://www.tigr.org/ software/tm4/ mev.html, TIGR, Rockville, MD, USA), and Genespring 6.0 (Silicon Genetics, Redwood City, CA, USA), after conversion to an appropriate format.

3.7.1 Statistical analysis

Statistical analysis was conducted in TMeV using SAM (original method by Tusher et al., 2001). Comparison of the various groups was carried out using all possible permutations and the S0 factor derived at the 5^{th} percentile (Quon et

al., 2001). This is preferable to the original Tusher et al method as this allowed for tighter control of the FDR. Genes were considered significantly differentially expressed at FDR 1% with fold change ≥ 2. Analysis of the data was conducted in parallel by Keith Vass in BICR, using ANOVA, implemented in the Bioconductor package for R.

3.7.2 Annotation

The resultant gene lists were annotated using the Affymetrix Netaffx analysis center (http://www.affymetrix.com/analysis/index.affx), and Expression Analysis Systematic Explorer (EASE, http://apps1.niaid.nih.gov/david/, Hosack et al., 2003). These tools allow for functional annotation and linkage to multiple databases The relative overrepresentation of biological process and molecular function Gene Ontology terms (Ashburner et al., 2000) within the gene lists was assessed by Fisher exact probability ($p<0.05$). This algorithm, which is very similar to a Chi-square test, assesses the frequency of a particular GO term in the given (statistically significant) list relative to the abundance of that term in all the annotated genes on the chip.

Chapter Four: Comparison of markers previously characterised in cultures

4.1 Introduction

Our series of primary cultures covers all stages of the development of oral cancer and provides a unique opportunity to study each stage within a defined system. It has also given us the ability to assess the importance of particular molecular changes in functional studies (Muntoni et al., 2003). However, the relationship of the pattern of gene expression seen in the cell cultures to that in the tissue of derivation had not been evaluated. This question was addressed by *in vivo* analysis of the well-characterised gene expression changes associated with the immortal phenotype, which have been previously published (McGregor et al., 2002).

The *in vivo* material used in these comparisons came from two sources:

1. Fresh tissue frozen at the time of establishment of the cultures and subsequently stored at -80ºC. The amount of total RNA recovered from the biopsies varied from 1μg to 19μg. Total cellular RNA was extracted from this tissue as RNA allowed the widest range of analyses of the limited amount of material using techniques and reagents available at the time.

2. Paraffin wax blocks of formalin fixed biopsy tissue. This material was used for IHC and extraction of genomic DNA after LCM.

One caveat in the comparison of the diagnostic biopsy of origin and the culture is that it is impossible to be certain of the spatial relation of the portion of the biopsy that gave rise to the culture and the relevant wax embedded tissue block. This is less of a problem with the fresh tissue left after the establishment of the culture. Since these samples were so small, it is reasonable to assume that it was in close proximity to the portion of the lesion that gave rise to the culture. This chapter assesses the agreement between the original tissue and the derived cell culture regarding the expression of p16, telomerase (catalytic component, hTERT and RNA template, hTR), p53 and retinoic acid receptor beta 2 (RARβ_2).

Overall methodological approach

The analysis of the biological material from the cultures and the tissues utilised techniques that are inherently semiquantitative in nature. The RNA analysis by RT-PCR required careful selection of cycle numbers and experimental conditions to ensure that the reaction was limited to the linear part of the PCR reaction curve. This allowed for semiquantitative comparison of gene expression in the samples. The analyses were conducted at least three times using different amounts of RNA from the cultures and varying reaction conditions to assess this. Once the conditions were optimised, the material from the biopsies was analysed and this was also repeated to ensure a representative and repeatable result was obtained. In each case, GAPDH was amplified from the same cDNA to ensure an equal amount of material had been used in each reaction. GAPDH is useful in the assessment of loading equality, as it does not change, regardless of the proliferative fate of the cells. Most of the primers used came from established methodologies in the literature. These are acknowledged in the appropriate section (see methods). The primers for p16 were selected using the primer design program Prime, with care taken to ensure that the fragment amplified was part of exon 1α of the CDKN2a locus, thus ensuring p16 specificity.

Each Western blot was repeated at least once. Total p38 was used to assess equality of whole cell extract loading. The activity of this protein, a constituent of the MAP kinase signalling pathway, is primarily regulated by phosphorylation. The expression of total p38 (regardless of phosphorylation state) is constant regardless of the proliferative state and fate of cells in culture, thus it is useful in assessment of protein loading in Western blotting.

The IHC protocol had been developed by Ken Parkinson's group in BICR (see methods). This was optimised as appropriate for each different antibody. For each tissue sample an unrelated primary IgG control was processed in parallel. This allowed for assessment of non-specific binding of antibody. The staining was repeated to assess consistency in the intensity and extent of staining. However, only sections that had been stained in the same run were directly compared. No quantitation of staining was conducted and the resulting assessment is descriptive.

4.2 p16

The p16 protein, is an inhibitor of the Cyclin D1/CDK4 complex. This complex phosphorylates retinoblastoma protein (Rb), allowing the cell cycle to progress. Thus, p16 acts as a cell cycle inhibitor. The gene for p16, CDKN2A, also encodes for p14ARF and is found at chromosome 9p21, a common site of LoH in HNSCC (see Figure 4.1). The level of p16 within the cell can be reduced by promoter methylation, deletion or mutation (Serrano, 1997; Yoo et al., 2000). In HNSCC, deletion of p16 exon 1α is common and this also occurs in 12% of dysplasias (Ali Shahnavaz et al., 2001). Our previous work has demonstrated a clear association of loss of expression of p16 and immortality (Loughran et al., 1996; McGregor et al., 2002; Munro et al., 1999).

4.2.1 Expression of p16 protein

Protein was extracted from a panel of mortal and immortal dysplastic cultures as previously described. The expression of p16 in the cultures was assessed by Western blotting (Figure 4.2). Expression of total p38 was measured to assess the equality of loading in the gels. In general, mortal dysplastic cultures expressed p16, whilst this was lost in the immortal cultures. There were two exceptions to the correlation of loss of p16 expression and the acquisition of immortality: D17 and D38. D17 has lost expression of p16 whilst only gaining a modest increase in proliferative lifespan, whilst D38 has retained p16 expression despite having gained the immortal phenotype. This confirmed the previously described pattern in the dysplasia cultures (McGregor et al., 2002).

The expression of p16 in the tissues of origin was assessed by IHC in 5µm sections cut from the original diagnostic biopsy tissue blocks (Figure 4.3). For each tissue section, an IgG control was used to assess non-specific binding. Biopsies that gave rise to mortal cell cultures had nuclear p16 staining throughout the viable layers of the epithelium. There was also p16 expression in the tissue that gave rise to D38, an immortal culture that has retained p16 expression. On comparison with the Western blots of protein from the cultures, the agreement was good; however, heterogeneity was evident in some of the tissue samples. This was most obvious in D17, where there were small areas of

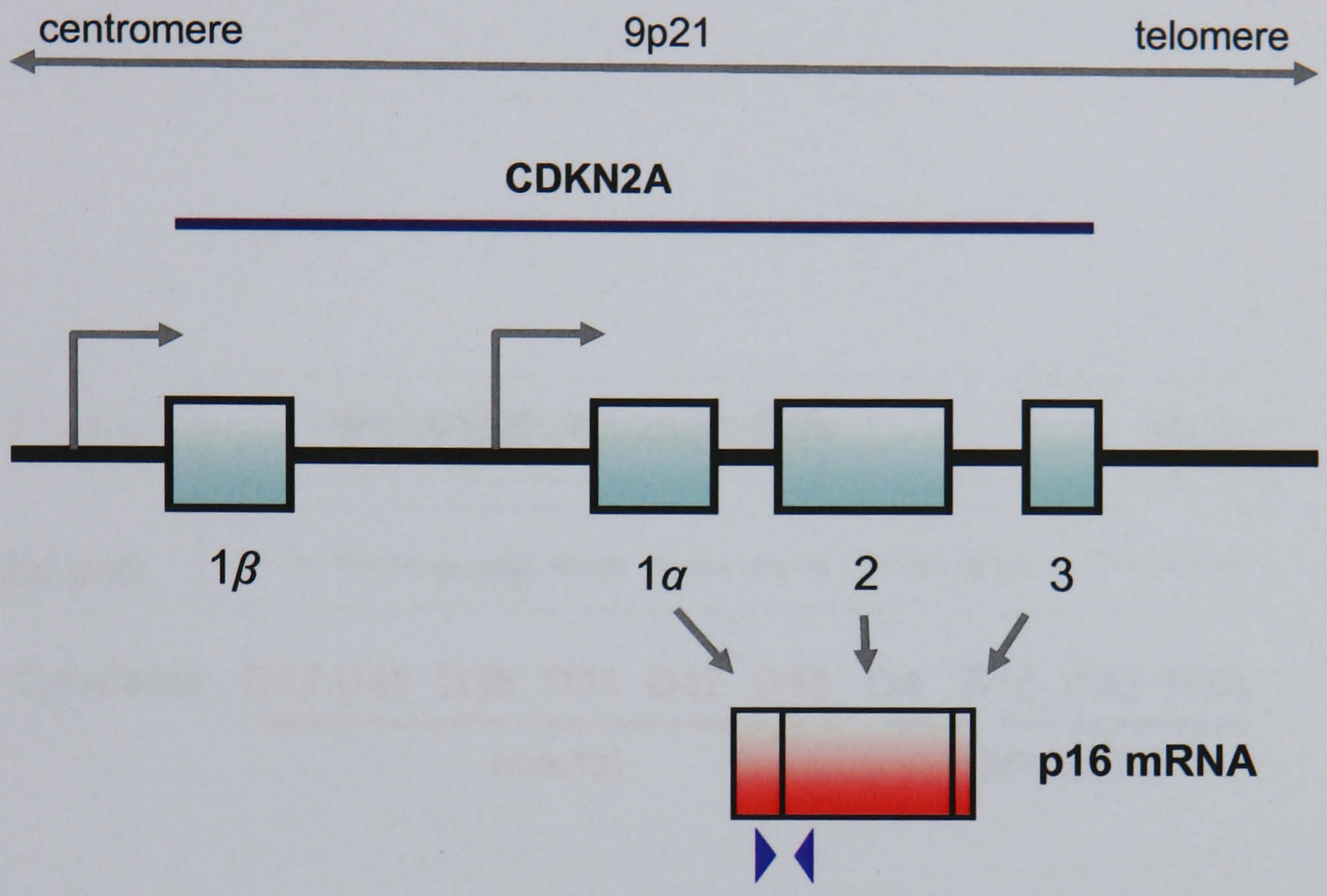


Figure 4.1 Genomic organization of the CDKN2A and gene on 9p21

The CDKN2A locus encodes two cell cycle regulators, p16 and ARF(p14), which are transcribed from separate promoters located upstream (5') of unique exons 1α and 1β respectively, a shared exon 2, with exon 3 present only in p16. The primers used for RT-PCR are marked in blue. These were designed to span the intron between exons 1α and 2, giving specificity for p16 and avoiding amplification of any contaminating DNA.

p16 inhibits the Cyclin D-CDK4/6 complex to prevent phosphorylation of Rb. ARF inhibits MDM2 mediated degradation of p53 and is induced by various oncogenic signals. ARF functions in overlapping pathways and links Rb and p53. The stabilization of p53 mediated by ARF can lead to growth arrest via p21, apoptosis and DNA repair. This figure has been adapted from Smeds et al, 2002 (Smeds et al., 2002).

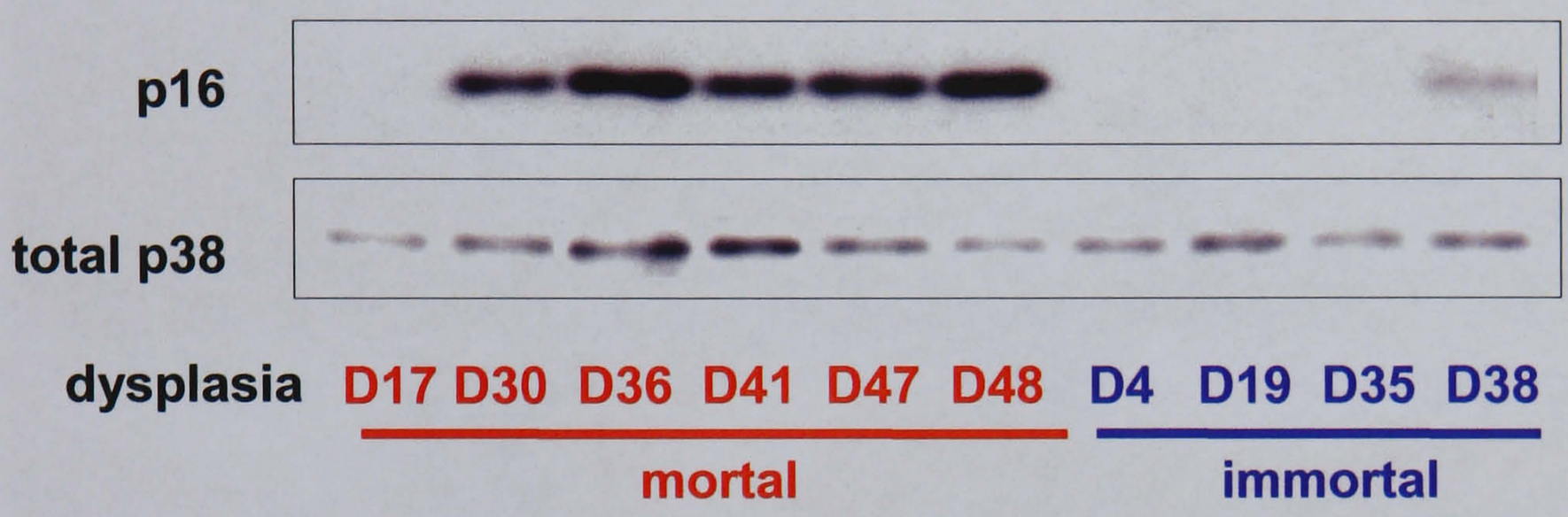


Figure 4.2 p16 immunostaining in mortal and immortal dysplasia cultures.

The panel shows Western blot of whole cell extract from cultured dysplastic oral keratinocytes using p16 antibody (F12). Total p38 was measured to assess loading of the samples

This result confirms that described by earlier work in the group (McGregor et al., 2002). The mortal dysplastic cultures express p16, except D17 which has an extended lifespan. The immortal cultures have lost the expression of p16, except D38 which is immortal and which is furthermore unusual in that it has also retained wild type p53.

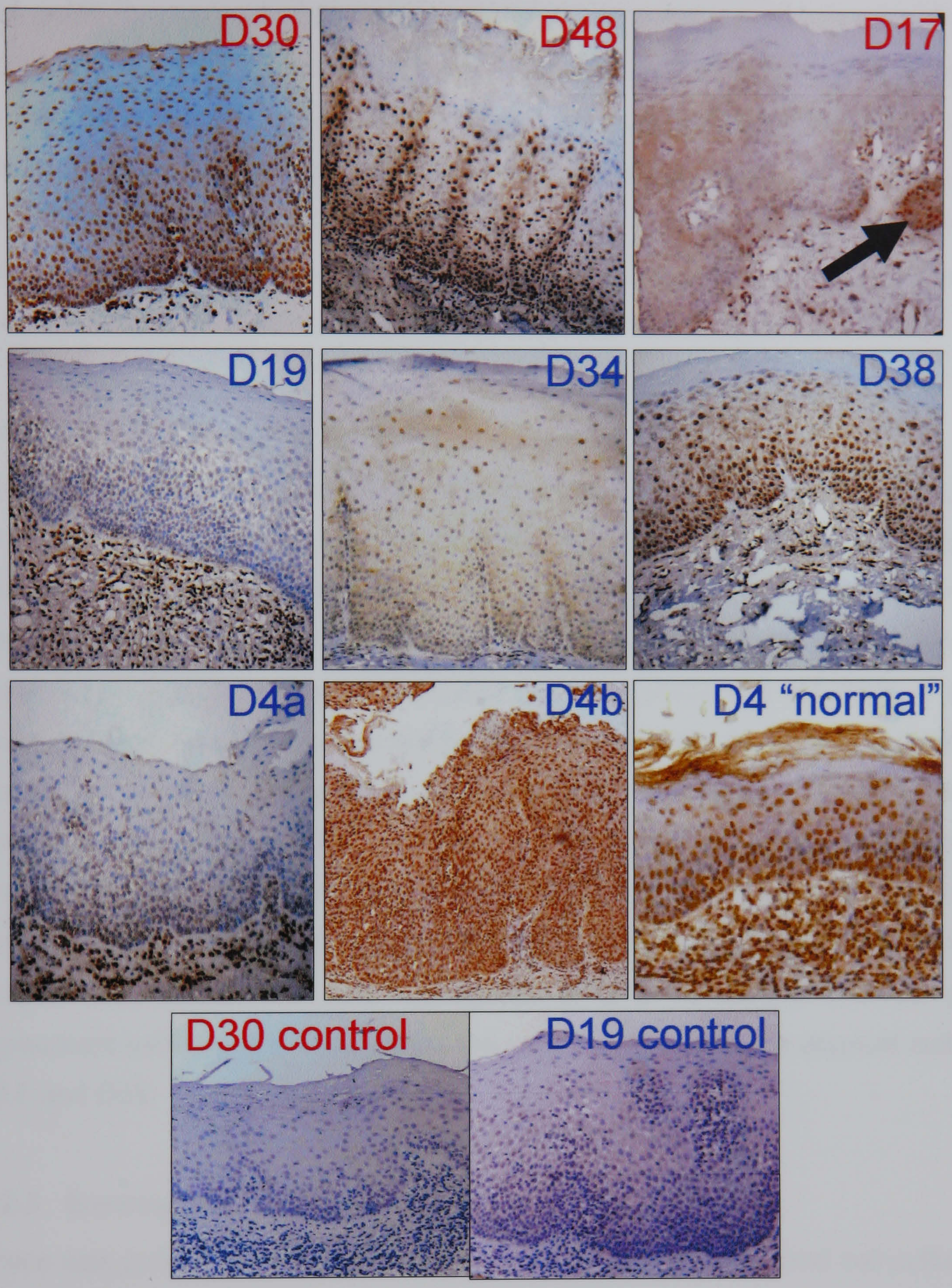


Figure 4.3 p16 immunostaining in the tissue of origin of the cultures

The panels shows p16 immunostaining in formalin fixed biopsy tissue from which the culture was derived. Negative controls, using IgG, showed no non-specific staining. These images are representative of two experiments. Cultures D30 and D48 are mortal and p16 expression is seen throughout the viable layers of the epithelium. D17 is also mortal, but has an extended lifespan. p16 expression is lost from large areas of the biopsy, but some p16 expression is retained (see arrow). D4, D19, D34 and show loss of p16 expression. However, the heterogeneity is the tissue from which D4 was derived is evident. D38 is atypical, having retained p16 expression in the culture and also in the tissue. Magnification x20, except D4 "normal" x40.

p16 positivity punctuated along the basement membrane (see arrow in Figure 4.3). The tissues that yielded immortal cell cultures showed complete loss of p16 expression in at least part of the biopsy. Tissue related to D4 was the most heterogeneous with areas of p16 positivity present alongside areas devoid of p16 expression (Figure 4.3). This tissue sample is an excision specimen and by far the largest piece of tissue examined. Therefore, this may give a realistic assessment of the heterogeneity within the lesions generally.

4.2.2 Expression of p16 mRNA

The previously published analyses of p16 in the cultures assessed the presence or absence of p16 protein (McGregor et al., 2002). Analysis of p16 transcription was important, as it had not been established if the lack of expression of protein seen in the immortal dysplastic cultures was also seen at a transcriptional level. Expression of p16 RNA was assessed by semiquantitative RT-PCR of RNA extracted from fresh tissue and the cultures (Figure 4.4). In order specifically to amplify p16 transcripts, primers were designed to span the intron between exons 1α and 2 (Figure 4.1). GAPDH mRNA levels were also measured to ensure comparable amounts of starting RNA were used. The panel of samples was smaller, than that seen in Figures 4.6, 4.8, 4.12 as some of the tissue RNA stocks were exhausted. The expression of p16 transcripts showed good agreement between the tissues and the cultures, including the atypical cultures D17 and D38.

4.2.3 Summary and discussion

There was good agreement of the expression of p16 in tissue and cell culture at mRNA and protein level. There was some heterogeneity evident, particularly in the larger tissue sections. Such differential staining raises the possibility of the presence of “mortal” and “immortal” keratinocyte clones within the same lesion, although the areas of differential staining were quite distinct. Whilst the method of Reinwald et al facilitates growth of cells from all stages of the development of keratinocyte neoplasia (Rheinwald & Green, 1975), the factors that dictate which cells from a biopsy grow in culture are unknown. It is possible that factors such as the doubling time of the cells, which is related to the time to establish in

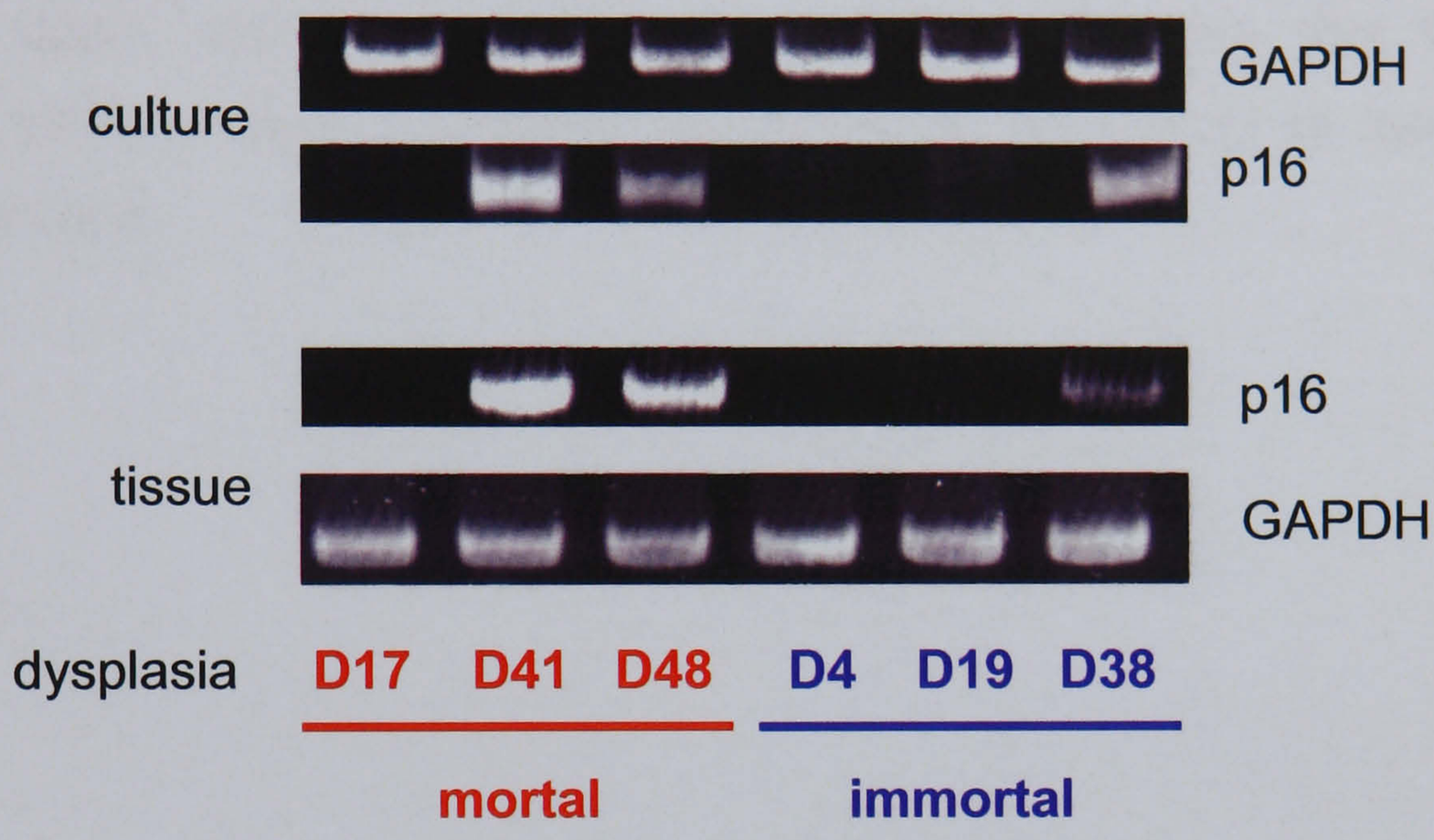


Figure 4.4 Comparison of p16 exon 1*α* expression in primary oral keratinocytes and their tissue of origin.

Expression of p16 was demonstrated by RT-PCR in a panel of mortal and immortal dysplasia cultures for which there was matched tissue RNA available. The figure shows 5% Acrylamide gel electrophoresis of p16 exon 1*α* RT-PCR products stained with ethidium bromide. GAPDH mRNA levels were also measured as a control. This data is representative of two experiments.

culture, will be important. However, since the portions of the biopsies used to establish the cultures were very small, the amount of heterogeneity therein may not have been significant.

Previously, it has been suggested that the high levels of p16 seen in cell culture are due to limitations of the culture system, and may be a response to the stress of inadequate conditions (Ramirez et al., 2001). However, this current work does not support that conclusion, as high levels of p16 were present in the tissues of origin and the corresponding culture, indicating that the p16 expression pattern seen in cultures is not merely the result of inadequate culture conditions

4.3 Telomerase

Increased levels of telomerase activity have been described in many cancers, including HNSCC. Previous work within the group has demonstrated that increased telomerase activity and levels of hTERT are associated with the immortal phenotype (McGregor et al., 2002). The telomerase holoenzyme consists of two main components, a catalytic domain with telomere-specific reverse transcriptase activity (hTERT) and an RNA template (hTR). There is much controversy in the literature as to the relative importance of these subunits in the regulation of telomerase activity. Direct measurement of telomerase activity may be assessed using the Telomerase Repeat Amplification Protocol (TRAP) assay. As this requires protein from the cells or tissues, it was not possible to assess telomerase activity in this manner.

4.3.1 hTERT

Splice deletions and insertions in hTERT transcripts result in a variety of alternative forms, which may vary in their activity (Yi et al., 2000). These various forms are summarised in Figure 4.5. Some of these alternatively spliced forms may retain very little or no telomerase activity (Colgin et al., 2000). The insertion variants all insert a premature stop codon, resulting in a truncated protein. These were not assessed in this study.

Initial experiments investigated the expression of all deletion and full length forms of hTERT using primers in a region common to all transcripts. Expression was assessed by semi-quantitative RT-PCR in the tissue and cultures (Figure 4.6). Total cellular RNA was extracted from the cultures and fresh tissue frozen at the time the cultures were established. The PCR primers used avoided regions of splice deletion (Dome et al., 1999). GAPDH mRNA levels were also measured to ensure comparable amounts of starting RNA were used. As previously described, hTERT expression in the mortal cultures was very low or absent, with higher expression evident in the immortal cultures. A small amount of hTERT expression was noted in D17. However, whilst the pattern of hTERT expression showed good agreement for the tissues related to the immortal samples, expression of hTERT was also seen in the tissue of origin of two of the mortal samples, D41 and D48.

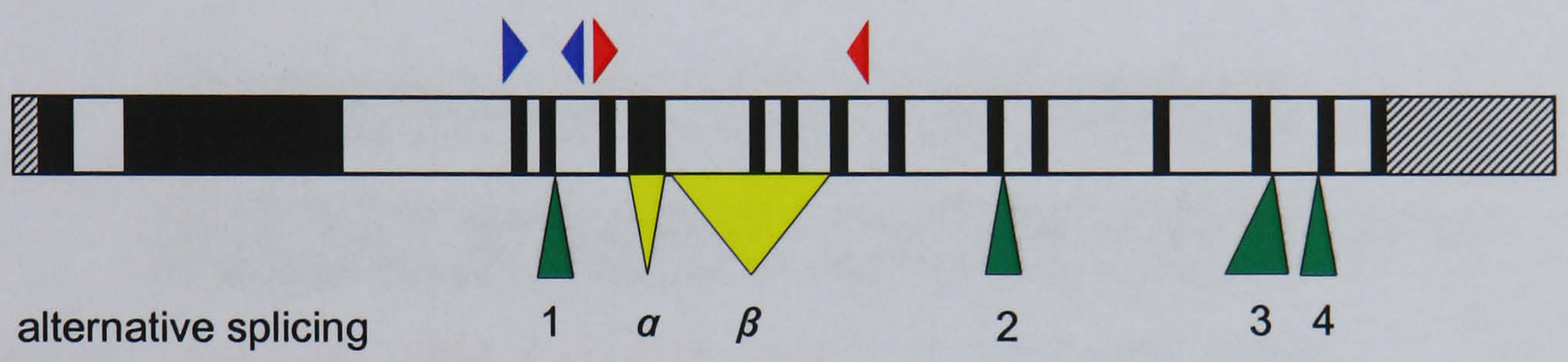


Figure 4.5 Genomic organisation of the hTERT gene

The figure shows the domains and variants of human telomerase catalytic subunit hTERT gene. Six alternate splicing sites are present (green arrows = insertions 1-4 and yellow arrows = deletions α and β). All four insertion variants generate premature stop codons. Adapted from Yi et al (2001) (Yi et al., 2001).

The primers used for semiquanitative RT-PCR are marked in blue (Dome et al., 1999). The primer set spans nucleotides 1789-1971 and does not amplify the region of either of the deletion variants of hTERT gene. It spans intron 4 to avoid analysis of contaminating genomic DNA. The primers used for analysis of the deletion splice variants are those described in Yi et al, (2001) and span nucleotides 2109-2531(marked in red) (Yi et al., 2001).

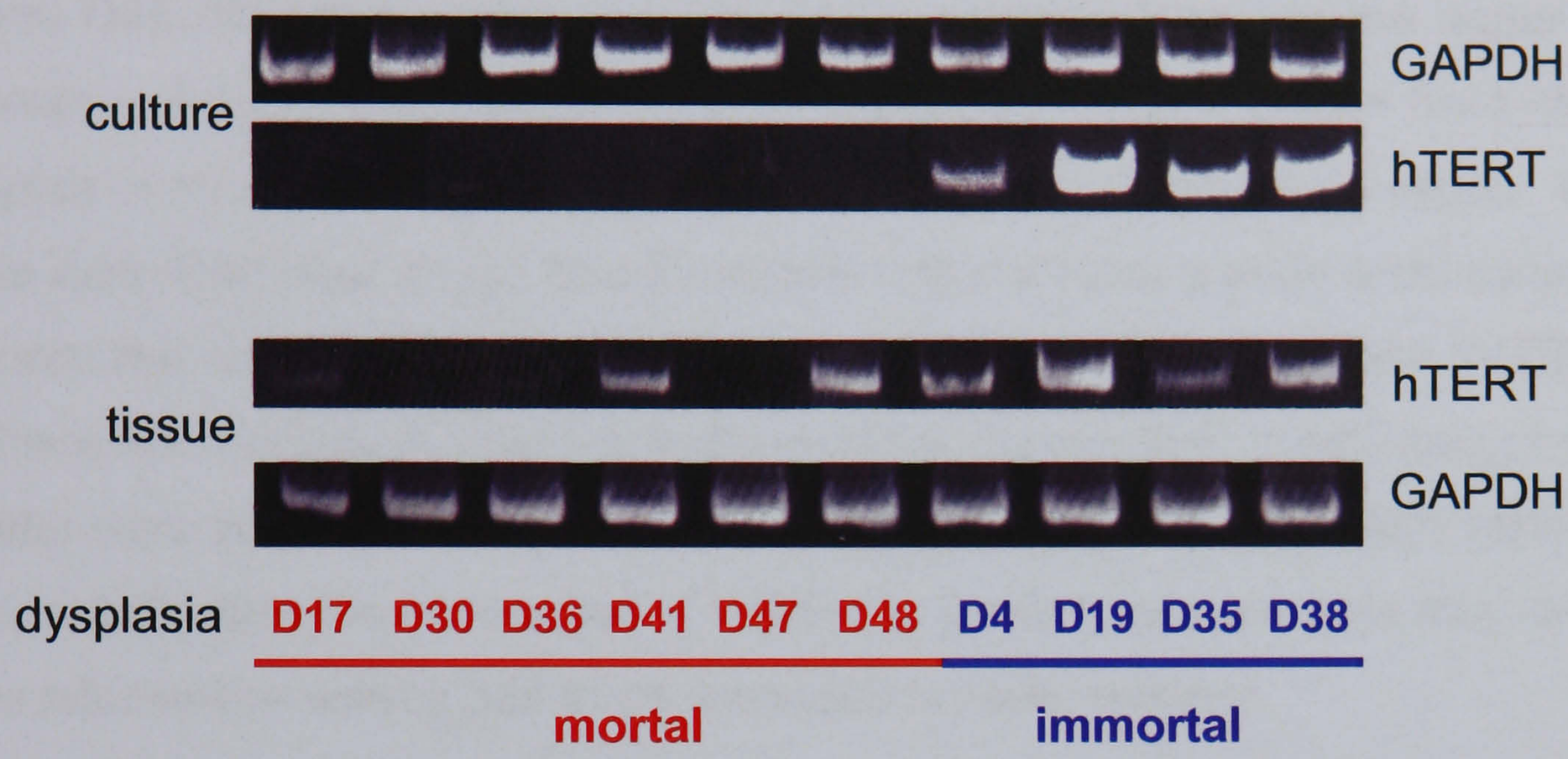


Figure 4.6 Comparison of hTERT RNA expression in primary oral keratinocytes and their tissue of origin.

Expression of hTERT was demonstrated by RT-PCR in a panel of mortal and immortal dysplasia cultures for which there was matched tissue RNA available. The figure shows 5% Acrylamide gel electrophoresis RT-PCR products stained with ethidium bromide. GAPDH mRNA was also amplified as a control. This data is representative of three experiments. Note that the tissue related to D17 does show hTERT expression albeit at a very low level.

The reasons for hTERT expression in D41 and D48 tissues are not clear. As the tissue was not microdissected in this experiment, this may represent tissue heterogeneity within the area of dysplasia. Alternatively, the possibility of contamination from cells in the lamina propria cannot be discounted, as high levels of telomerase activity have been reported in activated lymphocytes (Hiyama et al., 1995). However, the H&E stained tissue sections related to D41 and D48 did not reveal a prominent lymphoctyic infiltrate in the upper lamina propria. Additionally, as there is little evidence of contamination from other cell types in relation to p16 expression, contamination is an unlikely cause. Work to be described later shows that these two cultures have a gene expression profile more like normal cultures than the other mortal dysplastic cultures. Additionally, these are the only mortal dysplastic cultures derived from the tissues of patients who were non-smokers. Due to the varying activity of the deletion variants, the possibility that the expression of hTERT in these tissue samples may not relate to telomerase activity had to be assessed in some manner.

4.3.2 hTERT splice variants

As there was no possibility of direct assessment of telomerase activity using the TRAP assay, the deletion variants of hTERT were assessed. Admittedly, this only allows for inference of the likelihood of the hTERT expression in the tissues resulting in telomerase activity and the assessment is semiquantitative. RNAs isolated from HeLa and WI38 cells were used as positive and negative controls respectively. The data show that whilst in all cases the dominant form present was the β deletion (-β), full-length hTERT transcript was also present, the only exception being D17 tissue (Figure 4.7). The predominance of the β splice-deletion form is in agreement with the work of other groups (Yi et al., 2000). Thus, the hTERT expression demonstrated in the initial analysis of D41 and D48 was not solely due to the expression of an inactive splice variant. However, the uniform pattern seen in the tissues is not surprising, as there is no evidence in the literature for regulation of hTERT activity by alternative splicing in keratinocyte neoplasia *in vivo*.

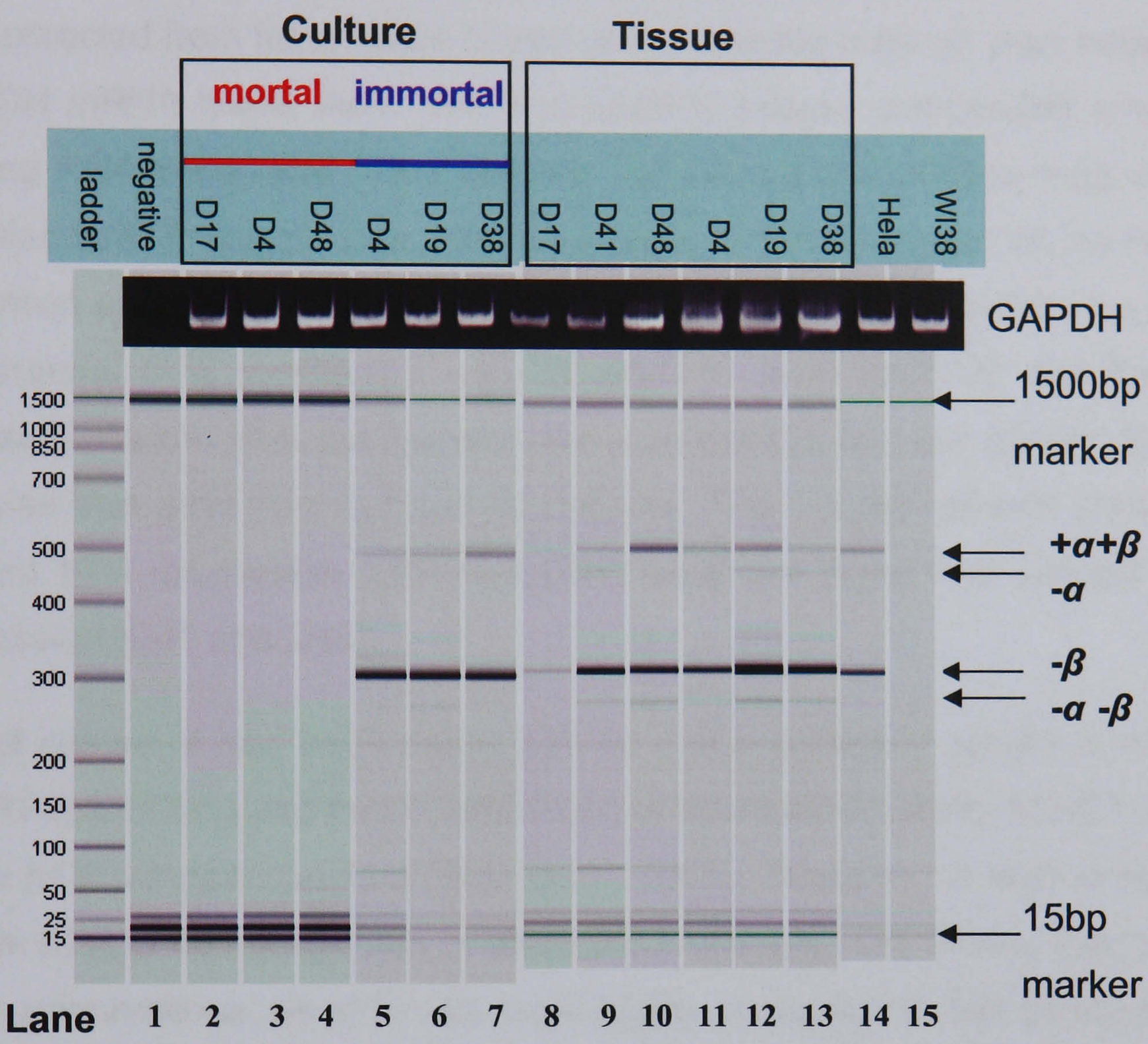


Figure 4.7 Agilent 2100 Bioanalyser "virtual gel" of hTERT mRNA deletion splice variants

The primers used were designed to detect the deletion splice variants of hTERT. The figure shows semiquantitative RT-PCR products analysed using a RNA 6000 nanochip on an Agilent Bioanalyser. The output can be visualised as a virtual gel. This demonstrated the presence of all four deletion splice variants of hTERT. HeLa and WI38 cells were used as positive and negative controls respectively.

$+\alpha+\beta$ = full length	-	active
$-\alpha$ = α splice variant		dominant negative (Colgin et al., 2000)
$-\beta$ = β splice variant		inactive
$-\alpha-\beta$ splice variant.		inactive

4.3.3 hTR

The expression of the telomerase RNA template (hTR) was assessed by semiquantitative RT-PCR in the tissue and cultures (Figure 4.8). Tissue RNA was extracted from fresh tissue frozen at the time the cultures were established. GAPDH mRNA levels were also measured to ensure comparable amounts of starting RNA were used. This analysis demonstrated that there was very little variation in hTR levels in the cultures, whether mortal or immortal, as has been described previously (McGregor et al., 2002). The exception to this was D41. In the tissues, hTR expression was detected in most samples, but there was greater variation, with the highest levels present in two out of four dysplastic biopsies that gave rise to immortal cultures. The "mortal" tissues showing the highest hTR expression (D30 and D36) were not those that showed hTERT expression (D41 and D48).

In cell culture, it has been demonstrated that telomerase activity is related to hTERT expression and that in alterations of telomerase activity hTERT reduces, whilst hTR remains constant (Shin et al., 2003). However, *in vivo* studies have shown hTR to be upregulated in some solid tumours. Our *in vivo* data supports these observations, as hTR was most highly expressed in two of the immortal dysplasias. However, the effect was not as clear as for hTERT.

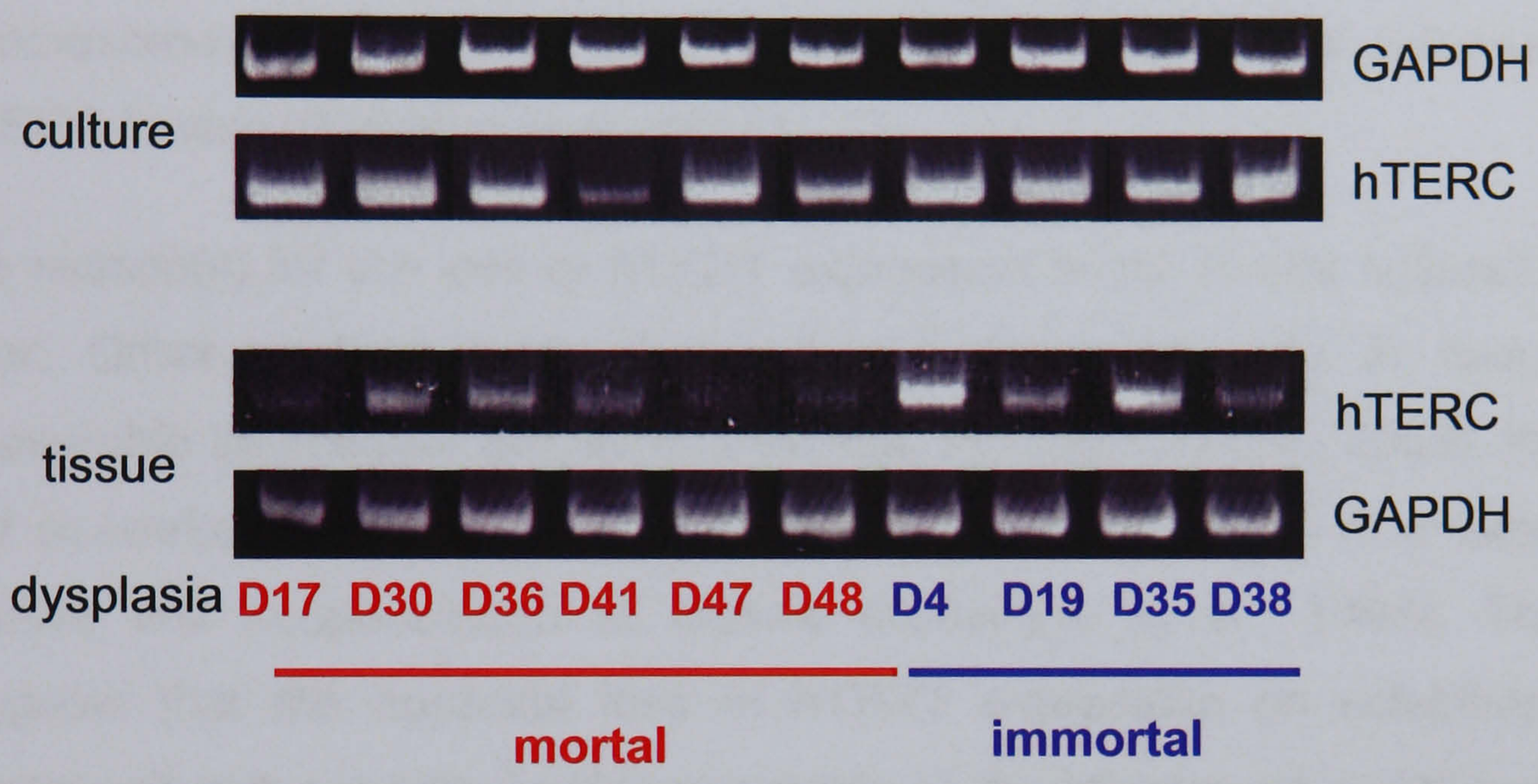


Figure 4.8 Comparison of telomerase template component (hTR) RNA expression in primary oral keratinocytes and their tissue of origin.

Expression of hTR was demonstrated by RT-PCR in a panel of mortal and immortal dysplasia culture for which there was matched tissue RNA available. The figure shows 5% Acrylamide gel electrophoresis RT-PCR products stained with ethidium bromide. GAPDH mRNA levels were also measured as a control. This data is representative of two experiments.

4.3.4 Discussion

The expression of hTERT in the immortal cultures reflected that seen in the tissues of origin. This suggests that the normal repression of hTERT expression had been lost in the subset of dysplastic tissues that gave rise to immortal cultures and this was maintained in culture. In contrast, the subgroup of dysplastic tissues that gave rise to mortal cultures generally showed no hTERT expression both *in vivo* and in culture. Why a minority of dysplastic lesions express hTERT *in vivo*, but not in culture is not entirely clear. This may be due to the persistence of, for example, the putative telomerase repressor on chromosome 3p. This is a common site of LoH in immortal cultures and in HNSCC *in vivo* (Partridge et al., 1994).

The reason(s) for the loss of hTERT expression in the mortal cultures are not clear. Other workers have shown that normal oral cells in culture lose measurable telomerase activity by passage 5 (Fujimoto et al., 2003). Kunimura and co-workers suggested that this relates to the rapid loss of a telomerase-positive cell subpopulation in culture (Kunimura et al., 1998). This work suggests that the apparent loss of hTERT expression on establishment of normal cell cultures may be due to dilution of the telomerase positive cells in a heterogeneous cell population. This hypothesis has not been rigorously demonstrated. Use of organotypic culture systems has also clearly demonstrated that telomerase activity reduces once cells are committed to terminal differentiation (Cerezo et al., 2003). However, as the monolayer culture system does not allow cells to differentiate to any degree, reduction of hTERT expression related to differentiation is an unlikely and in any case paradoxical, explanation for the phenomenon seen in our cultures.

Telomerase activity in normal cells in primary culture has also been related to the cell cycle (Kunimura et al., 1998). Telomerase increases in S phase and is dramatically reduced after S phase. In contrast, in immortalized cell lines telomerase activity remains constant with respect to the cell cycle, indicating that telomerase activity is deregulated in immortal cells (Holt et al., 1997). This may explain the good agreement of immortal cultures with tissues in our panel. Thus, whilst mortal oral keratinocytes may express hTERT *in vivo*, it is possible that only a small fraction of the mortal cells *in vitro* may be in S phase and

indeed some cells, even at low passage, may be exhibiting features of senescence. The observation that telomerase activity and hTERT expression reduces as cultured normal oral keratinocytes approach senescence has been attributed to methylation of the hTERT promoter as expression of hTERT was re-established by application of 5´Aza-2´-deoxycytidine (Shin et al., 2003).

The expression of hTR *in vivo* varies, with generally higher levels in the tissues that gave rise to the immortal cultures. This implies that the regulation of hTR expression may be different in culture from the *in vivo* situation. However, without direct measurement of telomerase activity, it is difficult to be sure of the relative importance of these subunits *in vivo*.

4.4 p53

Mutation of p53 has a well established role in the development of many cancers, including HNSCC. Cells lacking wild type p53 do not show G1 arrest in response to DNA damage and this leads to the accumulation of genetic abnormalities and also harbour defects in apoptosis. Earlier work in the group has demonstrated that p53 mutation is associated with the acquisition of immortality (McGregor et al., 2002).

4.4.1 p53 protein

p53 protein expression was measured in the cell cultures using Western blotting (Figure 4.9, lower panel). Expression of total p38 was measured to assess the equality of loading in the gel. The pattern of protein expression confirms that previously described (McGregor et al., 2002). In general, the expression in the mortal cultures was low, with the exception of D17, which had an elevated level of p53 (shown to be wild type McGregor et al., 2002). Table 4.1 shows that the protein level seen in the immortal cultures relates well to the previously published mutations in p53 (McGregor et al., 2002). The highest levels were seen in D19 and D34, both of which have homozygous mutations, whereas the protein level in D4, which had a heterozygous mutation, was lower. The frameshift mutation identified in D35 resulted in a truncated protein, which was not detected in the Western blot. The level in D38, which has wild type p53, was similar to that seen in the mortal cultures.

The expression of p53 protein in the tissues was assessed by IHC (Figure 4.9, top panel). This showed that, excepting D17, the tissues that gave rise to mortal cultures demonstrated low intensity of staining which was restricted to the basal and immediately suprabasal layers of the epithelium. In contrast, the expression of p53 in the tissues which gave rise to immortal cultures was higher in intensity and extended beyond the immediately suprabasal cells into the more superficial layers of the epithelium. This must be interpreted with some caution, as basal cell hyperplasia (BCH) is a common feature seen in reactive and preneoplastic changes in oral epithelium. The spatial effect seen may merely be a function of an increased basal compartment in these tissues.

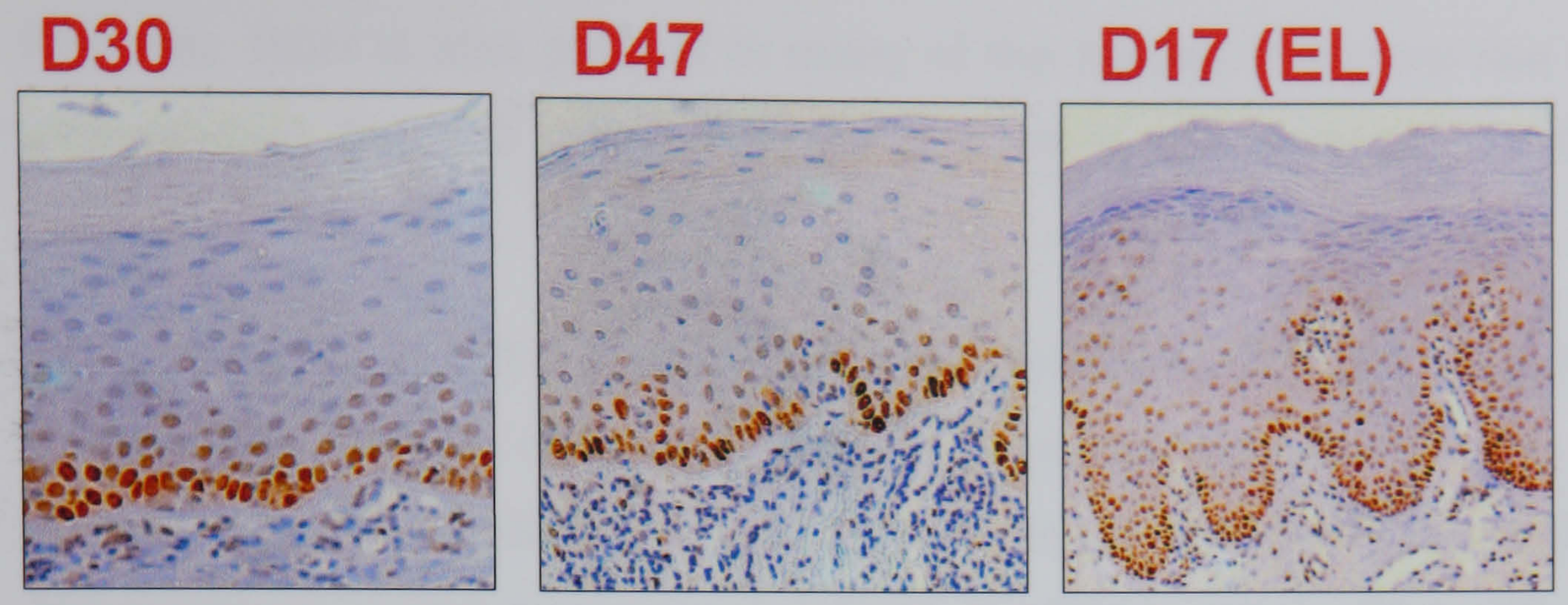


Figure 4.9 p53 (DO-1) immunostaining in primary oral keratinocyte cultures and their tissue of origin.

Upper panel shows p53 immunostaining in formalin fixed biopsy tissue from which the culture was derived. EL refers to the extended lifespan of D17. Negative controls (not shown), using IgG showed no staining.

Lower panel shows Western blot of whole cell extract from cultured dysplastic oral keratinocytes using p53 antibody (DO1). p38 was also measured to assess loading of the samples. The mutation in D35 resulted in a truncated protein and is shown here as absent. Magnification x20.

However, BCH is also present in many of the tissues that gave rise to mortal cultures and the p53 staining in the tissues is still restricted to the basal compartment.

The expression of p53 protein in the tissues related to D17 and D38 showed an intermediate pattern of staining which does not fit either of the patterns mentioned above. This may reflect heterogeneity within the tissue or the loss of p53 functions by the presence of defects at other point(s) within p53 regulated pathways, but without the increase in staining seen in a mutated and stabilised p53. The p53 protein results are summarised in Table 4.1.

	cultures			biopsy
dysplasia	growth	p53 gene status	protein WB	protein IHC
D17	mortal	wild type	mod	intermediate
D30	mortal	wild type	low	basal
D36	mortal	wild type	low	basal
D41	mortal	wild type	low	basal
D47	mortal	wild type	low	basal
D4	immortal	mutation, exon 5, stop codon	mod	upper layer
D19	immortal	mis-match mutation, exon 5	high	upper layer
D34	immortal	mis-match mutation, exon 4	high	upper layer
D35	immortal	frame-shift mutation, exon 6	absent	none specific
D38	immortal	wild type	mod	intermediate

Table 4.1 A comparison of the in vitro gene status of p53 with p53 protein expression in vitro and in vivo.

The cell culture p53 mutation data is from McGregor, Muntoni et al (2002) (McGregor et al., 2002). Protein expression in the cultures was assessed by Western blot (WB) as shown in Figure 4.9.

Protein expression in the tissues was assessed by immunostaining of formalin-fixed biopsy tissue (IHC), as shown in Figure 4.9.

4.4.2 p53 expression in organotypic culture

In order to assess the effect of reconstituting a stratified squamous epithelium on the expression pattern of p53, an organotypic culture system was developed. This used fibroblasts derived from an oral premalignant lesion (which also gave rise to the keratinocyte culture D19) set in a collagen gel matrix. The epithelial cells (2 mortal cultures and 2 immortal cultures), placed on collagen gels at the air interface, were fed from below. This resulted in the formation of a stratified squamous epithelium over about 11 days (Figure 4.10). Immunohistochemistry revealed that the organotypic cultures maintained the difference in their expression of p53 seen in monolayer culture, although it was not as clear as in the original tissues (Figure 4.10, compared with Figure 4.9). This demonstrated that the alterations in p53 expression were an intrinsic feature of the cells, as reconstitution of a stratified epithelium in organotypic culture showed a similar pattern of expression of p53 to that in the original biopsy.

4.4.3 p53 gene status

Sequencing of specific sections of the p53 gene was carried out in order to assess the genetic relationship between the immortal cultures and the biopsies. As p53 mutations had been previously described in the immortal cultures *in vitro*, sequencing of material from the *in vivo* samples was only conducted in these areas (McGregor et al., 2002). Initially cDNA derived from total cellular RNA by RT-PCR was sequenced. This demonstrated that the same mutation was present in the tissue related to D19, but was heterozygous (Table 4.2). The requisite mutation could not be demonstrated in D4. However, the mutation in D4 resulted in a stop codon and this may be removed by nonsense mediated mRNA decay (reviewed in Culbertson, 1999). There was insufficient RNA to gain satisfactory results from D34 and D35.

DNA was extracted from laser capture microdissected (LCM) tissue from formalin fixed sections of the original biopsies (Figure 3.1). This allowed for sampling of phenotypically different areas of epithelium. The heterozygous mutation found in D4 in culture was present in the dysplastic area of the tissue section, but was absent from "normal" adjacent epithelium (Table 4.2).

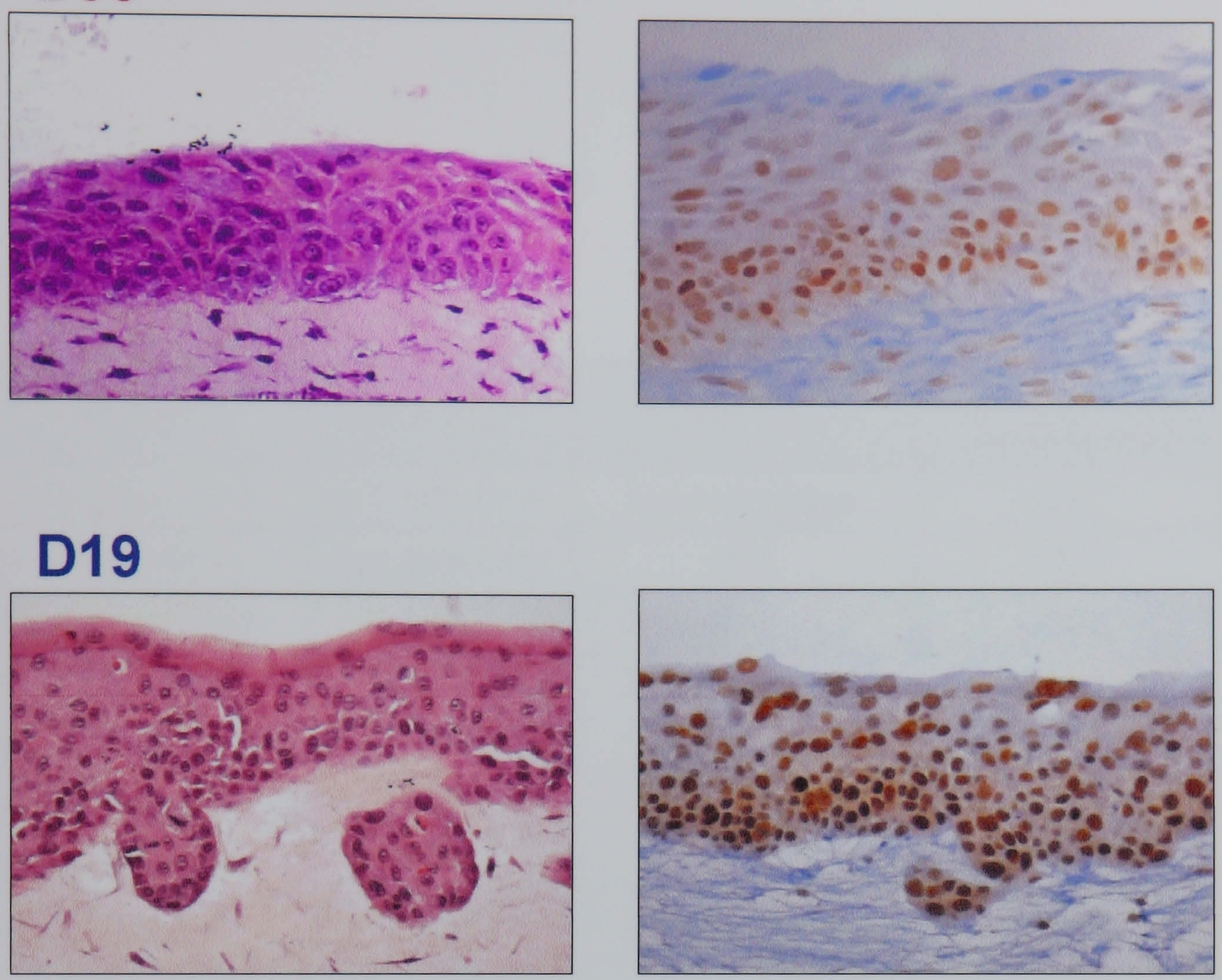


Figure 4.10 Formalin–fixed tissue derived from organotypic cultures of dysplastic oral keratinocytes D19 (immortal) and D30 (mortal).

Left panels formalin-fixed tissue stained with haematoxylin & eosin. Magnification x20

Right panels show p53 protein expression (immunostaining using antibody DO-1 with haematoxylin counterstain). Comparison with corresponding panels in Figure 16 shows that p53 expression in D30 extends more superficially in organotypic culture than in the tissue. However, on comparison with D19, a clear difference in expression pattern was maintained. Magnification x40

sample	mutation	zygosity	Same mutation as culture?
D19 biopsy RNA	NT 488 C>A	heterozygous	yes*
D4 biopsy RNA	not detected	N/A	N/A
D19 biopsy DNA	NT 488 C>A	heterozygous	yes
D4 biopsy DNA dysplastic	NT 489 C>A	heterozygous	yes
D4 biopsy DNA non-dysplastic	not detected	N/A	N/A
D9 biopsy DNA	NT 1009 C>T	heterozygous	yes
D20 biopsy DNA	NT 743 G>A	heterozygous	yes

Table 4.2 Comparison of p53 mutations *in vitro* and *in vivo* in D4, D19, D9 and D20.

The mutations were initially detected in cell culture (McGregor et al., 2002). Primers were designed to detect the specific mutation found in the culture and sequencing was performed on cDNA derived from

- total cellular RNA (from frozen tissue) – by RT-PCR
- DNA (from laser capture microdissection) – by PCR.

***Note**: The fact that the mutation in the biopsy is heterozygous may indicate some level of stromal or normal epithelial contamination or heterogeneity within the sample.

The mutation found in D19 in culture was also seen in the tissue, but was heterozygous. This may indicate some level of stromal/normal epithelial contamination or heterogeneity within the sample. The mutations previously seen in D9 and D20 were also present in the original biopsy tissue. Analysis of D34 and D35 is ongoing.

4.4.4 Summary and discussion

This analysis demonstrates that the differences in p53 expression seen in the culture were present in the tissue of origin. Whilst others have demonstrated that suprabasal p53 expression in dysplastic oral epithelium is associated with an increased risk of progression to carcinoma (Cruz et al., 2002; Cruz et al., 1998), this data is novel in that it relates the alterations in the intensity and spatial distribution of p53 staining to p53 mutation and immortality in cell culture. As the mutations seen in the cultures were also demonstrated in the tissues from which they were derived, this demonstrated a clear genetic relationship between the immortal cells in culture and their tissues of origin.

4.5 Retinoic acid receptor beta (RAR-β)

Retinoids can regulate gene expression and thus modulate differentiation and growth in cells (Lotan, 1996) and there has been much interest in the effects that deregulation of this system may have in carcinogenesis. Much interest has been expressed in their potential use in the treatment of dysplastic lesions; however, this modality has proven to be disappointing. Downregulation of RAR-β has been reported in many cancers, including HNSCC (Lotan et al., 1995). We have previously described constitutive expression of RAR-β in our mortal dysplastic cultures with complete loss of expression in our immortal dysplastic cultures (McGregor et al., 1997).

Multiple isoforms of RAR-β have been described. Among the four known RAR-β isoforms, RAR-β2 is thought to be the key tumour suppressor in humans (Lotan et al., 1995; Toulouse et al., 2000), but the possibility remains that RAR-β1 is also involved. The expression pattern of RAR-β1 suggests it is a fetal isoform (Toulouse et al., 1996), but no direct tests of the effect of the latter on tumour cell phenotype have been published. It is very similar to RAR-β3 which is not found in human cells. The fourth isoform, a truncated version of RARβ2 named RARβ4, is associated with tumorigenicity in humans and mice, so it is unlikely to have a tumour suppressor activity (Berard et al., 1994; Hayashi et al., 2003) The structure of isoforms RARβ2 and RARβ4 is shown in Figure 4.11.

Unfortunately, the commercially available RARβ primary antibodies appear neither specific nor reproducible in IHC or Western blot. Therefore, there is no data on the expression of RARβ protein in the cultures or the tissues.

4.5.1 RNA

The expression of RAR-β_2 was assessed by semi-quantitative RT-PCR in the tissue and cultures (Figure 4.12). Tissue RNA was extracted from fresh tissue frozen at the time the cultures were established. GAPDH mRNA levels were also measured to ensure comparable amounts of starting RNA were used. The pattern of expression in the cultures agrees with the pattern previously described by Northern blotting i.e. constitutive expression in mortal cultures with no expression in immortal cultures, except D38 (McGregor et al., 1997). However,

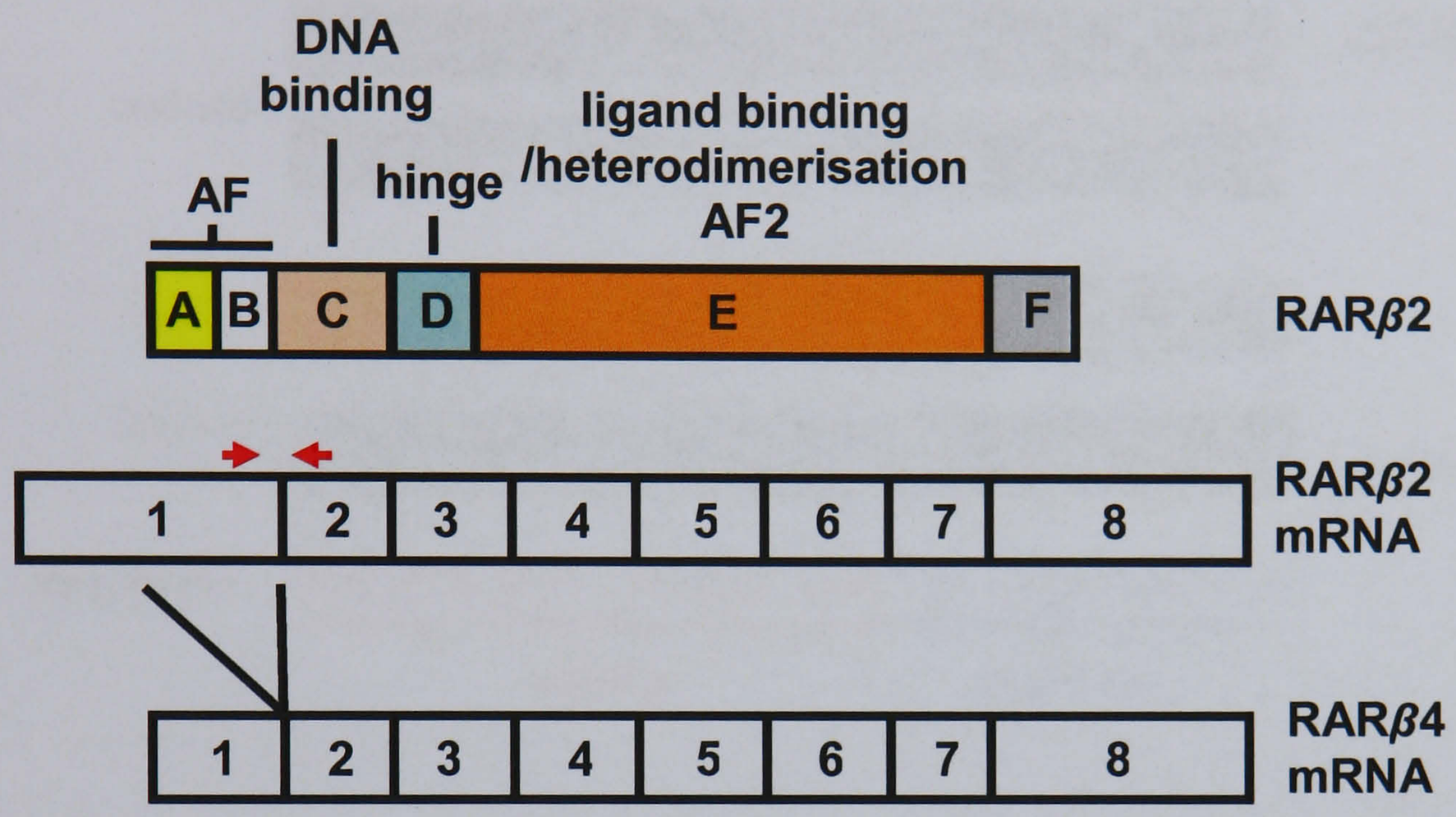


Figure 4.11 Diagram of RARβ2 and RARβ4 transcripts and RARβ2 protein functional domains

The most recent annotation of the RARβ locus and various isoforms is outlined in Ensembl transcript ID ENST00000330688 (http://www.ensembl.org). Much of the literature refers to the mRNA sequence of RARβ as exons 5-12. This diagram adopts the numbering sequence assigned in the Ensembl annotation (exons 1-8).

RARβ1(and RARβ3) differ from RARβ2 in their 5'UTR and A coding region. RARβ4 is generated by alternative splicing in exon 1 (Zelent et al., 1991). The RARβ2-specific primers used were those published by Sirchia et al (Sirchia et al., 2000). The positions shown by the red arrows.

The figure is adapted from Chen et al, 2002 (Chen et al., 2002).

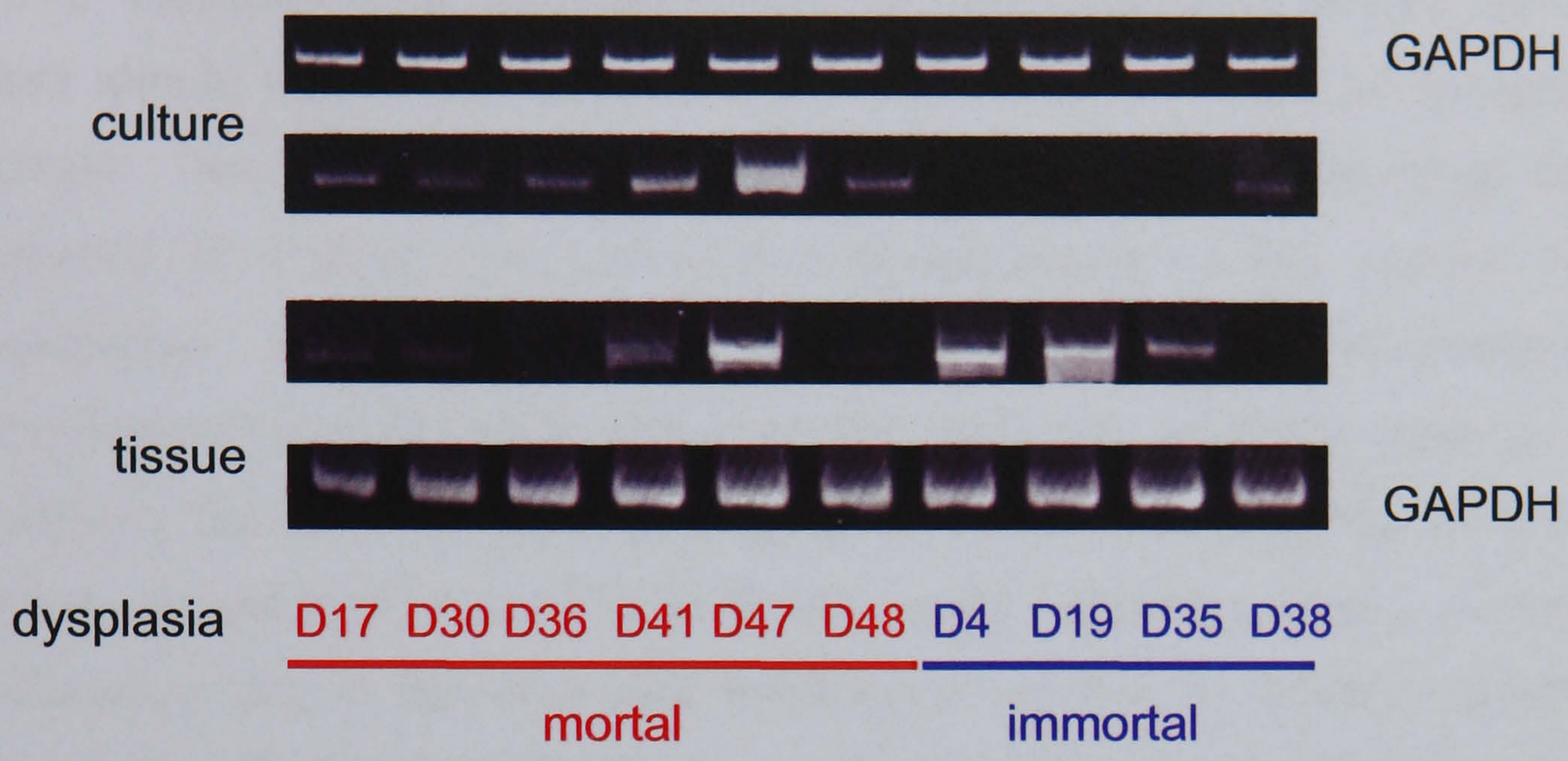


Figure 4.12 Comparison of retinoic acid receptor β2 (RARβ2) expression demonstrated by RT-PCR in a panel of cultures derived from dysplastic oral epithelium and their tissue of origin.

Expression of RARβ2 was demonstrated by RT-PCR in a panel of mortal and immortal dysplasia cultures for which there was matched tissue RNA available. The figure shows 5% Acrylamide gel electrophoresis of RT-PCR products stained with ethidium bromide. GAPDH mRNA levels were also measured as a control. This data is representative of two experiments.

Tissue expression of RARβ2 matched expression in the mortal cell cultures to some degree, but the immortal cell cultures did not match at all.

whilst RAR-β_2 was not expressed in the immortal cultures, it was expressed in three out of four dysplastic biopsies related to these cultures.

Mesenchymal contamination is a possible explanation, as fibroblast cultures express high levels of RAR-β in the presence of retinoic acid (Crowe et al., 1991). However, this observation has not been confirmed *in vivo*, and as this effect should have been seen in the "mortal" tissues, such an explanation is unlikely. Alternatively, heterogeneity in gene expression resulting from the presence of mortal and immortal subpopulations in the tissues may be responsible. This explanation assumes that, whilst mortal and immortal cells were present in the tissue, the immortal cells preferentially grew in culture. However, the lack of heterogeneity of p16 and p53 staining, particularly in tissues related to D19 and D35 mitigates against this conclusion. A more likely explanation lies in the now well established relation of RAR-β expression to squamous cell differentiation (Lotan, 1996). Therefore, the discrepancy in the tissues of origin may be explained by changes in RAR-β expression associated with terminal differentiation in the tissues. Such changes may not be apparent in monolayer culture of immortal cells.

4.5.2 RARβ2 expression in organotypic culture

In order to assess the influence of differentiation on the RAR-β_2 expression in the immortal cell cultures, an organotypic culture system was utilised. The expression of RAR-β_2 in the stratified squamous epithelium formed over a period of 11 days was assessed by semiquantitative RT-PCR of total cellular RNA (Figure 4.13). GAPDH mRNA levels were also measured to ensure comparable amounts of starting RNA were used. The differences in expression of RAR-β_2 between mortal and immortal dysplasias in monolayer culture were also seen in organotypic culture, indicating that a change in the activation of the terminal differentiation pathway did not result in the re-expression of RAR-β_2 in immortal cells. Thus, a difference in differentiation state was not the sole reason for the poor agreement of the immortal culture findings with their tissue of origin.

However, the organotypic culture system does not fully reconstitute *in vivo* conditions. Many other cell types are present within the lamina propria that may

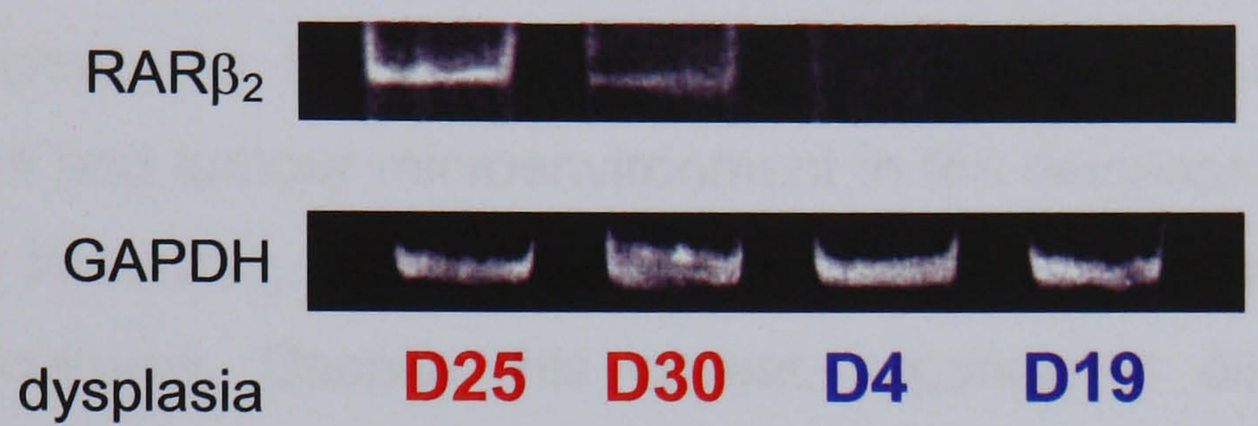


Figure 4.13 RAR*β*2 expression in organotypic oral keratinocyte cultures assessed by RT-PCR

Expression of RARβ2 was demonstrated by RT-PCR in a panel of two mortal (D25 and D30) and two immortal cell cultures (D4 and D19) grown in an organotypic culture system. The figure shows 5% Acrylamide gel electrophoresis of RARβ2 RT-PCR products. GAPDH mRNA was also amplified as an expression control.

The figure shows that growth of these primary cultures in a system which allows differentiation to occur did not result in a change in the expression of RARβ2 in the mortal or immortal cultures.

contribute to variations in the oxidative state. In addition, the serum used in the feeding medium contains an undefined level of retinoids. It may be interesting to repeat the experiment with varying concentrations of retinoids, in order to recreate a physiological retinoid gradient across the epithelium.

4.5.3 Discussion

The agreement of RAR-β expression in the cultures and tissues was poor, particularly in the tissues that gave rise to immortal cultures. The reasons for this are unclear. As the pathways and interactions related to the role of differentiation and tumour microenvironment in the development of HNSCC are undoubtedly important, it is a disadvantage that these cannot be examined in monolayer cultures. Despite this caveat, organotypic culture showed that induction of differentiation did not result in the expression of RAR-β in the cultures. The reasons for this are not clear

These results have implications for the extrapolation of *in vitro* gene expression data to the *in vivo* state. Validation of the markers related to the pathways described above (and perhaps others) must be conducted with care, bearing in mind the possibility that the *in vivo* correlation of the expression of such genes with that in the monolayer culture may be poor.

Chapter Five: Microarray study

5.1 Introduction

Analysis of the global gene expression profile of the cell cultures, both mortal and immortal was conducted using the Affymetrix U133A and U133B genechips. This allowed further analysis of the differences in gene expression seen on comparison of cultured mortal and immortal dysplastic oral keratinocytes. This data was further augmented by analysis of normal oral keratinocyte cultures and those from carcinomas, both primary tumours and metastases, a full list of which is seen in Table 3.1. The range of samples analysed allowed for fuller characterisation of events at various stages of the development of oral squamous cell carcinoma. These results refer primarily to the differences associated with keratinocyte immortality.

All preparatory work for the microarray analysis was carried out at the BICR. This included culture of the cells, harvest and isolation of RNA, and the various stages of analysis and labelling to produce the labelled, fragmented cRNA ready for hybridisation to the genechip arrays. The fragmented cRNA was sent to the Microarray Core facility at the Paterson Institute for Cancer Research, where the hybridisations and scanning were carried out. The RNA was only processed if the stringent quality controls set by the PICR were met (see materials and methods). A test array was used for at least one sample in every batch sent. The raw data was returned to the BICR for subsequent analysis.

5.1.1 Transformation and normalisation

All of the raw microarray data was analysed as shown in Figure 5.1. The matrix was log2 transformed to improve the data distribution. This allows easier assessment of the degree to which different genes alter their transcription. The data was normalized using the Robust Multichip Average (RMA) method (Irizarry et al., 2003) implemented in RMAExpress http://stat-www.berkeley.edu/users/bolstad/RMAExpress/RMAExpress.html.

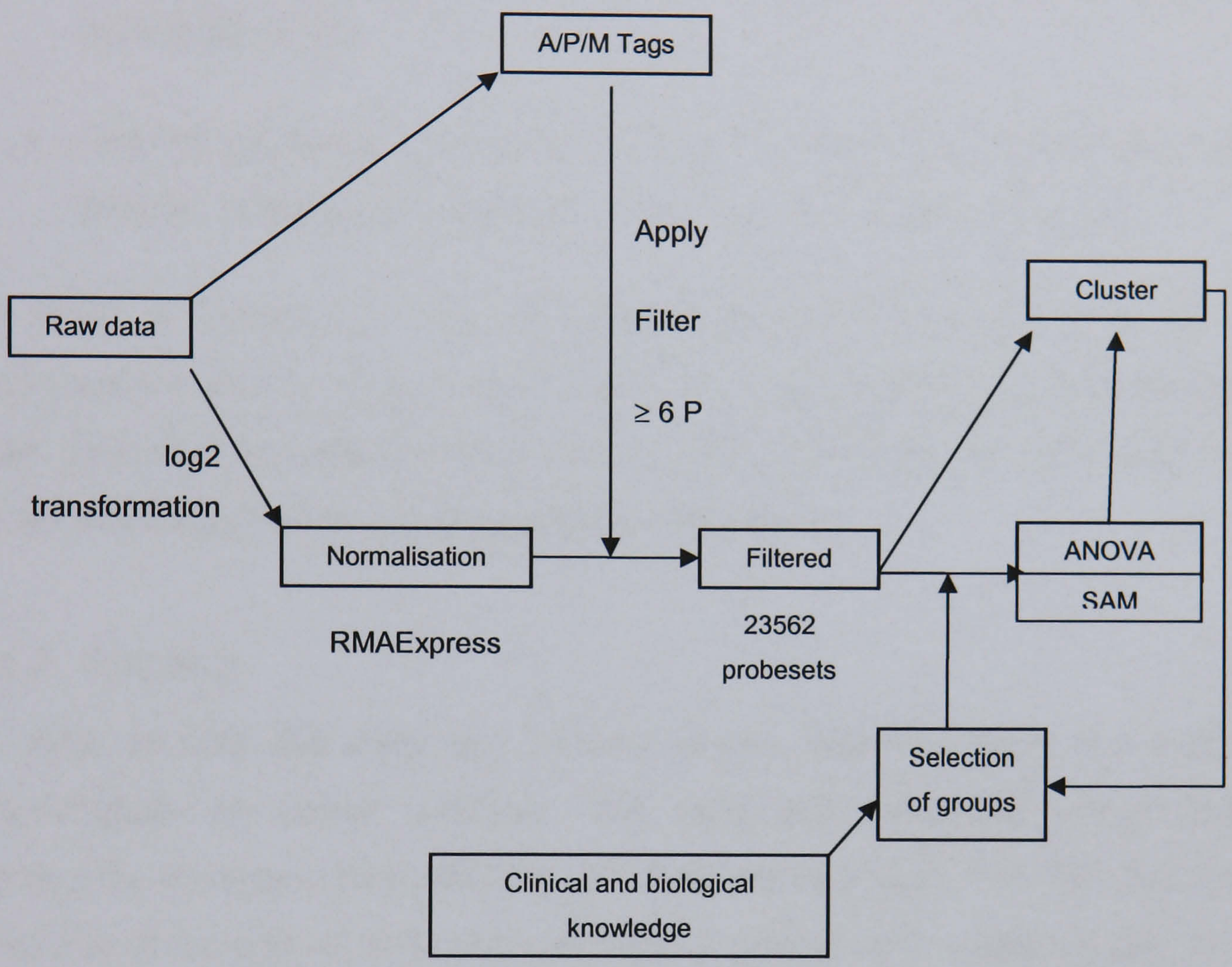


Figure 5.1 Schematic flow diagram of microarray data analysis

This diagram shows the standardised process which was developed for data analysis for all samples in the main study. The entire group of filtered genes are hereafter referred to as “all present genes”.

RMA performs the following operations:

- Probe-specific background correction to compensate for non-specific binding using perfect match (PM) values alone (mismatch (MM) values are not utilised).

- Probe-level multichip quantile normalization to unify PM distributions across all chips

- Robust probeset summary of the log normalized probe level data by median polishing, resulting in a single value for the probe set.

As RMA is based on rank normalisation, the distribution in each sample becomes the same. This results in a compression of the dynamic range of the data. This may be limiting in terms of the statistical analysis of the data, but may be an advantage when using clustering algorithms.

5.1.2 Filtering

In order to filter the data and remove probe sets that were not contributing meaningfully to further analysis, the data was analysed using Affymetrix Microarray Analysis Suite (MAS) 6.0. This utilises both the PM and MM data from the array and on this basis assigns a present (P), marginal (M) or absent (A) flag to each probe set in a particular sample. These flags were used to filter the data, as only probesets which were judged "present" in 6 or more samples were used in further analysis. In the main study, this reduced the number of probesets forwarded to further analysis from 44930 to 23562.

5.1.3 Data clustering

Hierarchical cluster analysis was used to arrange genes according to similarity in pattern of gene expression. This uses standard statistical algorithms and the output is displayed graphically, conveying the clustering and the underlying expression data. Clustering methods can be divided into two classes, supervised and unsupervised. In supervised clustering, vectors are classified with respect to known reference points. In unsupervised clustering, no predefined references are used (as in hierarchical clustering). Eisen recently reviewed various methods (Eisen et al., 1998).

Hierarchical clustering is widely used to find patterns in multidimensional datasets, especially for microarray data. The object of this algorithm is to compute a cluster dendrogram that assembles all gene expression elements into a single tree. The dendrogram produced is a binary tree in which each data point corresponds to terminal nodes and distance from the root to a subtree indicates the similarity of subtrees – highly similar nodes or subtrees have joining points that are farther from the root. It must be remembered that at the highest levels of the hierarchy the branches are very dependent on the lower links, thus they are less reliable. Almost all hierarchical agglomerative algorithms are safe when the interest is placed on the small, low levels of the hierarchy, but they become unsafe at high levels. The groups of genes derived from the cluster analysis showed great similarity to clinical and biological knowledge of the cultures in terms of type (normal, dysplastic or carcinoma) and replicative fate (mortal or immortal).

5.1.4 Statistical analysis

Statistical differences in gene expression were assessed using the Statistical Analysis of Microarray (SAM) algorithm (Tusher et al., 2001). The multiple hypotheses tested on analysis of microarray data mandates control of the type I error rate (false positives). In SAM this is defined as the False Discovery Rate (FDR). SAM identifies genes with statistically significant changes in expression by assimilating a set of gene-specific t tests. Each gene is assigned a score (delta, Δ) based on its change in gene expression relative to the standard deviation of repeated measurements for that gene (permutations). Genes with scores greater than a threshold, Δ, are deemed significant. The threshold can be adjusted to identify smaller or larger sets of genes. The percentage of such genes identified by chance is the FDR.

5.2 Pilot study

The pilot study assessed several important variables that may impinge on the gene expression profile. The 3T3 feeders are required for many of these cells to survive, but removal of the feeders may have differential effects depending on cell type, as some of the carcinomas will grow in the absence of an irradiated 3T3 feeder layer. In addition, there was concern that an immediate stress response may alter the GEP, as the feeders were being removed using PBS/EDTA. Assessment of the effects of serum replenishment on the gene expression profile and of two different operators growing the cells, preparing and labelling the RNA, was also required.

As it was unclear whether the method of preparation of the cells would have an effect on the gene expression profile, or affect the normal and SCC cultures differently, different protocols for the removal of the feeder cells were assessed:

A. Removal of irradiated 3T3 feeders 24 hours prior to RNA extraction. The cells were fed for the intervening period. This allowed for much quicker extraction of RNA after removal of the medium, but cells may respond differently without feeders present.

B. Removal of the feeders immediately followed by RNA extraction. This minimised variation in culture conditions, but increased the possibility of an immediate stress response.

The cluster dendrogram in Figure 5.2 shows a clear difference between the gene expression profiles of the normal culture (FNB5) and carcinoma (BICR 56) as assessed by hybridisation to the Affymetrix U133A genechip. It also demonstrated that the agreement of the technical replicates was good, despite that fact that the labelling of the replicates was carried out by two different operators (the author and Janis Fleming). On this basis, the subsequent main microarray study used duplicate technical replicates. The good reproducibility of the GEP of the technical replicates in the pilot study was also seen throughout the main study (data not shown). The differences in the GEP seen due to alternative timings of feeder removal in prep method were greater than between different operators using the same protocol (Figure 5.2).

In order to establish whether any 3T3 feeders remained on the plate and were differentially contributing to the GEP, cRNA prepared from irradiated 3T3 feeders was analysed on an Affymetrix U133A genechip and is also seen in the dendrogram in Figure 5.2. This shows that the gene expression profile of the irradiated 3T3s was very different from the keratinocytes, regardless of how they were prepared. The 20 highest expressed genes in the 3T3s are shown in Table 5.1, in comparison with the levels seen in FNB5 and BICR 56 (by both methods of 3T3 removal). Very few genes were more highly expressed in the irradiated 3T3 feeders than in the keratinocyte cultures. Furthermore, only one of these genes (GNAS) showed any differential expression between the two methods of 3T3 removal.

Application of SAM to the two timings of 3T3 removal yielded a list of consistently differentially expressed genes seen in Figure 5.3 (FDR = 1%, fold change ≥ 2). This list includes genes involved in cell-to-cell communication, such as interleukin 8, several interleukin receptors and also genes involved in adhesion, such as cadherin 11 and integrin alpha 2. Many of these genes have a role in communication between the fibroblasts and the keratinocytes, which may indicate a role in aiding the survival of the cultures.

Using 2-factor ANOVA, a small group of genes was identified in which the different methods had a differential effect on the cultures. This showed that the effect on the normal cultures of early removal of 3T3s was greater than in the carcinoma culture, as the method A normal samples cluster differently to the method B normal samples ($p<0.001$). The group of genes differentially affected by the early removal of the 3T3s is shown in Figure 5.4. However, the group of genes is small and the fold changes of the genes involved were not large, so the differential effect is subtle.

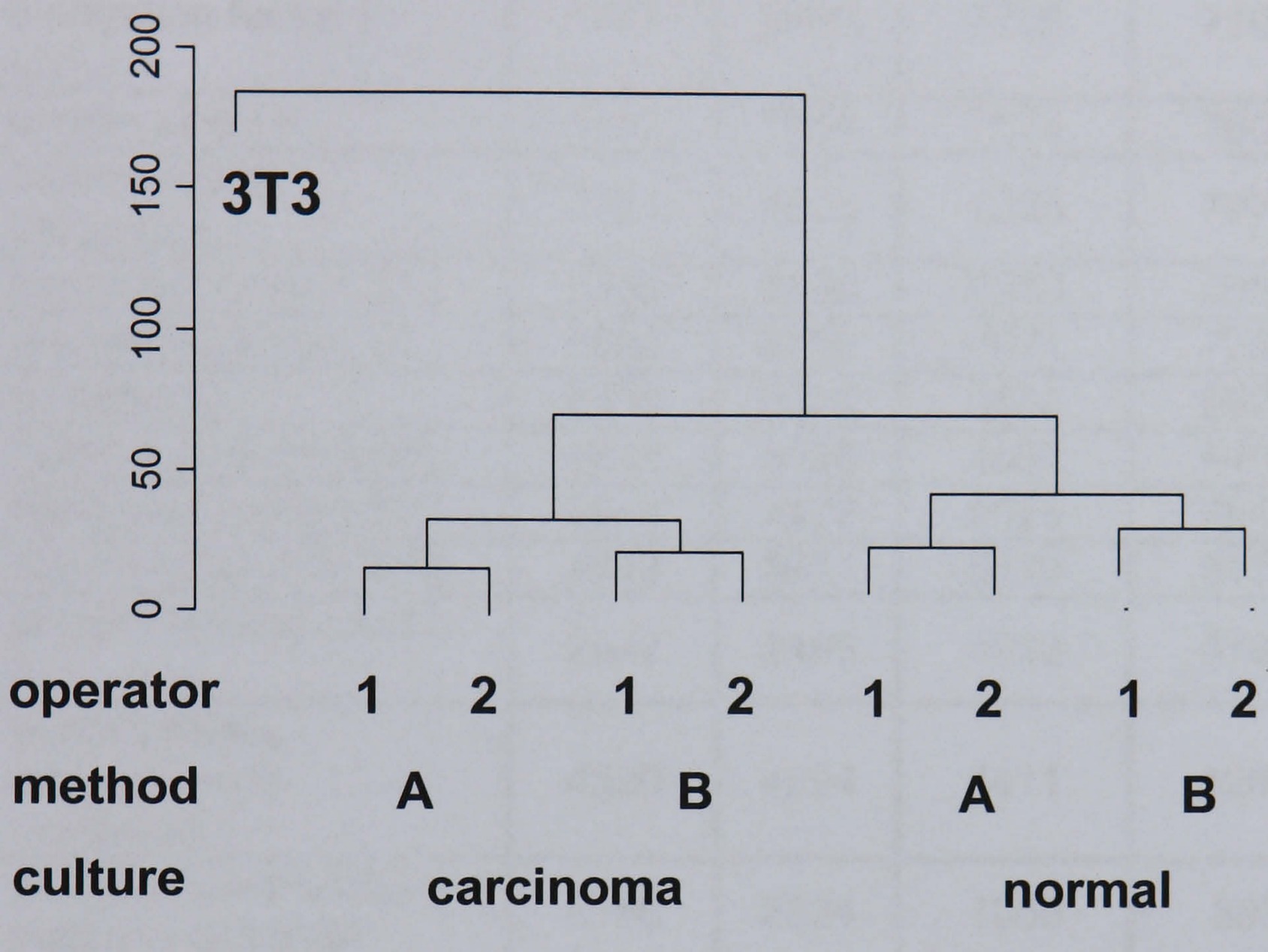


Figure 5.2 Hierarchical cluster of the GEPs of cultures used in the of microarray pilot study.

The cluster was constructed using the normalised gene expression data for all genes and the euclidean distance measure with complete linkage. The pilot study included 9 Affymetrix U133A genechips, including one irradiated 3T3 sample. Three variables were assessed.

1. Two operators (KH/JF) grew parallel cultures of each sample.
2. Two methods of 3T3 feeder removal:
   A. removal of 3T3 and re-feeding with medium 24 hours prior to RNA extraction.
   B. removal of 3T3s immediately prior to RNA extraction.
3. Two samples: one normal (FNB5) and one carcinoma (BICR56).

Gene	FNB5 A	FNB5 B	BICR56 A	BICR56 B	3T3
GNAS complex locus	893	340	1111	360	9617
eukaryotic translation elongation factor 1 alpha 1	7221	6888	7706	7105	7393
tubulin alpha 6	7337	7084	7574	7605	7211
tubulin, alpha, ubiquitous	7411	6925	7925	7974	6546
ribosomal protein S10	5530	4880	6383	6059	6515
ribosomal protein S2	7029	6650	7921	7262	6342
ubiquitin C	6539	6548	6953	6974	5934
cofilin 1 (non-muscle)	4816	5028	4692	4464	5404
ribosomal protein S27	2645	2470	2925	2641	5313
ribosomal protein S10	4713	3951	5119	5136	5240
poly(A) binding protein 1	2847	2466	4013	3706	5052
tumor protein, translationally-controlled 1	4390	4394	4411	4204	5016
eukaryotic translation initiation factor 5A	3166	2824	1068	593	4977
nuclease sensitive element binding protein 1	3019	2557	3630	3739	4829
ribosomal protein S12	4659	4545	4839	4520	4646

Table 5.1 Assessment of contamination of keratinocyte cultures with possible 3T3 genes.

This table outlines a comparison of the 15 most highly expressed genes in irradiated 3T3 feeders, with the expression of these genes seen in BICR56 and FNB5, by both timings of 3T3 removal, A and B. The figures shown are the mean values for the technical replicates.

Apart from one gene (GNAS) there was no systematic difference in the expression of these genes in the keratinocyte cultures. This indicated that differential 3T3 contamination was unlikely to be contributing to the GEP of the keratinocyte cultures.

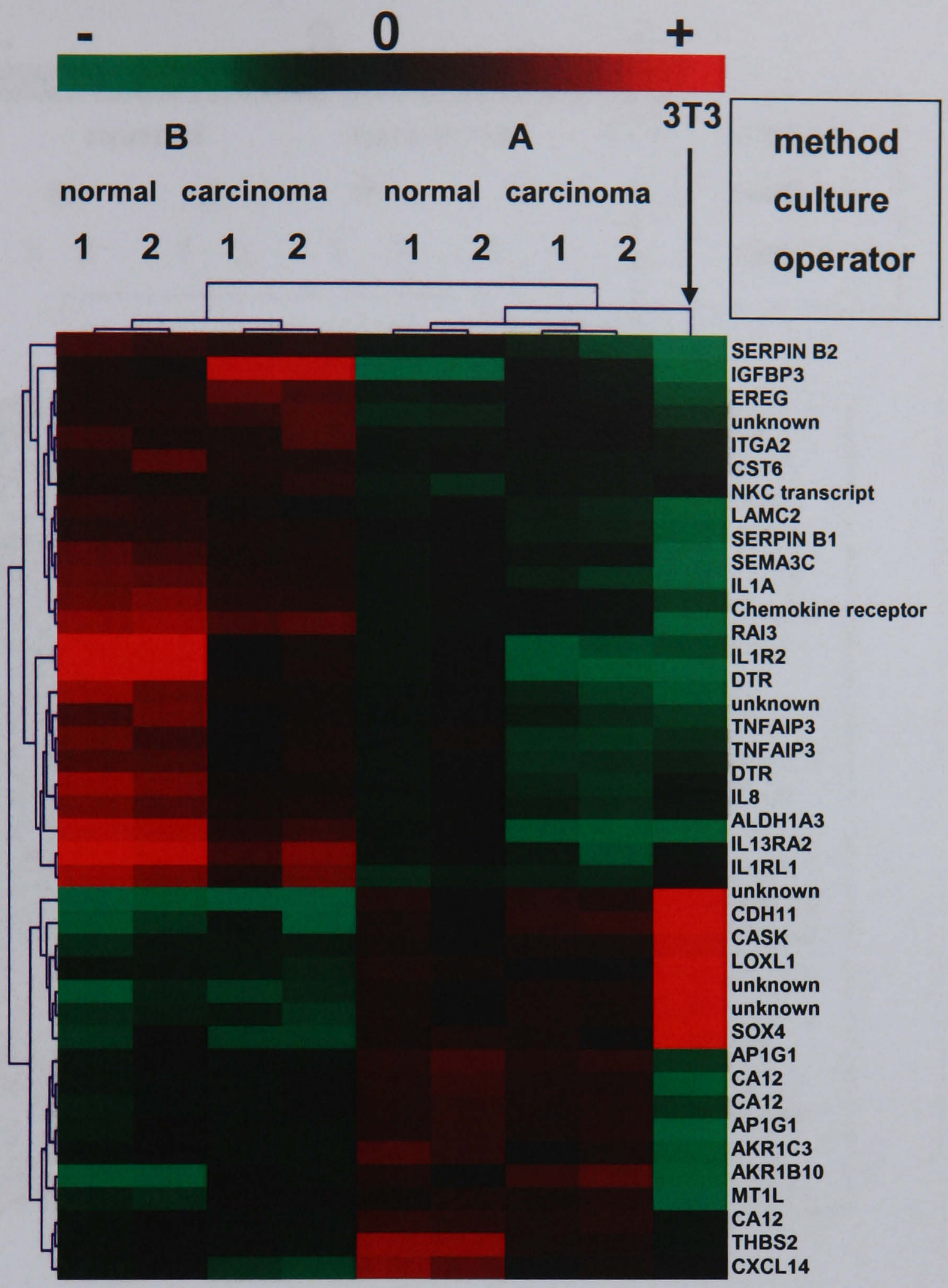


Figure 5.3 Graphical representation of genes significantly differentially expressed between methods A and B.

The figure shows the expression of those genes with red representing overexpression and green underexpression relative to the median expression of the gene across the dataset. Genes and samples have been hierarchically clustered using euclidean metric and complete linkage.

Genes were selected using SAM (FDR 1% and fold change $\geq$2). The official gene symbol is shown on the right. The genes selected are a group of genes whose pattern of expression is related solely to the timing of the removal of 3T3 feeders. These genes are primarily related to cell to cell communication (16/28 by Gene Ontology) and adhesion (7/28 by Gene Ontology).

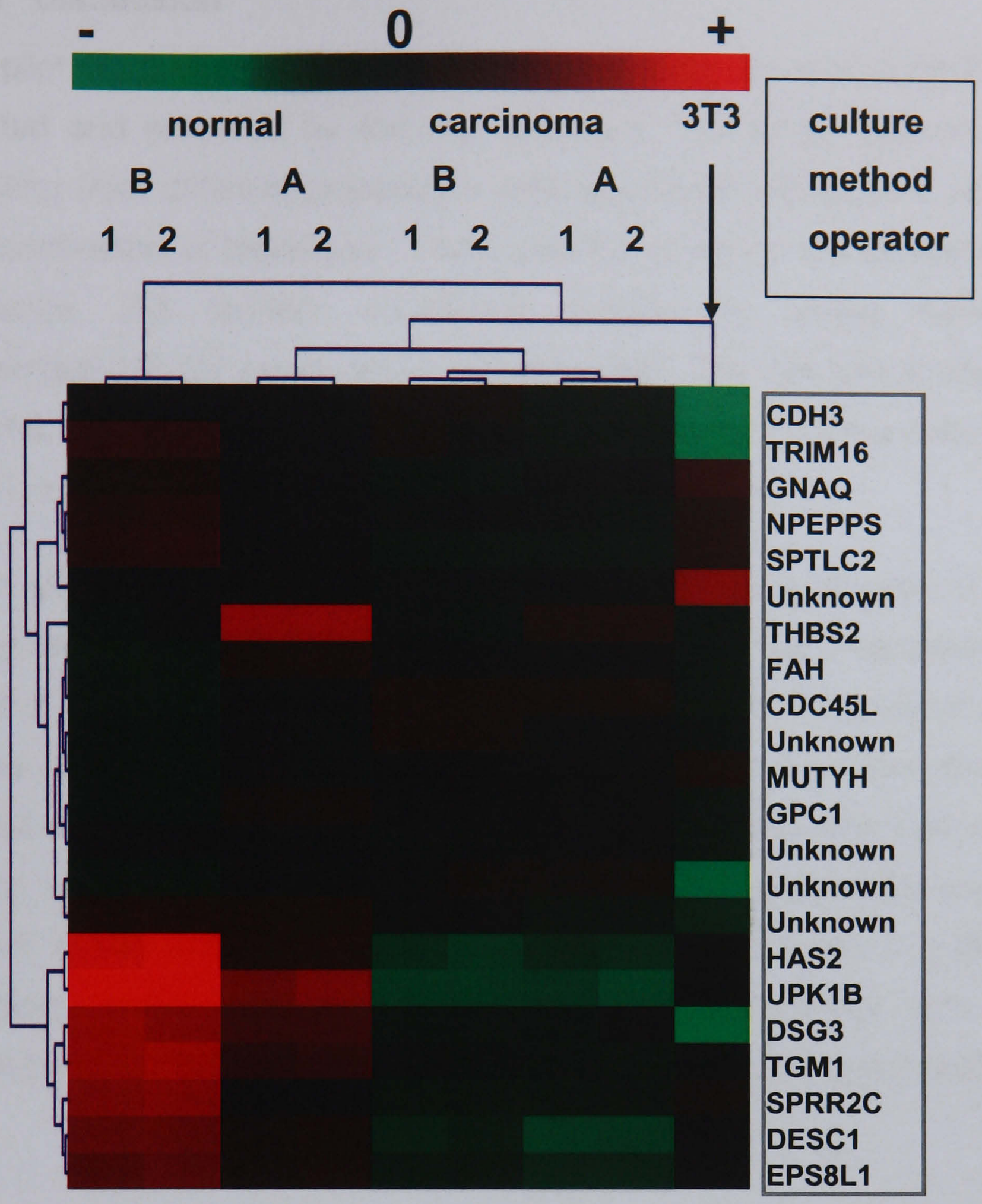


Figure 5.4 Graphical representation of genes significantly differentially expressed by sample type and by method.

The genes shown were selected by 2 factor ANOVA ($p<0.001$). Genes and samples have been hierarchically clustered using euclidean metric and complete linkage.

This demonstrates that a small number of genes show a pattern of expression which changes only in the normal cultures upon differing timing of feeder removal. Thus, the early (24 hour) removal of the 3T3 feeders has a small, but significant differential effect on the normal cells, but not the carcinomas. The genes perform various functions such as metabolism (12/21 by gene ontology), and cell growth and maintenance (5/21). The changes were not marked, but the list does include one potential marker of immortality (UPK1B, see Table 5.5).

5.2.1 Discussion

The pilot study demonstrated that there was little difference in GEP of the RNA isolated and prepared by the two operators. The larger differences in GEP resulting from different preparation methods clearly indicated a need for strict standardization of technique. There was no evidence that contamination from irradiated 3T3 feeders contributed significantly (either consistently or differentially) to the observed keratinocyte GEP. The different timing of removal affected expression of a small number of genes in the normal culture, with little effect on the carcinoma culture.

Such analysis demonstrated the need for strict standardization of the culture and preparation protocols. The standard protocol for the preparation of all RNA used in the main microarray study was removal of 3T3 feeders immediately before RNA extraction. This was preferred because it avoided the differential effects on gene expression in the cultures that are more dependent on the 3T3 feeder layer for survival. Such standardization also aligned the methodologies of cell culture and preparation of the cells for extraction of protein for the validation steps later and allowed for direct comparison with the earlier published data that had been gained from cells grown using this protocol.

5.3 Main study

The main microarray study utilised Affymetrix U133A and B genechips. Gene expression profiles were gained from cell cultures derived from all stages of SCCHN development – normal, dysplastic, primary carcinomas and metastases. This included a number of cultures (both mortal and immortal) derived from biopsies where the patient subsequently developed a carcinoma at the same site. The clinical and biological details of all cultures used in the study are in Table 3.1. The cells were grown to a standardized protocol (see pilot study) and harvested at approximately 60% confluence.

5.3.1 Comparison of normal cultures with all carcinomas cultures

A cluster dendrogram of the normal cultures and all of the carcinomas, both mortal and immortal, is shown in Figure 5.5. The cluster was constructed using the mean value of gene expression in the two technical replicates since the agreement between the GEPs of the two technical replicates was good (see pilot study and Figure 5.2). The dendrogram demonstrated a clear difference in the gene expression profile of mortal and immortal carcinomas. The normal group displayed the least heterogeneity, with the mortal carcinomas closely related to the normal cultures, but forming a distinct group. The immortal cultures clustered together, again demonstrating some heterogeneity. The culture in crisis, BICR7, was related to, but distinct from, the immortal carcinoma cultures.

Application of SAM to compare the mortal and immortal carcinoma groups yielded a list of 1251 genes expression differences significant at a FDR of 1% and fold change ≥ 2 (Table 5.2). In contrast, comparison of the normal with the mortal and immortal carcinomas gave lists of gene expression differences of 472 and 716 genes respectively (using the same parameters). Surprisingly, the overlap between the latter two lists of gene expression changes was only 11 genes, indicating that the mortal and immortal carcinoma cells may have arisen via divergent pathways. However, such seeming large numbers of differences in gene expression may also occur in relation, for example, to a change in activity of a single transcription factor or additional chromosomal material gained by passage through crisis.

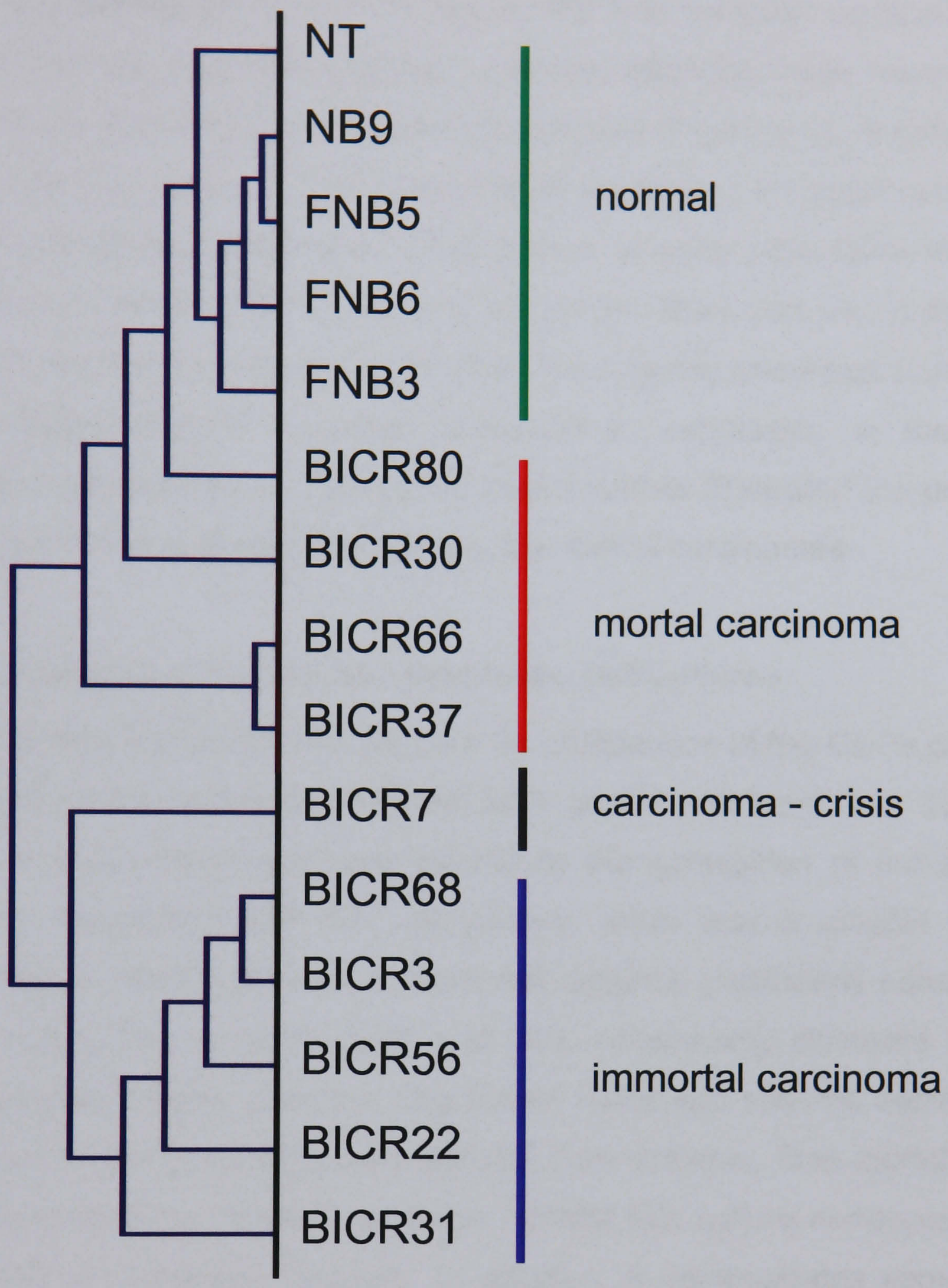


Figure 5.5 Hierarchical cluster based on the gene expression profile of normal and carcinoma keratinocyte cultures.

The cluster was calculated using the mean of two technical replicates for each cell culture, all present genes, and using the euclidean distance measure with complete linkage. The similarity/difference between two samples is based on the total distance travelled on the horizontal plane.

The normal samples cluster together, with the mortal carcinomas most closely associated. The immortal carcinomas are quite different in terms of GEP. BICR7 (in crisis) does not cluster closely with any of the samples

Further analysis using more stringent criteria (1% FDR and a fold change of ≥ 5) reduced the list from 1251 to 155 differences, as shown in Figure 5.6. These genes clearly distinguished not only the mortal and immortal carcinomas, but also the mortal and immortal dysplastic cultures, although some heterogeneity was present, particularly among the mortal samples (Figure 5.6). In general, the genes fall into two groups – those more highly expressed in mortal carcinomas and those in immortal carcinomas. The pattern of gene expression within the mortal samples showed that, for many of the genes, there was also a difference between the normal and mortal carcinomas. Thus, genes underexpressed in the normal cultures showed a further reduction in expression in the mortal carcinomas and vice-versa. This phenomenon further illustrated the possibility of divergence in the pathways to immortal and mortal carcinomas.

5.3.2 Comparison of normal and dysplastic cell cultures

Figure 5.7 shows the cluster dendrogram for comparison of the GEPs of normal cultures and all the dysplastic cultures, both mortal and immortal. The main difference in gene expression was related to the acquisition of the immortal phenotype. Compared with the carcinomas, there was a greater level of heterogeneity in relation to the dysplastic cell cultures, particularly amongst the mortal samples. Two samples (D41 and D48) consistently clustered with the normal samples. These were the only mortal dysplastic cultures derived from non-smokers, whilst the others were derived from smokers. One mortal sample (D17) clusters with the immortal samples. Whilst this culture senesces, it only does so after an extended lifespan. In addition, it demonstrates some of the features associated with immortal cells, namely loss of p16 and RAR-β. Clearly in addition to these changes, it had an overall GEP similar to the immortal cultures (see comments later). Within the immortal samples, D35 was somewhat different in pattern of gene expression. This patient subsequently developed a carcinoma at the same site within six months of the initial biopsy. Thus, it is possible that invasive SCC was present in another part of the lesion at the time of initial assessment. The GEP of the other immortal cultures from patients who developed a carcinoma at the same site (D19 and D20) did not cluster consistently with the carcinomas.

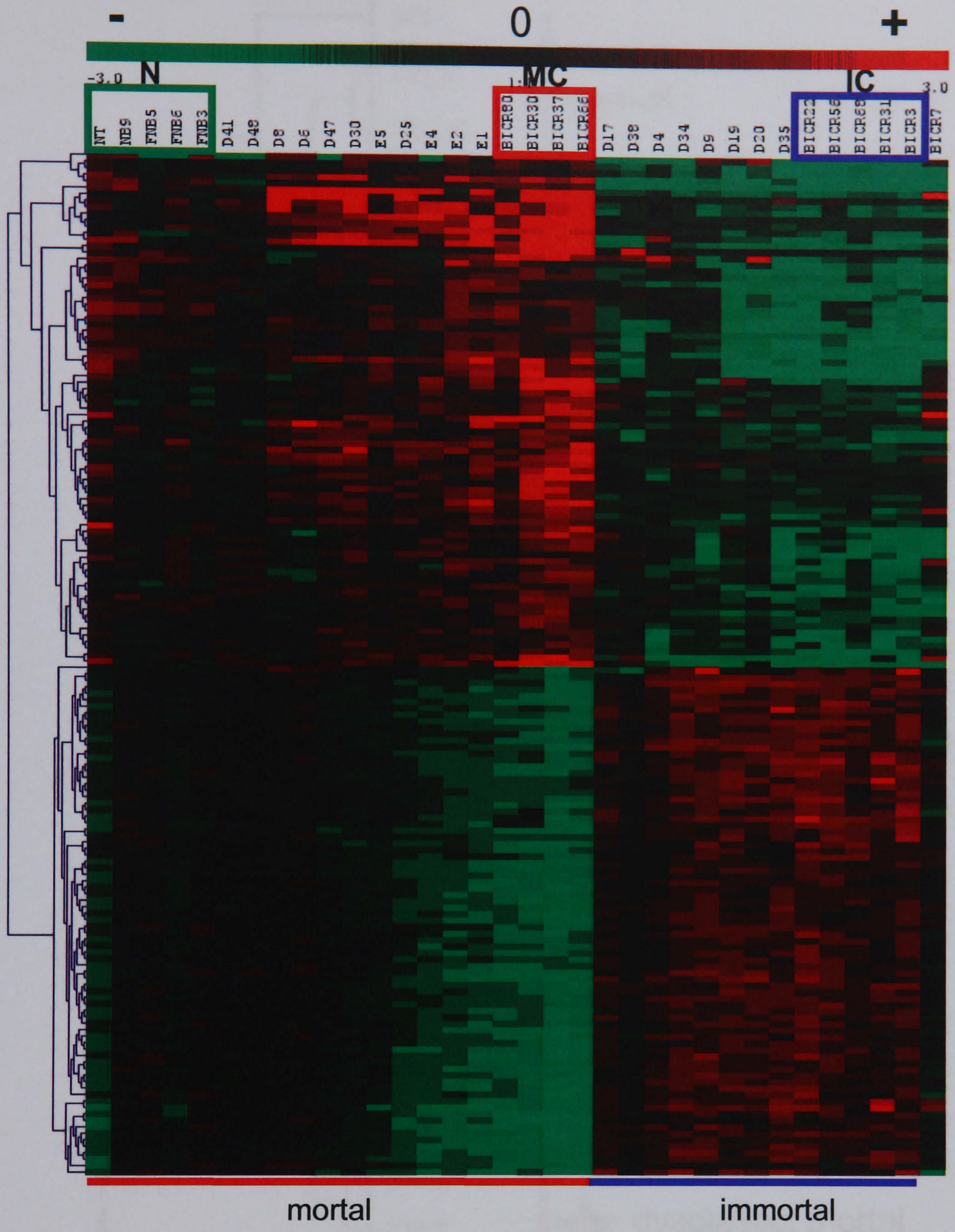


Figure 5.6 Graphical representation of genes significantly differentially expressed between mortal and immortal carcinoma samples.

Genes were selected using SAM (FDR $\leq 1\%$) and ≥ 5 fold difference in expression between mortal and immortal carcinomas, giving a total of 155 genes. The figure shows the expression of these genes in all samples (normal, dysplasia and carcinoma). Red represents relative overexpression and green relative underexpression as shown in the visual scale. Genes have been hierarchically clustered using euclidean metric and complete linkage. This demonstrates that some of the genes which differentiate mortal and immortal carcinomas also differentiate mortal and immortal dysplastic cell cultures.

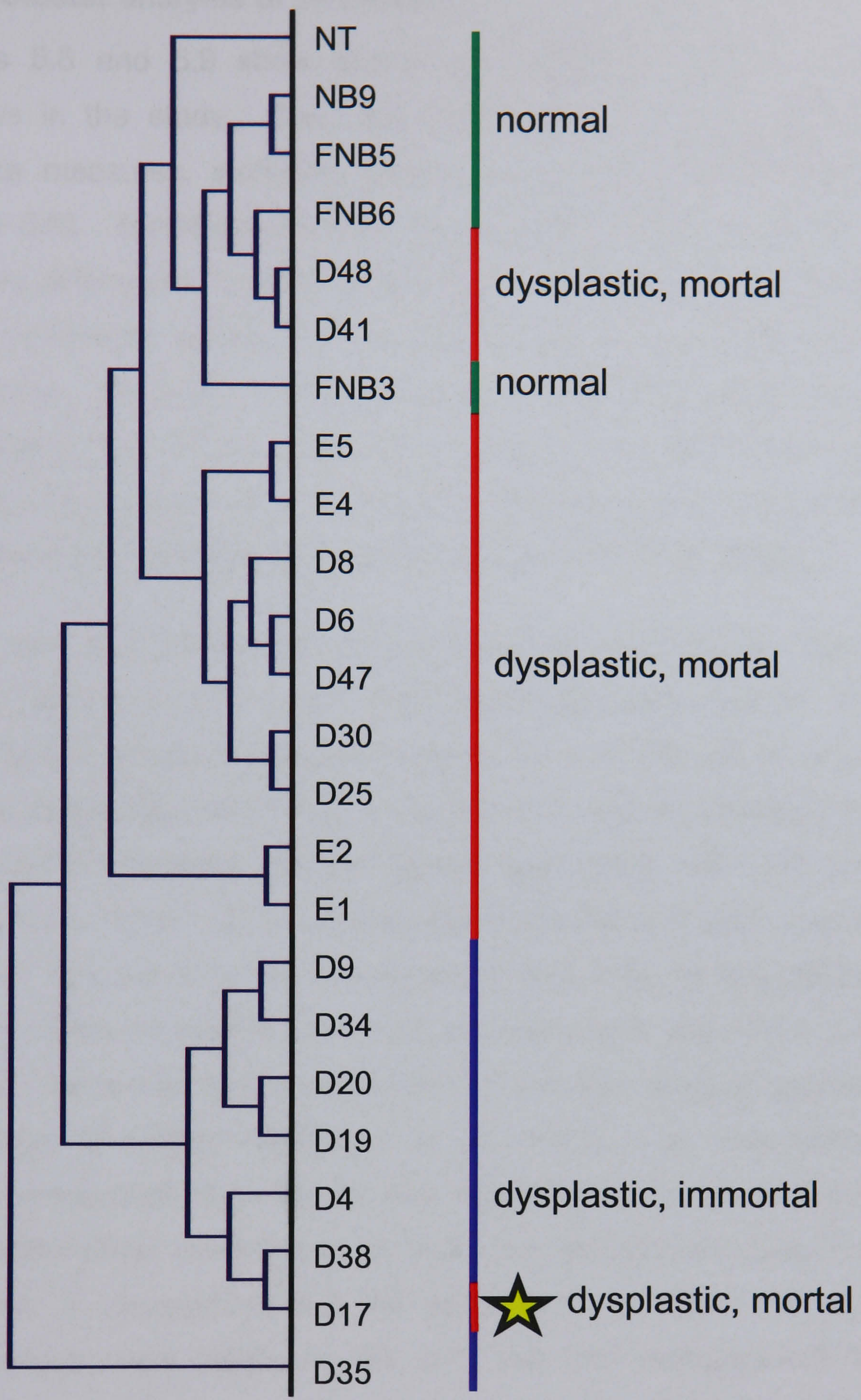


Figure 5.7 Hierarchical cluster based on the GEP of dysplastic and normal keratinocyte cultures.

The cluster was calculated using the mean of the two technical replicates for each cell culture, all present genes, and using the euclidean distance measure with complete linkage.

The normal cultures cluster together along with D41 and D48. The other mortal dysplastic cultures cluster together except D17 (marked with a star) which clusters with the immortal cell cultures.

5.3.3 Cluster analysis of all samples

Figures 5.8 and 5.9 show cluster dendrograms constructed using all the samples in the study. The matrix has been clustered using two different distance measures, euclidean distance (Figure 5.8) and cosine correlation (Figure 5.9). Euclidean distance is the most common method employed to measure differences between the points in n-dimensional space that represent gene expression values. It clusters based on absolute values of gene expression. However, a more appropriate metric for clustering of microarray data may be the cosine correlation, related to the angle between the vectors, since it allows comparison of trends in change of gene expression amongst samples rather than their absolute values (Causton et al., 2003).

There were few differences in gene expression relationships identified by the different distance metrics, but some interesting points emerge. The euclidean metric placed immortal dysplastic cultures D4 and D38 with the mortal cultures, in close association with D17. However, the cosine correlation metric placed D17 with the immortal cultures closely associated with D38 and BICR7 (a carcinoma in "crisis"). This indicates that the patterns of gene expression in D17 and D38 had some common features to that seen in immortality and crisis, whilst in terms of overall gene expression levels it was more like the mortal cultures. There was also evidence that D4 changed in early pass culture, losing expression of RAR-β (McGregor et al., 1997). This may indicate that D4 progressed through crisis shortly after establishment in culture and may account for a similar effect in relation to its position in the different cluster dendrograms. However, on comparison with the dendrogram in Figure 5.7, it is clear that these effects were subtle, as D4, D17 and D38 clustered with the immortal samples, despite the use of the euclidean distance metric. The pattern in Figure 5.9 also indicates that the gene expression patterns associated with crisis (as seen in BICR7) may have been present to some degree in D17 and D38. The euclidean metric clustered the mortal carcinomas close together, whereas with the cosine correlation, there was greater heterogeneity, particularly in the mortal carcinomas.

The main difference in GEP seen in both clusters was associated with the acquisition of immortality. In addition to this, some consistent subgroups were identified within the mortal and immortal groups.

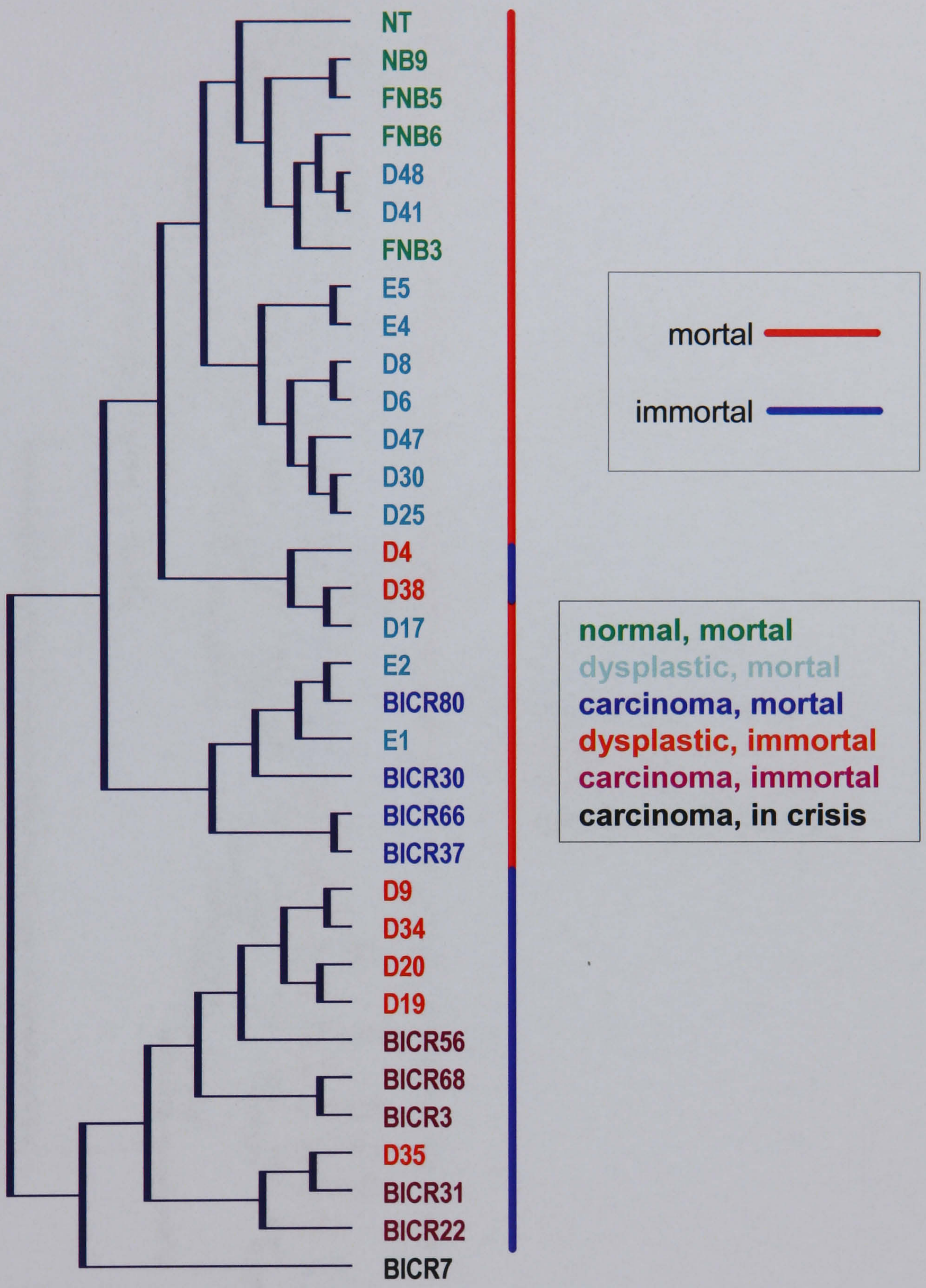


Figure 5.8 Hierarchical cluster based on the GEP of all samples using euclidean distance.

The cluster is based on the mean values of technical replicates, using all present genes. Distance metric = euclidean, with complete linkage. The euclidean distance metric uses absolute values of gene expression to calculate the cluster dendrogram.

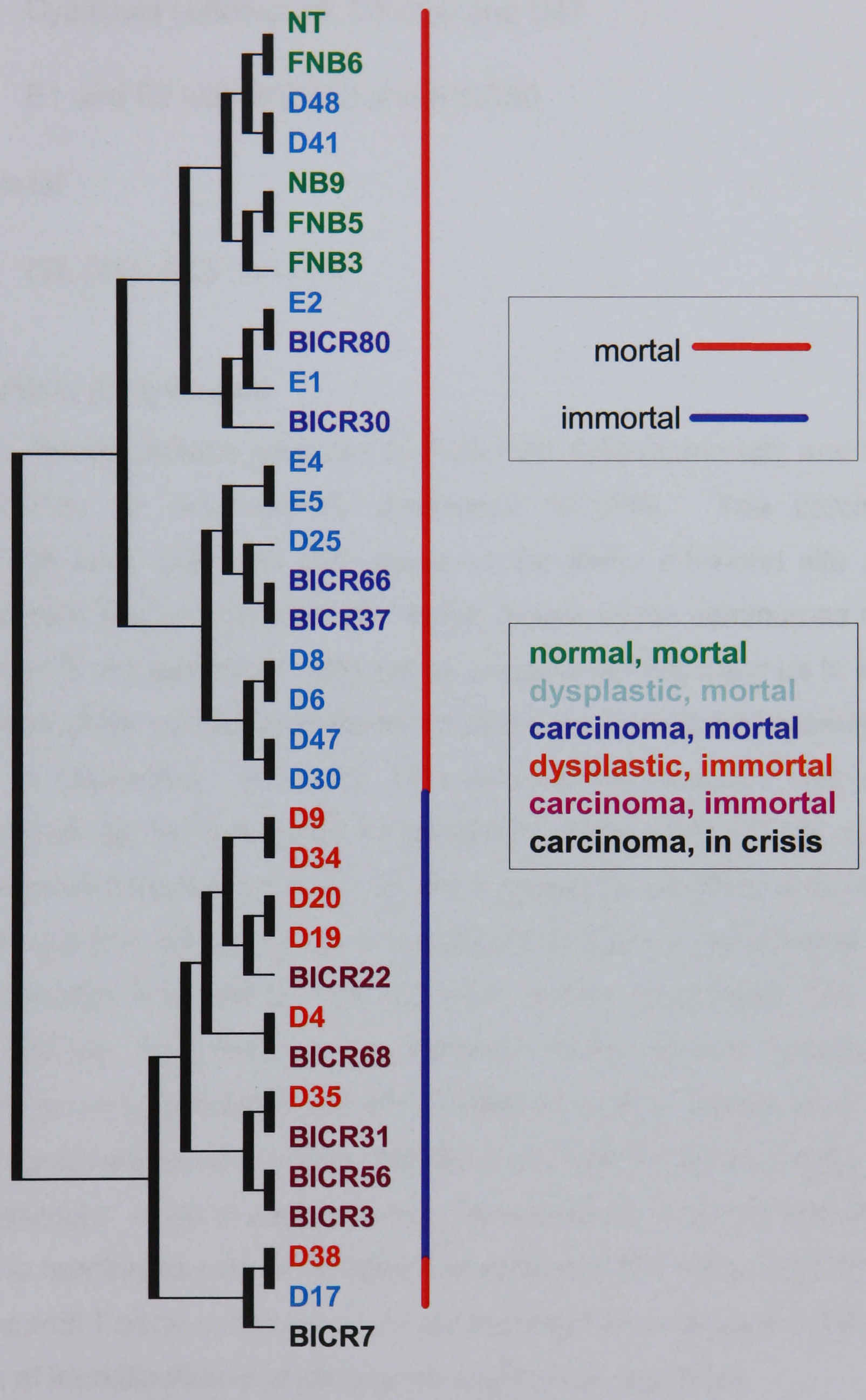


Figure 5.9 Hierarchical cluster based on the GEP of all samples using cosine correlation

The cluster is based on the mean values of technical replicates, using all present genes. distance metric = cosine correlation linkage = complete. The cosine correlation metric groups samples according to trends/pattern of expression rather than absolute value.

- **Mortal:**
    - All normal cultures with D41 and D48
    - Dysplasia cultures D6, D8 D30 and D47
    - E1 and E2 with BICR30 and BICR80
- **Immortal**
    - D9, D34, D19 and D20

5.3.4 Dysplasia progression

The patients whose biopsies gave rise to D19, D20, D35 (immortal), and E2, E4 and E5 (mortal) all subsequently developed SCCHN. The carcinomas associated with D19, D20 and D35 arose at the same intra-oral site as the original dysplastic biopsy, whereas the clinical details of the carcinomas related to E2, E4 and E5 are less clear. Microarray analysis did not allow us to identify clear groupings of the dysplastic cultures derived from lesions that subsequently progressed to carcinoma. However, D35 consistently clustered with certain immortal carcinomas, but there was no consistent relationship of D19 and D20 to the carcinomas (Figures 5.8 and 5.9). As a group, the erythroplakia cultures (E1, E2, E4 and E5) did not show a consistent pattern of relationship to the carcinoma cultures, however E1 and E2 were closely associated with mortal carcinoma cultures, regardless of the distance metric used to construct the cluster dendrogram (Figures 5.8 and 5.9). However, as the clusters were based on the entire gene expression profile, this does not rule out the possibility that a "high risk signature" of gene expression in the dysplastic cultures that clinically progressed to carcinoma may be hidden in a subset of the data. Paul Drake, a Clinical Research Fellow in the group, is conducting further analysis of the data with the aim of identification of such a gene expression signature.

5.3.5 Identification of the genes associated with the immortal phenotype

Based on the groups derived from the cluster analysis and from clinical and biological knowledge, various groups of the cultures were selected for analysis by SAM. Statistical analysis was also performed using ANOVA, with good agreement of the list of genes selected by the two methods (Figure 5.10). The

number of statistically significant differences in gene expression between the groups selected is shown in Table 5.2 (upper and lower). The FDR of 1% was selected as this was deemed to give a working list of a manageable size, whilst maintaining a high level of stringency. In addition, the fold change cut off was set at ≥2 for practical reasons including limiting the size of the gene lists and allowing for some possibility of validation of the differences identified by independent methods. D17 was excluded from the mortal dysplastic group since, as indicated previously, this appears to have an overall GEP very similar to the immortal dysplastic samples (Figures 5.8 and 5.9).

The method for selection of the genes associated with immortality is shown in Figure 5.11. This selected consistent differences in gene expression that are associated with the immortal phenotype, whether at the dysplasia or carcinoma stages. This required comparison of the normal cultures with the immortal dysplastic culture and the immortal carcinomas (comparisons 1 and 2). Additionally, these differences in gene expression had to be present on comparison of the mortal and immortal dysplastic cultures (comparison 3). Initial criteria also excluded genes that differed on comparison of the normal cultures and mortal carcinomas. On close inspection of the expression pattern of these genes this was seen to be inappropriate as the differences noted in many of these genes were paradoxical to the differences associated with immortality. Thus, these genes were retained in the list. Examples of genes retained include cdc20 and AF1Q in Figures 5.13 and 5.14.

This analysis yielded a list of 55 Affymetrix probesets, representing 49 different genes (Figure 5.12). Of these genes, 27 were expressed at a higher level in immortal samples and 22 genes were expressed at a lower level in immortal samples than mortal. The mean expression level for the different biological groups (with standard error) is shown in Figures 5.12 and 5.13. This demonstrates the variability within the groups, even though the statistical stringency in the initial SAM analysis/ANOVA was high. Certain genes have extremely large standard errors, particularly within the mortal carcinoma group in Figure 5.13. As it was less likely that these genes would be good markers of the immortal phenotype, none of these markers was selected for the subsequent validation steps.

Figure 5.10 Comparison of the size and agreement of gene lists obtained by SAM and ANOVA

This Venn diagram illustrates the agreement between ANOVA ($p<0.01$) and SAM (FDR 1%) in the comparison of normal v immortal carcinoma (in both cases fold change > 2 fold). In this instance, the ANOVA list is almost completely a subset of the SAM list.

	normal	MD-D17	MDT	MC	ID	IDT	IC
normal	-	192	32	472	124	693	716
MD-D17		-	55	239	218	573	731
MDT			-	5	576	1056	1239
MC				-	1196	1300	1251
ID					-	7	4
IDT						-	0
IC							-

	Normal	all MD-D17	MC	all ID	IC
normal	-	36	472	264	716
all MD-D17		-	75	unknown	764
MC			-	1358	1251
all ID				-	30
IC					-

Table 5.2 (upper and lower) Number of statistically significant differences in gene expression in comparison of all groups.

The numbers of gene expression differences were generated using SAM at FDR1% and fold changes ≥2. MD = mortal dysplasia, MDT = mortal dysplasia which progressed to carcinoma, MC = mortal carcinoma, ID = immortal dysplasia, IDT = immortal dysplasia which progressed to carcinoma, IC = immortal carcinoma. The allMD-D17 v all ID could not be completed due to a lack of sufficient computing power at BICR.

D17 was left out of these analyses since, as previously indicated, this appears to have a GEP very close to the immortal dysplasias overall (Figures 5.8 and 5.9 and comments in the text).

The largest number of significant differences in gene expression were found on comparison of mortal carcinomas and immortal dysplasias which progressed to carcinoma .

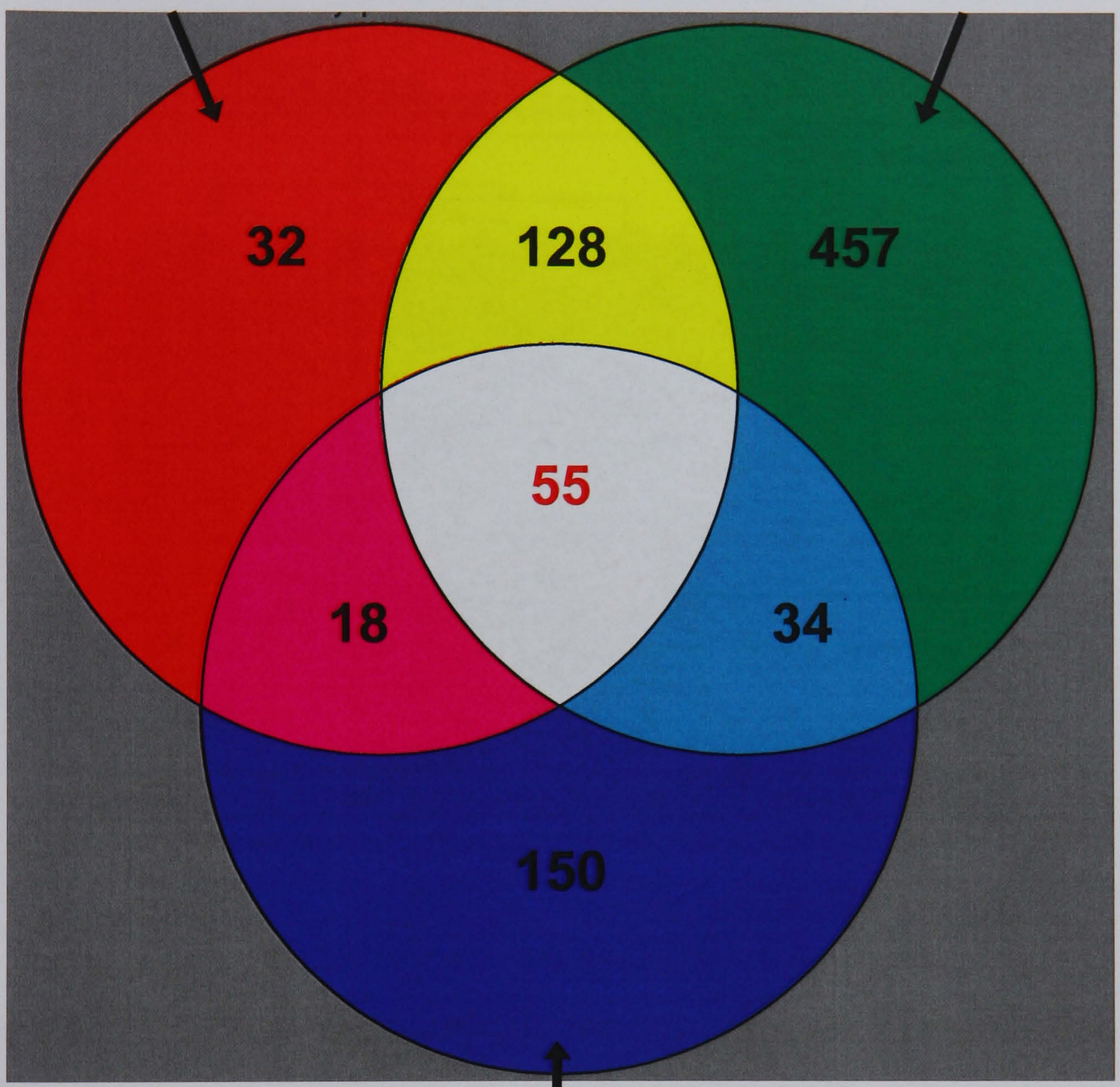


Figure 5.11 Venn diagram illustrating the method of identification of genes associated with immortality

Gene lists derived from the statistical analysis of comparisons of various groups were loaded into Genespring®. Only genes which showed consistent differences in the normal-immortal dysplasia and normal-immortal carcinoma comparisons and were also present in a comparison of mortal-immortal dysplasias were selected. The 55 probe sets identified relate to 49 individual genes (Figure 5.12).

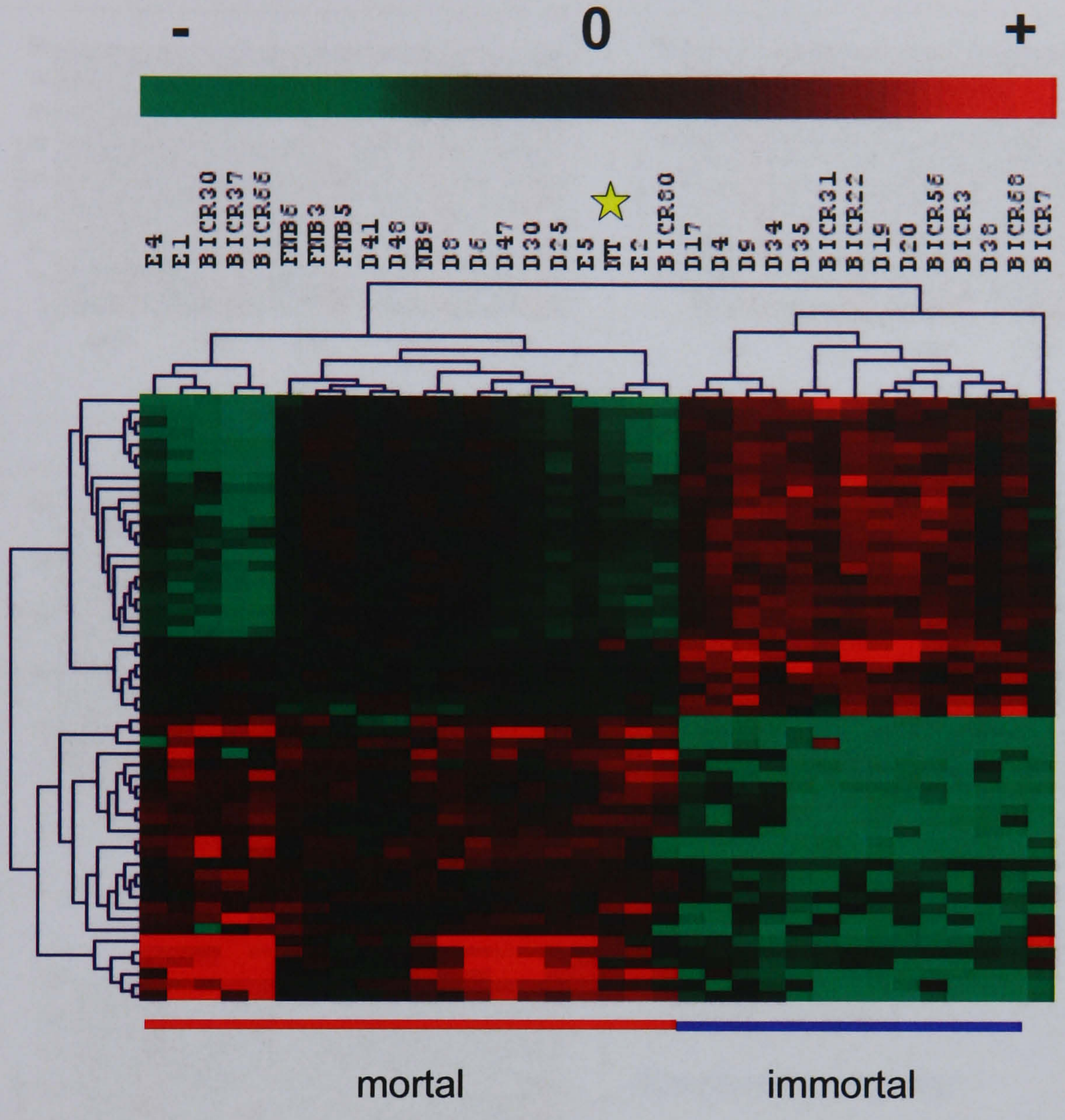


Figure 5.12 Graphical representation of expression of 55 candidates.

The 55 probe sets were derived as shown in Figure 5.11. The diagram shows the genes and samples clustered using the euclidean distance metric and complete linkage. Red indicates relative overexpression, and green relative underexpression – see visual scale.

The genes divided into two clear groups with reciprocal patterns related to the immortal phenotype. The samples clustered very similarly to that seen in earlier figures, however NT (marked) no longer clusters with the other normal samples. All of the immortal dysplasia cultures that progressed to carcinoma (D19, D20 and D35) were closely associated with immortal carcinoma cultures.

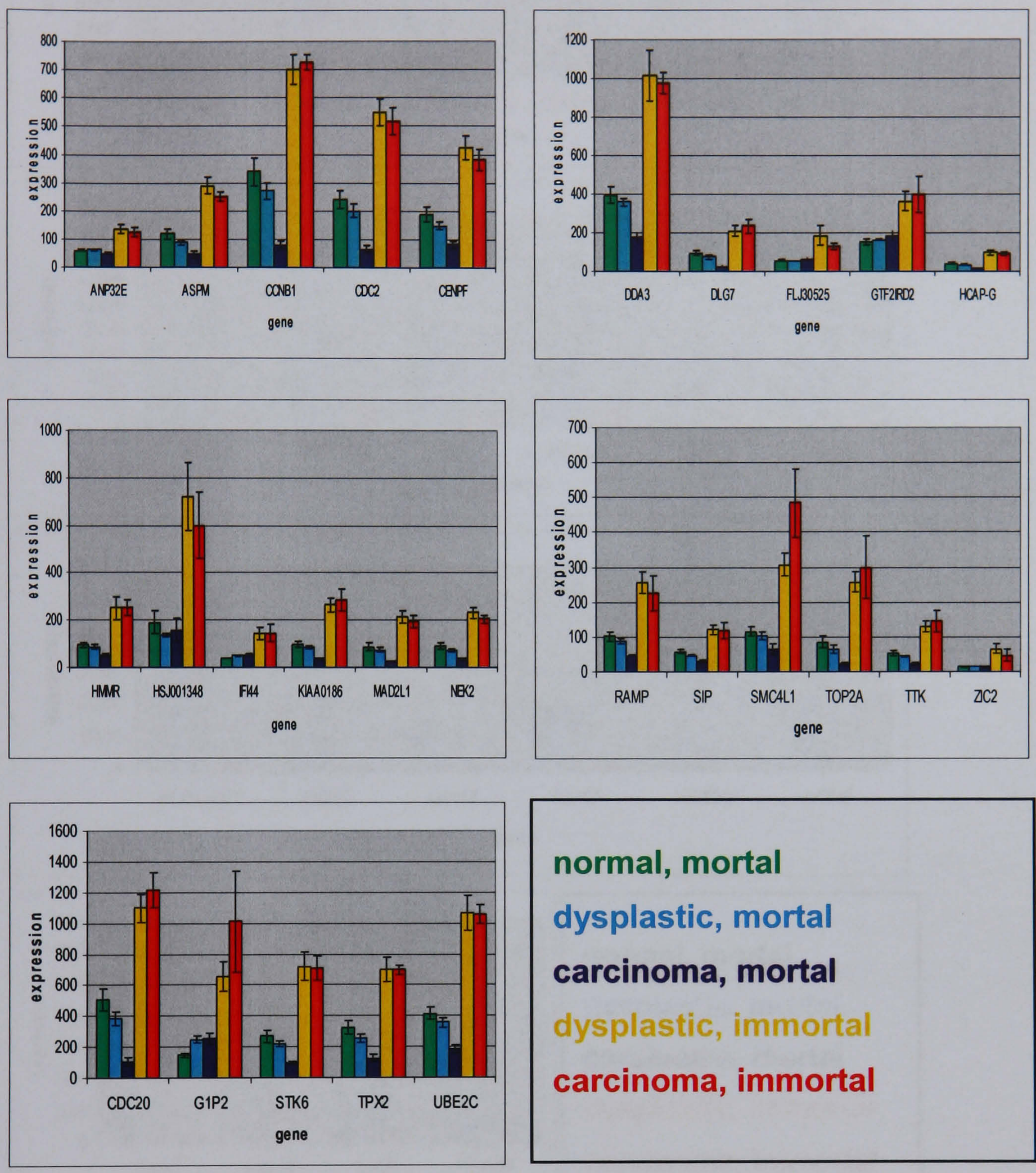
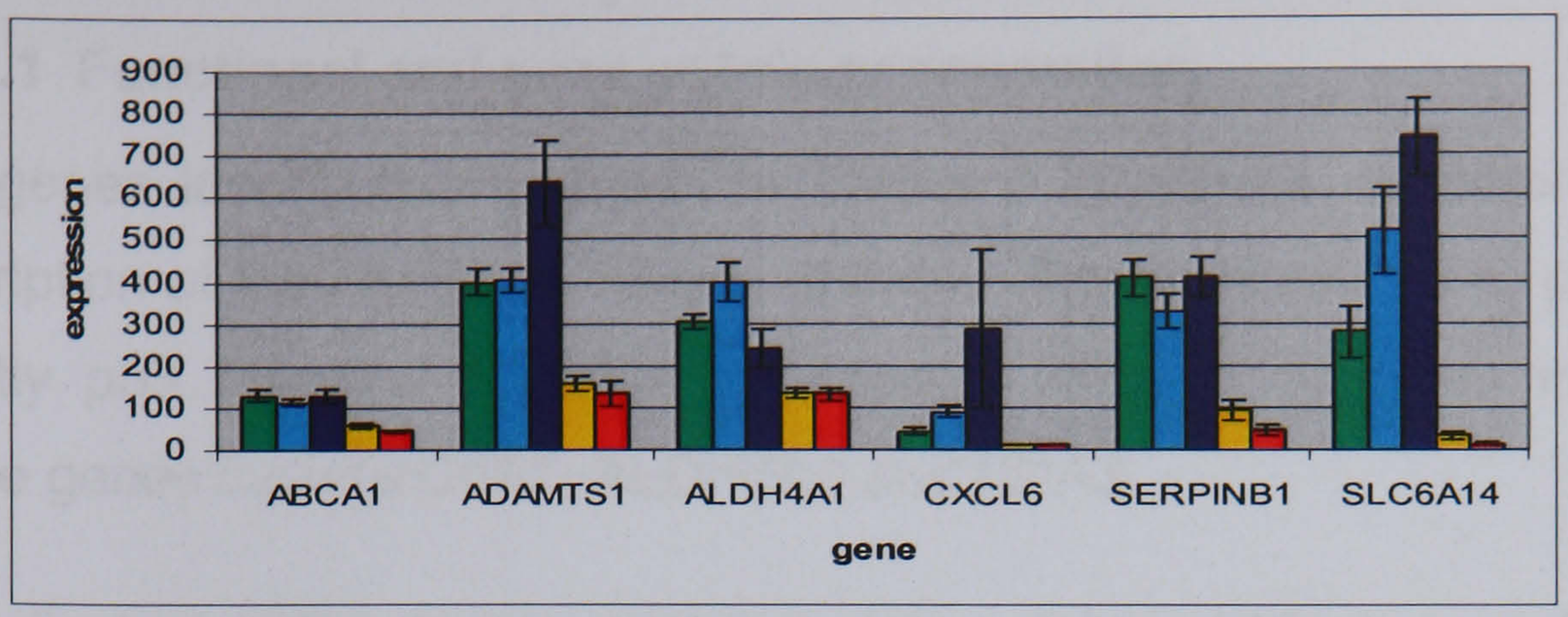
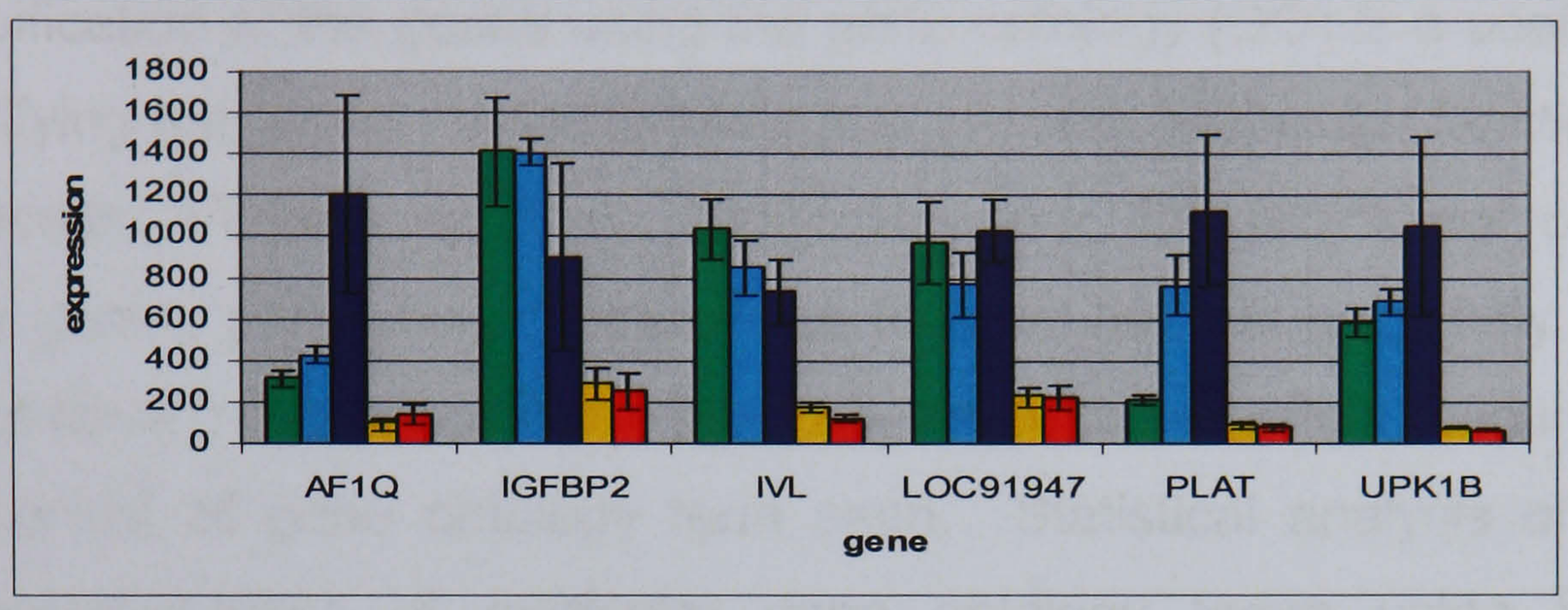
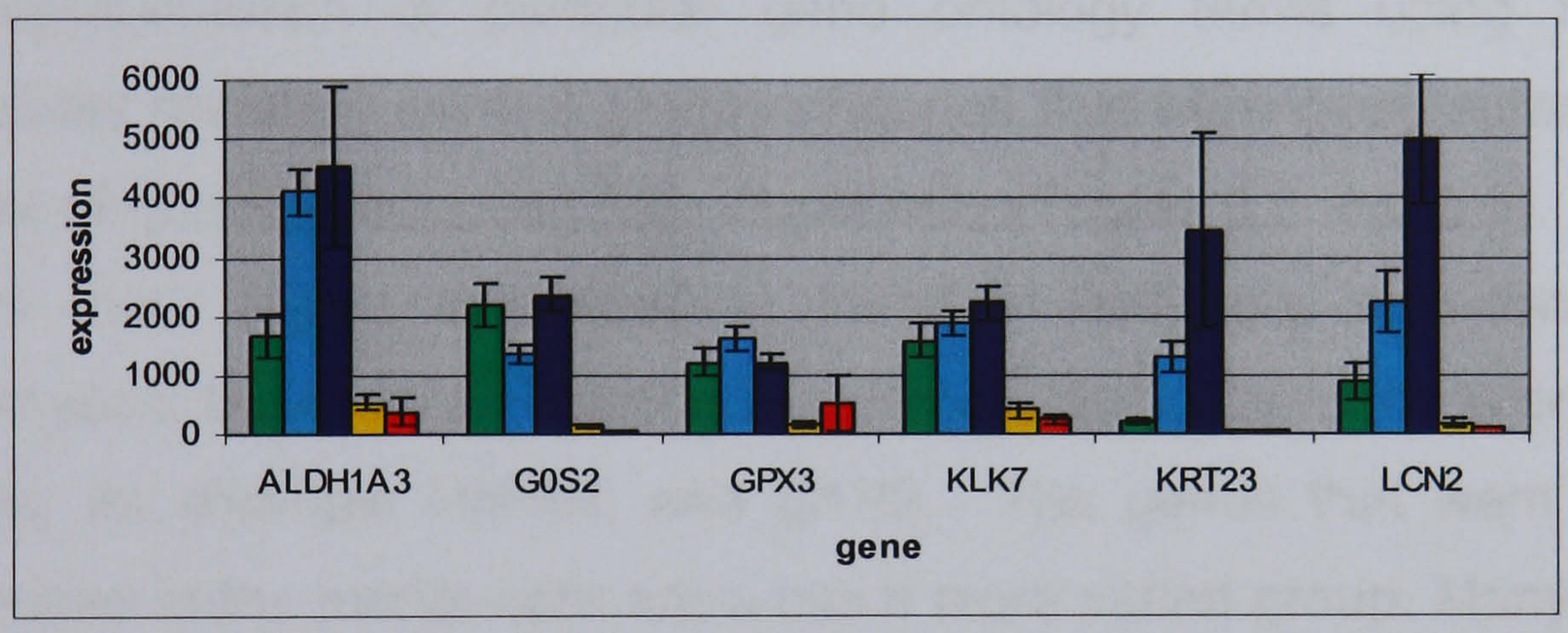


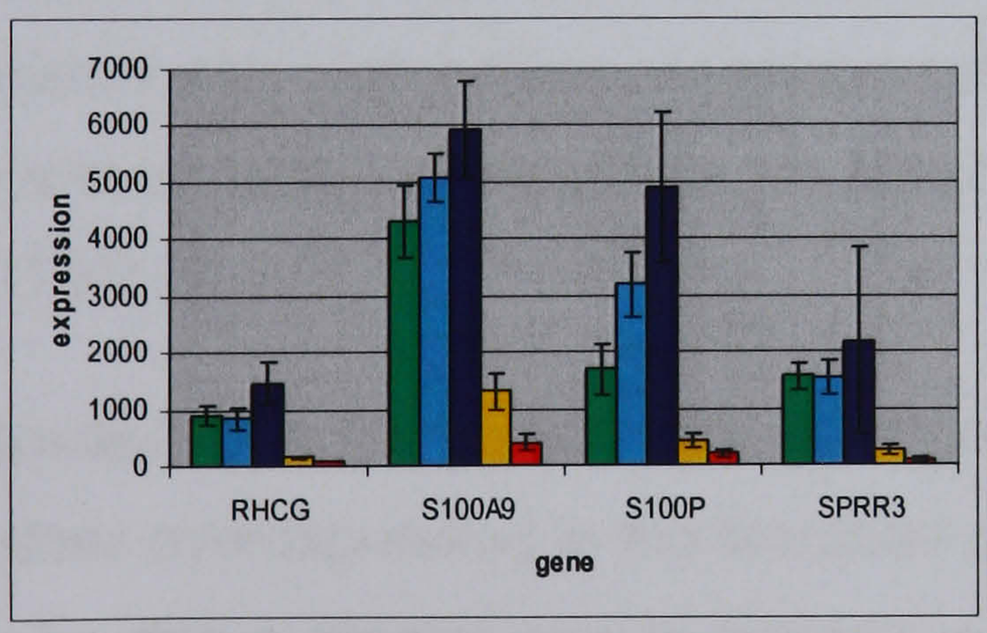
Figure 5.13 Affymetrix U133A genechip expression levels of 27 genes overexpressed in immortal samples.

Genes were selected as outlined in Figure 5.11. Expression level is the normalised mean Affymetrix expression level for each group with the standard error of the mean also shown. In addition to demonstrating overexpression in the immortal samples, many of these genes showed a reduction of expression on comparison of the normal and mortal samples.









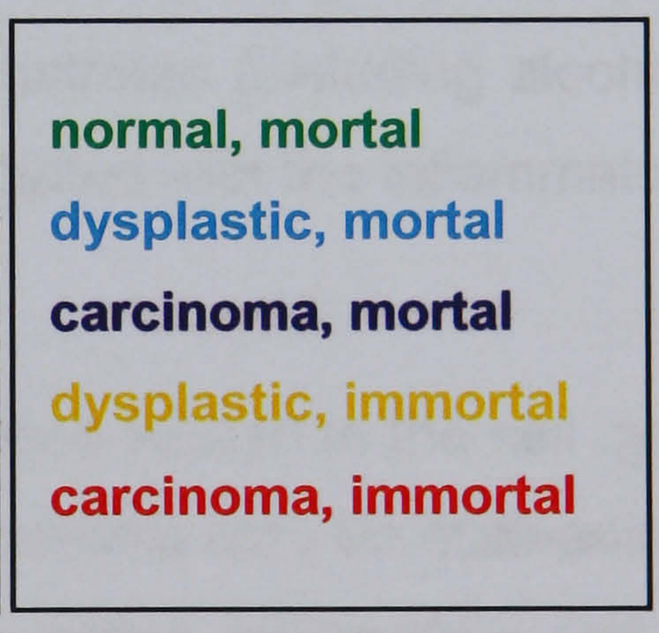


Figure 5.14 Affymetrix U133A genechip expression levels of 22 genes underexpressed in immortal samples.

Genes were selected as outlined in Figure 5.11. Expression level is the normalised mean Affymetrix expression level for each group with the standard error of the mean also shown. Whilst these genes demonstrate consistent underexpression in the immortal samples, the standard errors, particularly in the mortal samples were often large, indicative of the heterogeneity within these samples.

5.3.5.1 Functional and gene ontology annotation.

The genes identified are shown in Tables 5.3 and 5.4, together with a brief description of their function. Some of these genes are related to p53, either as directly p53 responsive genes or indirectly through unknown intermediates. These genes include G0S2, ALDH1A3 and DDA3.

Classification of the genes using the gene ontology (GO) is a useful method of classifying the genes related to biological process, molecular function or cellular component (Ashburner et al., 2000). However, the GO is not complete and many genes, particularly those whose function has not been fully investigated, do not have a GO annotation. This may introduce a certain amount of bias into the spread of gene ontology term seen. Statistical analysis of the relative overrepresentation of particular gene ontology terms using Fisher exact probability revealed several groups of genes that were over represented in the subset of genes associated with immortality (Tables 5.5 and 5.6). Many of the genes more highly expressed in immortal cells are associated with cell proliferation, but there are other genes whose biological function does not relate to this, for example HMMR, and G1P2. The genes that were more highly expressed in the mortal cells are a much more varied group. Many of these are associated with metabolism of various substrates (including alcohol, retinoids, lipids and proteins), whilst others are associated with the inflammatory response (e.g. CXCL6).

It is possible that the preponderance of genes related to the cell cycle amongst the genes overexpressed in the immortal cultures may be misleading. This may partly be due a greater growth fraction or higher proliferative rate than mortal cultures, as the doubling time is shorter for immortal cultures than for mortal cultures (data not shown). Alternatively, the mortal cultures may contain a senescent subpopulation even though the cultures were used at the earliest passage possible. However, there are many other genes in these lists that have not been linked to the cell cycle and these may prove to be proliferation independent markers of the immortal phenotype. However, the cells that are immortal may have consistent changes within the regulatory mechanisms of the cell cycle and as such, these genes may be important and should be assessed

Symbol	Gene Name	Alias	Genbank	Biological function
ANP32E	acidic nuclear phosphor-protein 32 family, member E	LANP-L	NM_030920	function unclear, inhibits protein phosphatase 2A
ASPM	abnormal spindle-like, microcephaly associated		NM_018123	microtubule-based process
CCNB1	Cyclin B1		NM_031966	regulation of cell cycle - G2/M transition/mitosis, complexes with cdc2
CDC2	cell division cycle 2	CDK1	NM_001786	mitosis, protein amino acid phosphorylation, start control point of mitotic cell cycle
CDC20	cell division cycle 20 homologue	p55CDC	NM_001255	regulation of cell cycle, ubiquitin-dependent protein catabolism
CENPF	centromere protein F	mitosin	NM_016343	DNA replication and chromosome cycle, regulation of mitosis, chromosome separation
DDA3	differential display, activated by p53	CKS1B, CDC28	NM_001826	regulation of CDK activity, cytokinesis
DLG7	discs, large homolog 7	HURP	NM_014750	cell-cell signalling
FLJ30525	hypothetical protein		NM_144584	function unknown
G1P2	interferon alpha-inducible protein	IFI15	NM_005101	cell-cell signalling, immune response
GTF2IRD2	GTF2IRD2		AY312853	transcription factor
HCAP-G	chromosome condensation protein G	CAP-G	NM_022346	mitotic chromosome condensation
HMMR	hyaluronan-mediated motility receptor	RHAMM	NM_012485	cell motility
HSJ001348	cDNA for differentially expressed CO16 gene		NM_017527	function unknown
IFI44	interferon-induced protein 44	MTAP44	NM_006417	invasive growth microtubule-based process, response to viruses
KIAA0186	hypothetical protein		NM_021067	
MAD2L1	mitotic arrest deficient-like 1 (yeast)	HSMAD2 MAD2	NM_002358	mitotic chromosome segregation, mitotic spindle checkpoint
NEK2	NIMA-related kinase 2	NLK1	NM_002497	cytokinesis, meiosis, protein amino acid phosphorylation, regulation of mitosis
RAMP	RA-regulated nuclear matrix-associated protein	L2DTL	NM_016448	function unknown
SIP	Siah-interacting protein	CACYBP	NM_014412	centromere/kinetochore complex maturation
SMC4L1	structural maintenance of chromosomes 4-like 1	CAP-C	NM_005496	chromosome organization, condensation, and segregation, transport
STK6	serine/threonine kinase 6	ALK	NM_003158	mitosis, protein amino acid phosphorylation
TOP2A	topoisomerase II alpha		NM_001067	DNA topological change
TPX2	TPX2, microtubule-associated protein homolog		NM_012112	mitosis
TTK	TTK protein kinase	ESK, MPS1L1, PYT	NM_003318	mitotic spindle assembly and checkpoint, positive regulation of cell proliferation, protein amino acid phosphorylation
UBE2C	ubiquitin-conjugating enzyme E2C	ubch10	NM_007019	Cyclin catabolism, mitosis, regulation of cell proliferation, regulation of DNA repair, ubiquitin-dependent protein catabolism
ZIC2	Zic family member 2	HPE5	NM_007129	brain development

Table 5.3 Functional annotation of 27 genes overexpressed in immortal cell cultures.

The biological function noted is that designated by the gene ontology and is a composite from all three of the annotation networks of the gene ontology.

Symbol	Gene Name	Alias	Genbank	Biological functions
ABCA1	ATP-binding cassette, sub-family A, member 1	CERP	NM_005502	cholesterol metabolism, phagocytosis, protein targeting
ADAMTS1	a disintegrin-like and metalloprotease with thrombospondin type 1 motif, 1	METH1	NM_006988	integrin-mediated signalling pathway, negative regulation of cell proliferation, proteolysis and peptidolysis
AF1q	ALL1-fused gene from chromosome 1q		NM_006818	cell growth and maintenance
ALDH1A3	aldehyde dehydrogenase 1 family, member A3	ALDH6	NM_000693	alcohol and lipid metabolism
ALDH4A1	aldehyde dehydrogenase 4 family, member A1	ALDH4	NM_003748	proline biosynthesis/catabolism
CXCL6	chemokine (C-X-C motif) ligand 6	GCP-2	NM_002993	chemotaxis, cell-cell signalling, inflammatory response, signal transduction
G0S2	putative lymphocyte G0/G1 switch gene		NM_015714	regulation of cell cycle
GPX3	glutathione peroxidase 3		NM_002084	response to lipid hydroperoxide
IGFBP2	insulin-like growth factor binding protein 2	IBP2	NM_000597	regulation of cell growth
IVL	involucrin		NM_005547	differentiation
KLK7	kallikrein 7		NM_005046	epidermal differentiation, proteolysis and peptidolysis
KRT23	keratin 23	HAIK1	NM_015515	cytoskeleton organization and biogenesis
LCN2	lipocalin 2 (oncogene 24p3)	NGAL	NM_005564	transport
LOC91947				signal transduction, sensory perception
PLAT	plasminogen activator, tissue	T-PA	NM_000930	blood coagulation, protein modification, proteolysis and peptidolysis
RHCG	Rhesus blood group, C glycoprotein	RHGK	NM_016321	transport
S100A9	S100 calcium binding protein A9	CAGB; MIF	NM_002965	cell-cell signalling, inflammatory response
S100P	S100 calcium binding protein P		NM_005980	calcium binding, motility
SERPINB1	serine proteinase inhibitor, clade B (member 1	MNEI;PI 2	NM_030666	regulation of proteolysis and peptidolysis
SLC6A14	solute carrier family 6 member 14	ATB(0+)	NM_007231	amino acid metabolism, neurotransmitter transport
SPRR3	small proline-rich protein 3		NM_005416	cornfied envelope. differentiation
UPK1B	uroplakin 1B		NM_006952	urothelial differentiation

Table 5.4 Functional annotation of 22 genes underexpressed in immortal cell cultures.

The biological function noted is that designated by the gene ontology and is a composite from all three of the annotation networks of the gene ontology.

GO biological process group	No. of genes	Fisher exact probability, p	examples
cell proliferation			
cell cycle	15	3.7x10-14	MAD2L1, cdc20
mitosis	11	5.5x10-17	STK6, CAPG,
G2/M transition	2	0.004	DDA3, CyclinB1
G1	1	0.03	Cdc2
metabolism			
protein modification	5	0.03	STK6, NIMA
ubiquitin-dependent protein catabolism	2	0.02	UBE2C, cdc20
DNA metabolism			
regulation of DNA repair	1	0.01	UBE2C
DNA topological change	1	0.01	TOP2A
cytoskeleton organization	3	0.02	ASPM, IFI44

Table 5.5 Classification of genes upregulated at immortality by GO biological process.

The analysis demonstrates several significantly over represented groups of genes, as assessed by Fisher exact probability. Only groups over represented with $p<0.05$ were considered significant. Groups are presented as a primary term (shaded pale blue) with "child" terms underneath. The primary term may not itself be significantly over represented. A particular gene with multiple functions may belong to more than one group.

GO biological process group	No. of genes	Fisher exact probability, p	examples
metabolism			
alcohol metabolism	2	0.04	ALDH1A3 ALDH4
amino acid metabolism	2	0.03	SLC6A14
protein catabolism	4	0.02	KLK7, PLAT
immune response			
inflammatory response	2	0.03	CXCL6 S100A9

Table 5.6 Classification of genes down-regulated at immortality by GO biological process.

The analysis demonstrates several significantly over represented groups of genes, as assessed by Fisher exact probability Groups are presented as a primary term (shaded pale blue) with associated child terms underneath (if appropriate). The primary term may not itself be significantly over represented. The analysis demonstrates several significantly over represented groups of genes, as assessed by Fisher exact probability. Only groups over represented with $p<0.05$ were considered significant.

in the validation steps. The above provisos must be noted, particularly when validating these markers *in vivo*.

5.3.6 Discussion

The pilot study was vital in aiding our understanding of the effects that subtle changes in experimental protocol could have on the gene expression profile. It confirmed that strict standardization of the protocol was required. The pilot study also demonstrated the differential effect that the removal of irradiated 3T3 feeders may have on the GEP, particularly on the normal cultures.

Microarray analysis of samples from all stages of the development of HNSCC revealed that the main differences in gene expression were associated with the immortal phenotype. Within the main division of the samples into mortal and immortal, many of the samples clustered according to their clinical groups. Exceptions to this included the consistent clustering of D41 and D48 with the normal samples. It was noted earlier that these patients were non-smokers and that the tissues from which these cultures were derived expressed hTERT at a high level. This may indicate that these cultures are fundamentally different from the other mortal dysplastic cultures. Comparison with the expression of hTERT in the tissues related to the normal samples may shed some light on this. However, no tissue from the normal biopsies was retained (either frozen or formalin fixed), so this analysis is not possible.

The microarray analysis allowed for the generation of a list of novel candidate genes associated with the immortal phenotype. Many of the genes that were overexpressed in the immortal cultures are related to control of the cell cycle. All these genes require to be validated to confirm the accuracy of microarray data and assess if the differences seen at the level of transcript are also seen after translation.

Chapter Six: Validation of microarray results

6.1 Expression of well characterised markers

Initial validation was restricted to the well-characterised markers in the cultures such as hTERT, RAR-β, and p16. The expression of these markers is outlined in Figure 6.1.

6.1.1 hTERT

The upper panel of Figure 6.1 shows the expression of hTERT as detected by the Affymetrix U133A genechip. Only one of the two hTERT probe sets on the U133A genechip passed the filtering level set in the initial analysis (i.e. $\geq$6P). One culture, which had hTERT ectopically expressed by retroviral vector (D17 EST) was added to this graph as a positive control. This demonstrates that the Affymetrix U133A probe set 207199_at does detect hTERT, but only when present at a high level, such as follows ectopic expression. Otherwise, there is little discernable difference in the expression of hTERT in the mortal and immortal samples. There is no correlation between this result and that found by quantitative RT-PCR (McGregor et al., 2002).This indicates that the probe set is not sensitive enough to measure accurately the expression of hTERT in these keratinocyte cultures.

6.1.2 RAR-β

The lower panel of Figure 6.1 shows the expression of RAR-β as detected by the only one of three probe sets that passed the initial filtering criteria. This demonstrates a good agreement with the previously published Northern blot data (McGregor et al., 1997). The level of expression is low in D41 and D48, but this is appropriate considering their similarity to the normal cultures that has been demonstrated by the GEP. Unfortunately Northern blot analysis of RARβ expression in these samples has not been conducted.

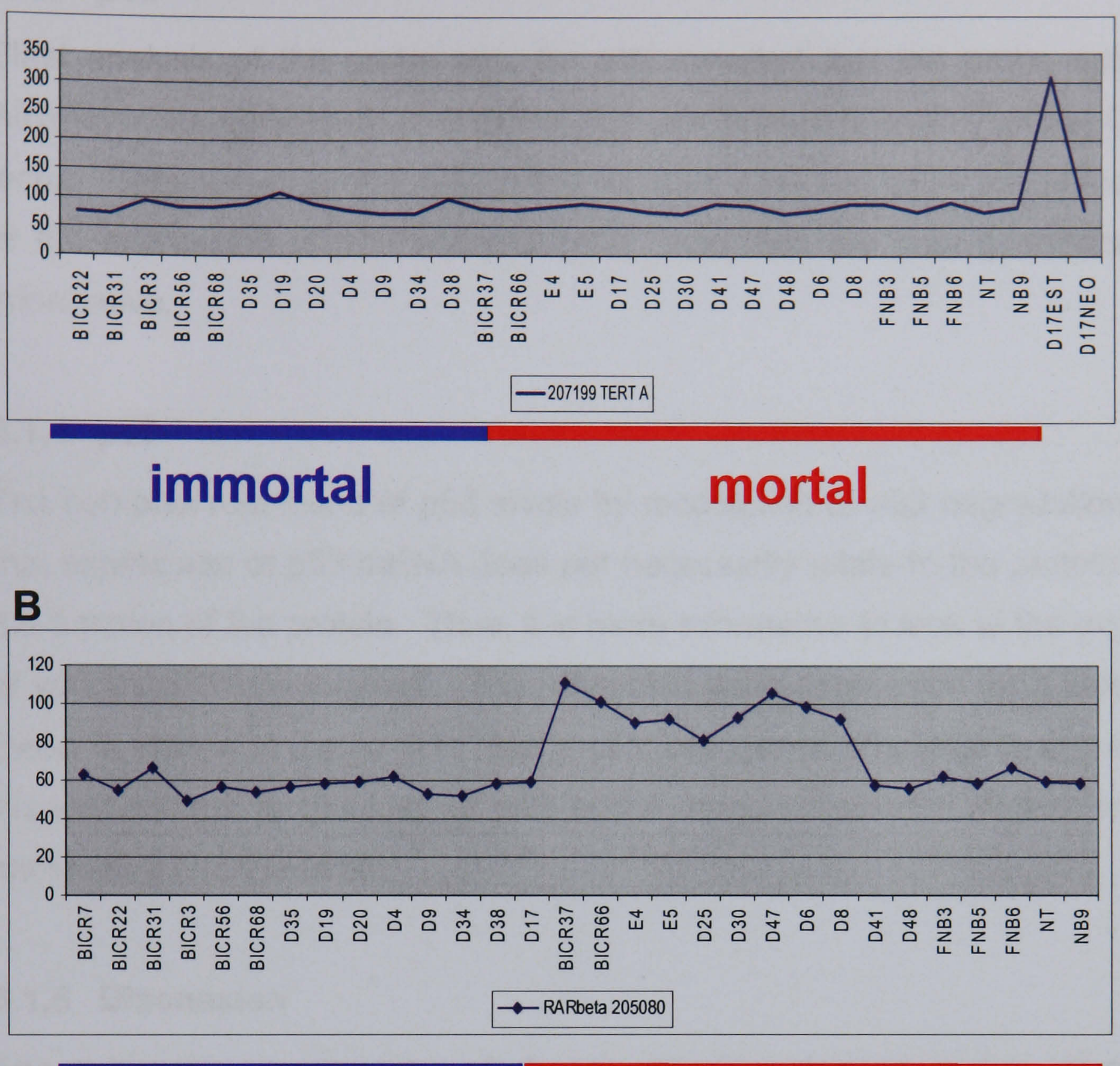


Figure 6.1 Expression of "known" markers on Affymetrix U133A genechip

Panel A shows expression of hTERT as measured by oligo-nucleotide probe set 207199_at. D17 with ectopically expressed hTERT (D17EST) and the empty vector control (D17NEO) were included as controls.

Panel B shows expression of RARβ as measured by oligonucleotide probe set 205080_at. The other RARβ probe set is not informative and was not passed as "present" in the initial filtering of the data.

Two of the three p16 probe sets were designated as present in the initial filtering. However, the oligonucleotide probe sets for the measurement of p16 on the U133A genechip were not informative.

6.1.3 p16

Blast analysis of the probe sets for p16 revealed that the probe sets which Affymetrix state measure p16 expression are located in exon 2 of the CDKN2a locus. Selection of probe sets in this location does not allow for differentiation of the expression of p16 and p14 (ARF) and thus the data generated is not informative.

6.1.4 p53

The complex regulation of p53 levels by modulation of p53 degradation means that expression of p53 mRNA does not necessarily relate to the protein level or the function of the protein. Thus, it is more informative to look at the expression of various p53 target genes. The Affymetrix gene expression for a selection of these is shown in Table 6.1. Many of these genes changed in a predictable manner related to the loss of p53 in the immortal cultures. However, others showed no change at all.

6.1.5 Discussion

The microarray expression of these well-characterised genes agrees well expression as assessed by other methods (see chapter 4). However, this analysis has highlighted the limitations of microarray analysis with regard to sensitivity, specificity and accurate design and annotation of the probe sets. It is also not possible to comment meaningfully on proteins for which expression level or activity is controlled mainly by post-translational events.

GENE	Normal	MD	MC	ID	IC
p21WAF1	836 ± 34	803 ± 37	1183 ± 197	472 ± 154	393 ± 38
Cyclin G2	296 ± 30	261± 15	438 ± 81	166 ± 28	110 ± 17
Ribonucleotide reductase M1	322 ± 34	332 ± 21	156 ± 12	580 ± 86	596 ± 48
Ribonucleotide reductase M2	691 ± 130	703 ± 93	102 ± 27	1130 ± 210	1105 ± 101
GADD45	901 ± 119	634 ± 44	1231 ± 132	490 ± 90	485 ± 78
tp53IP3	490 ± 104	607 ± 35	1044 ± 93	351 ± 79	176 ± 30
PERP	2109 ± 185	1676 ± 133	2230 ± 106	1655 ± 217	1147 ± 96

Table 6.1 Mean Affymetrix expression level of selected known p53 target genes in various groups of samples.

The numbers shown in the table are normalised and represent the mean for the various groups with standard error of the mean also shown. MD = mortal dysplasia, MC = mortal carcinoma, ID = immortal dysplasia and IC = immortal carcinoma. No significant differences in expression of other p53 target genes, such as PUMA, MDM2, BAX, PIDD, FAS, were seen.

6.2 Validation of novel candidate markers of immortality in vitro by western blotting

The candidate markers of immortality were validated by Western blotting. Ultimately, this was preferred to validation at mRNA level by quantitative RT-PCR as only markers that show differences at protein level can be considered to harbour gene expression changes that may alter the phenotype of the cell. Even so, many functional changes are post-translational and are unlikely to be identified by Western blotting. Additionally, the expression of some genes may be regulated by alterations in the pattern of degradation of the encoded proteins. The use of Western blotting restricted the validation process to those genes for which antibodies were commercially available. The Western blots, together with the Affymetrix expression data, for a selection of these validated are seen in Figures 6.2 (genes more highly expressed in immortal cells) and 7.2.2 (genes more highly expressed in mortal cells).

In general, the protein expression of genes which were more highly expressed in immortal cultures , agreed with the Affymetrix expression level. There was very little expression of these genes in the mortal cultures (Figure 7.2.1). The expression levels in the atypical mortal culture D17 were very similar to that seen in the immortal samples. This is in keeping with earlier comments on the pattern of gene expression in this culture.

The heterogeneity within the mortal samples, which was alluded to earlier, was evident in those in the genes that were overexpressed in the mortal cultures (Figure 6.3). Additionally, low levels of expression of proteins encoded by these genes in the immortal cultures, as predicted by the Affymetrix expression level, were not uniformly seen. This seems to be particularly true for D9 and D34 (bearing in mind that D38 is also atypical). The Western blots indicated that few of the markers showed an “all-or-nothing” loss or gain of the expression of the particular gene, as was the case with p16.

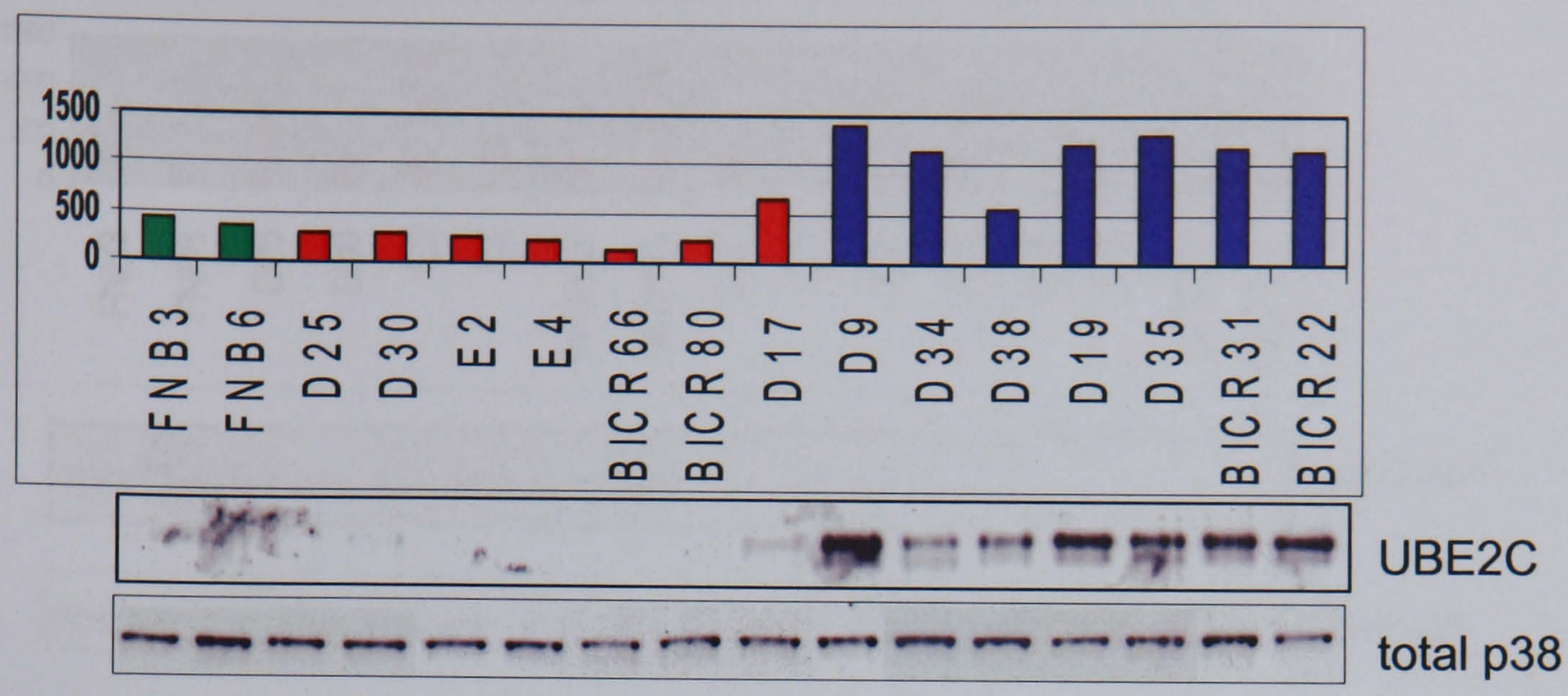
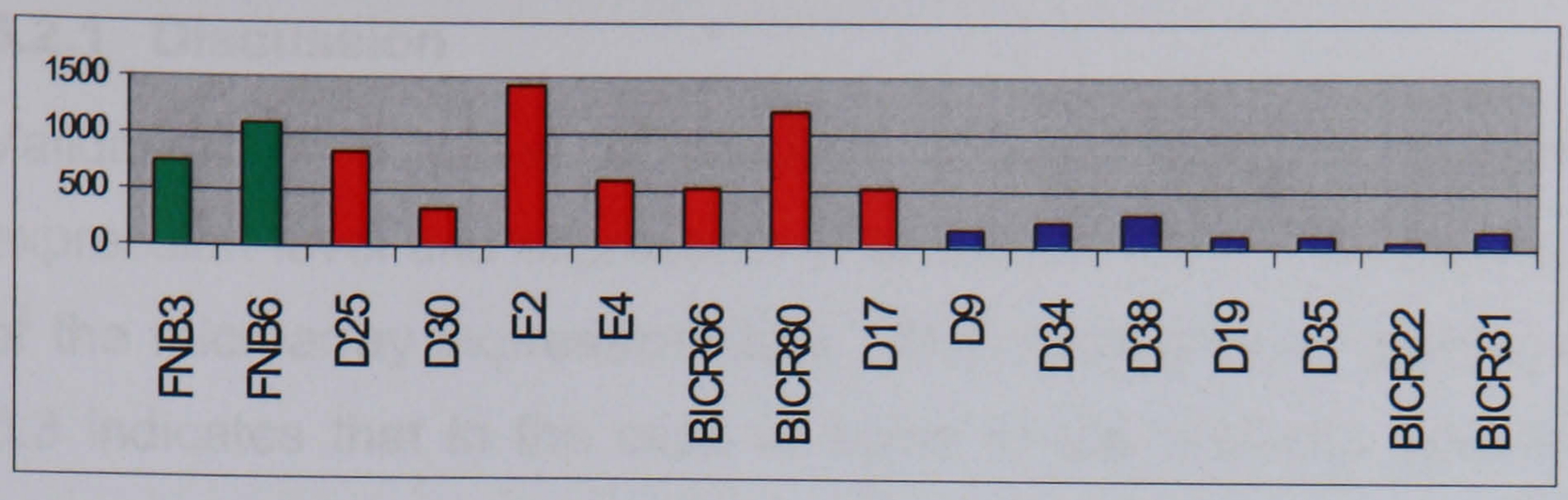
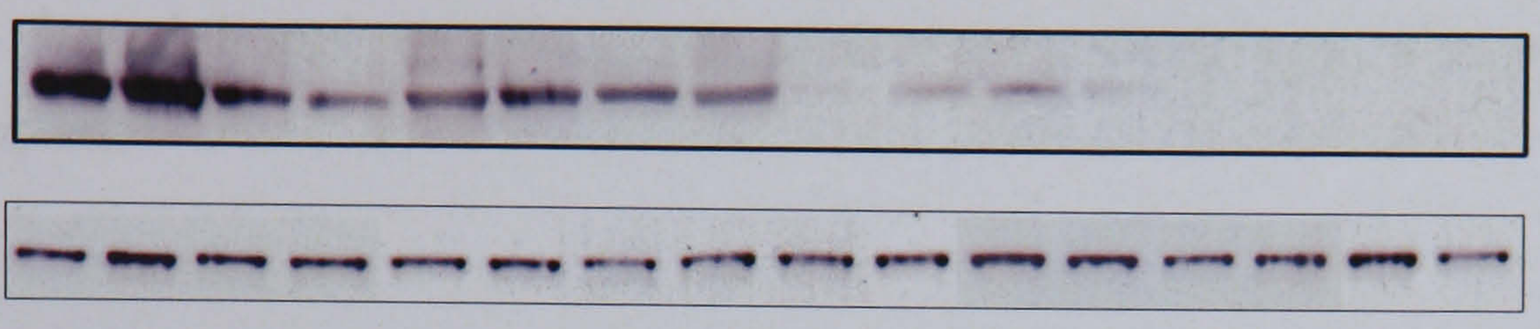
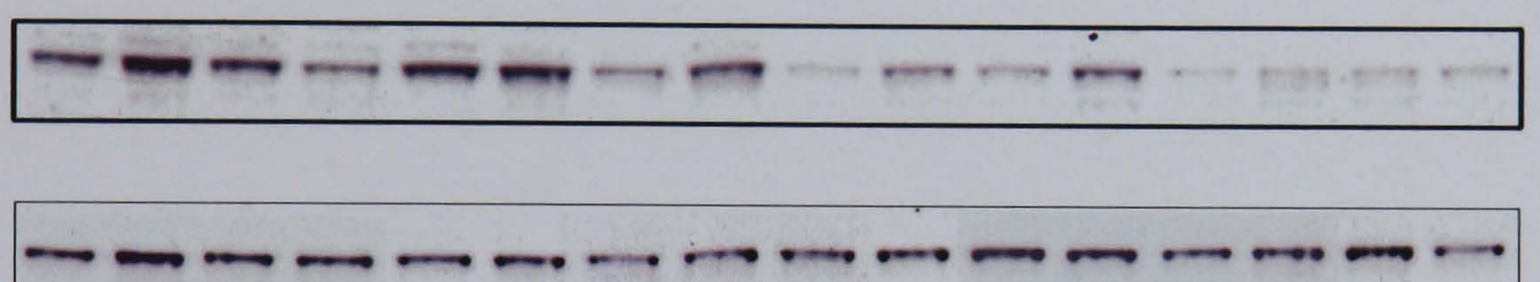


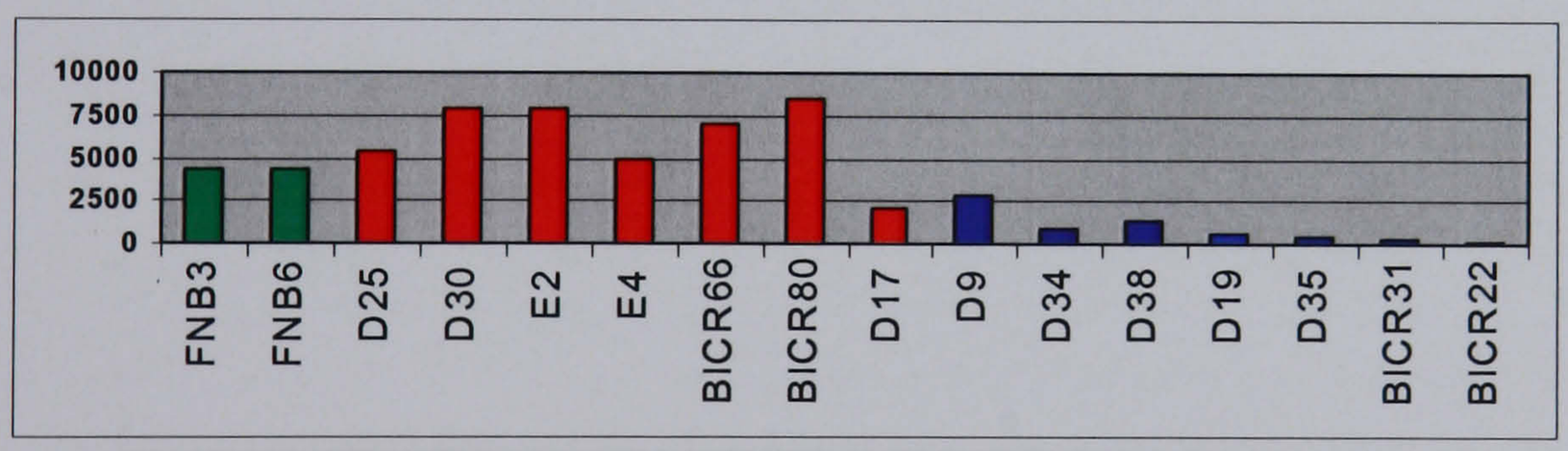
Figure 6.2 Validation of selected genes which were overexpressed in immortal samples.

Western blots of whole cell extract from a panel of normal, dysplastic and carcinoma keratinocyte cultures. The Y-axis refers to the normalised Affymetrix expression level for the mean of the technical replicates. Bars in the graphs coloured green represent normal samples, red bars mortal dysplasia/carcinomas and blue bars immortal dysplasias/carcinomas. The expression of these proteins matches the RNA transcript level as assessed by microarray. The expression of some of these genes in D17 is similar to that seen the in immortal dysplastic cultures. This work was completed by the author and Dr J Thurlow.









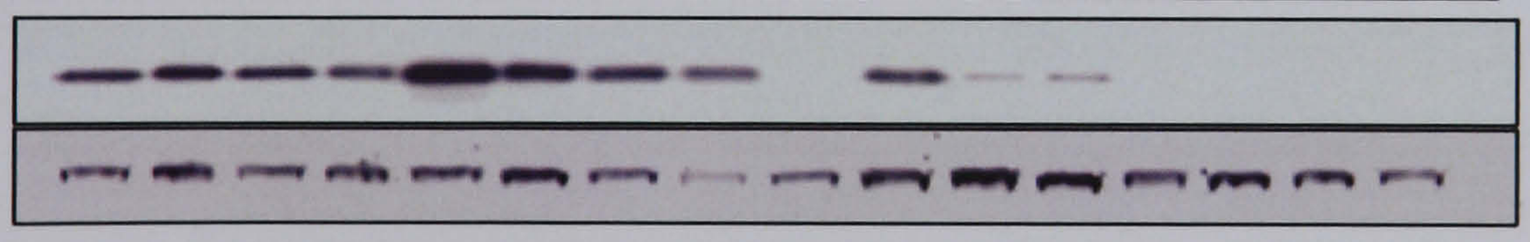


Figure 6.3 Validation of selected genes which were underexpressed in immortal samples.

Western blots of whole cell extract from a panel of normal, dysplastic and carcinoma keratinocyte cultures. The Y-axis refers to the normalised Affymetrix expression level for the mean of the technical replicates. Bars in the graphs coloured green represent normal samples, red ones mortal dysplasia/carcinomas and blue immortal dysplasias/carcinomas. The pattern of protein expression matched the RNA transcript level only to some degree. In particular, some of the immortal cultures (D9 and D34) expressed more protein than the RNA analysis would indicate. This work was completed by the author and Dr J Thurlow.

6.2.1 Discussion

Validation of a panel of markers showed good agreement in the RNA expression level and expression of protein *in vitro*. This confirms the accuracy of the microarray expression data. The heterogeneity seen in figures 6.2 and 6.3 indicates that in the case of some of the markers, post transcriptional or post translational modifications may have modulated the levels of protein detected.

6.3 Validation of candidate markers of immortality *in vivo* by IHC

The expression of some of the markers validated in western blot was also assessed in the original tissue sections by IHC in 10 of the carcinomas: four "mortal", five "immortal" and one in "crisis". A selection of the markers validated in the carcinomas is shown in Figures 6.4 and 6.5. p53 was added to the panel as the p53 status of the culture had been fully investigated, with p53 mutation and alteration in expression showing clear association with immortality (Burns et al., 1993). The carcinomas were utilised in preference to the dysplasia tissues, as the difference in expression level of the novel immortality markers, as measured by the arrays, was maximal in comparison of mortal and immortal carcinoma cultures. Assessment of the expression of these proteins *in vivo* is subject to very similar caveats to those outlined for validation by western blotting, in addition to the subjective nature of assessing expression of proteins by IHC.

The tissues from which the immortal carcinomas were derived showed relatively uniform staining of the markers of immortality particularly at the advancing edge of the tumour (Figure 6.5). In most cases, the tissues showed fairly uniform p53 expression, together with strong Cyclin B1 expression, especially at the invading edge of the HNSSC nests. In contrast, these HNSCC nests showed only a small proportion of cells expressing S100A9 or involucrin. There was, however, a measure of heterogeneity in all of these tumours. This was most pronounced in BICR56, which showed areas of well differentiated nests of tumour, which centrally showed a mortal staining pattern. However, the expression of the characteristically immortal markers was clearly present in the basal layer of these nests, indicating that they were not mortal in character, but immortal with retained ability to differentiate. In general, the more poorly differentiated the tumour, the more uniform the immortal staining pattern. BICR7, which has an overall GEP very similar to that of the immortal carcinomas showed a characteristically immortal staining pattern (Figure 6.5).

The tumours that gave rise to mortal carcinoma cultures were much more heterogeneous, with varying proportions of characteristic mortal and immortal staining patterns (Figure 6.4). Thus, some areas showed staining

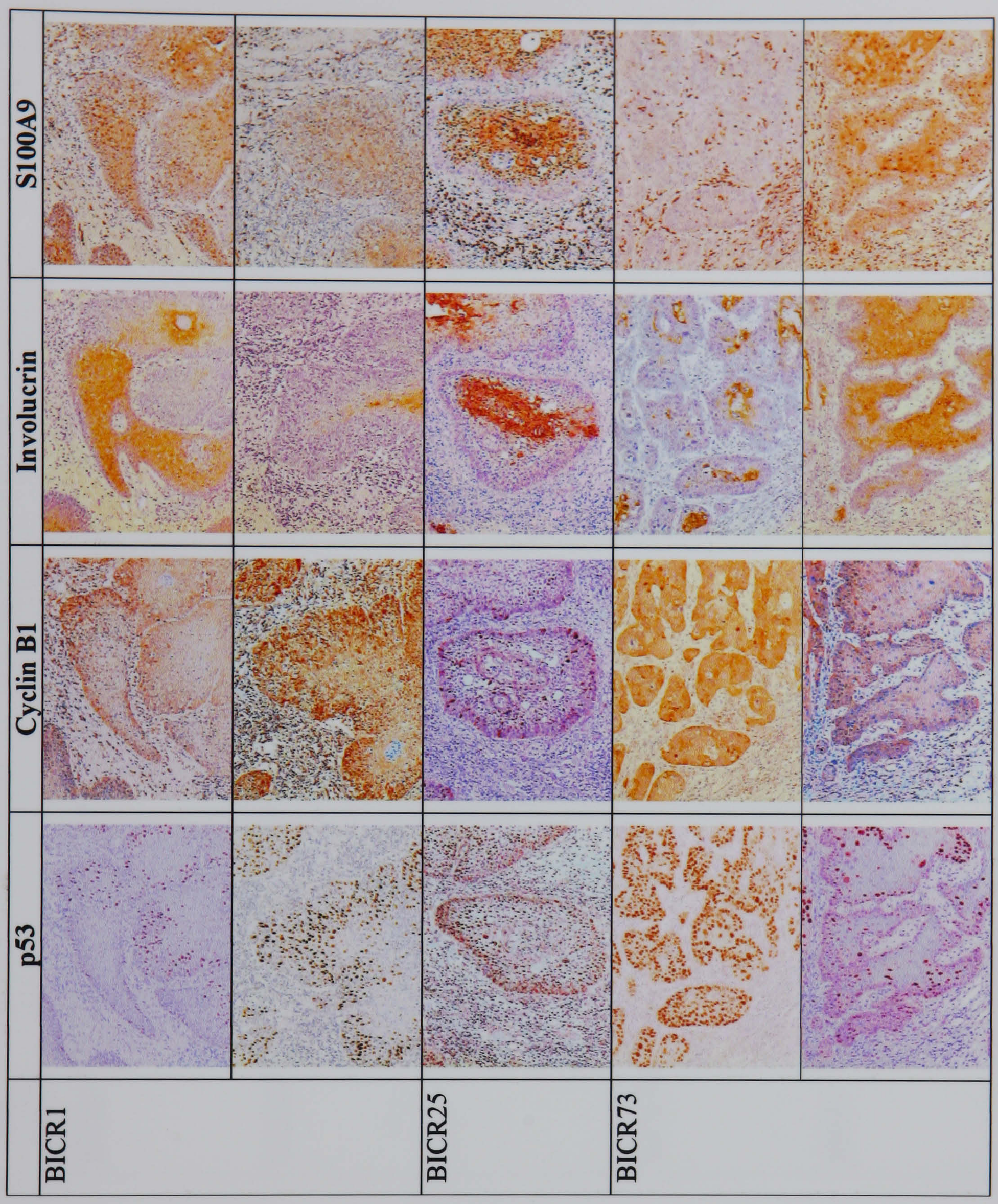


Figure 6.4 Validation of selected candidate immortality genes by immunohistochemistry in tumours which gave rise to mortal cultures.

The panels show p53, Cyclin B1, Involucrin and S100A9 immunostaining in formalin-fixed biopsy tissue from which the cultures were derived. Negative controls (not shown), using IgG showed no staining. The panels indicate the level of heterogeneity present in the tumours which gave rise to the mortal carcinoma cultures. One tumour, BICR25, showed a uniform mortal staining pattern. This work was conducted on adjacent sections where possible and completed by the author, Dr J Thurlow and Ms J Fleming.

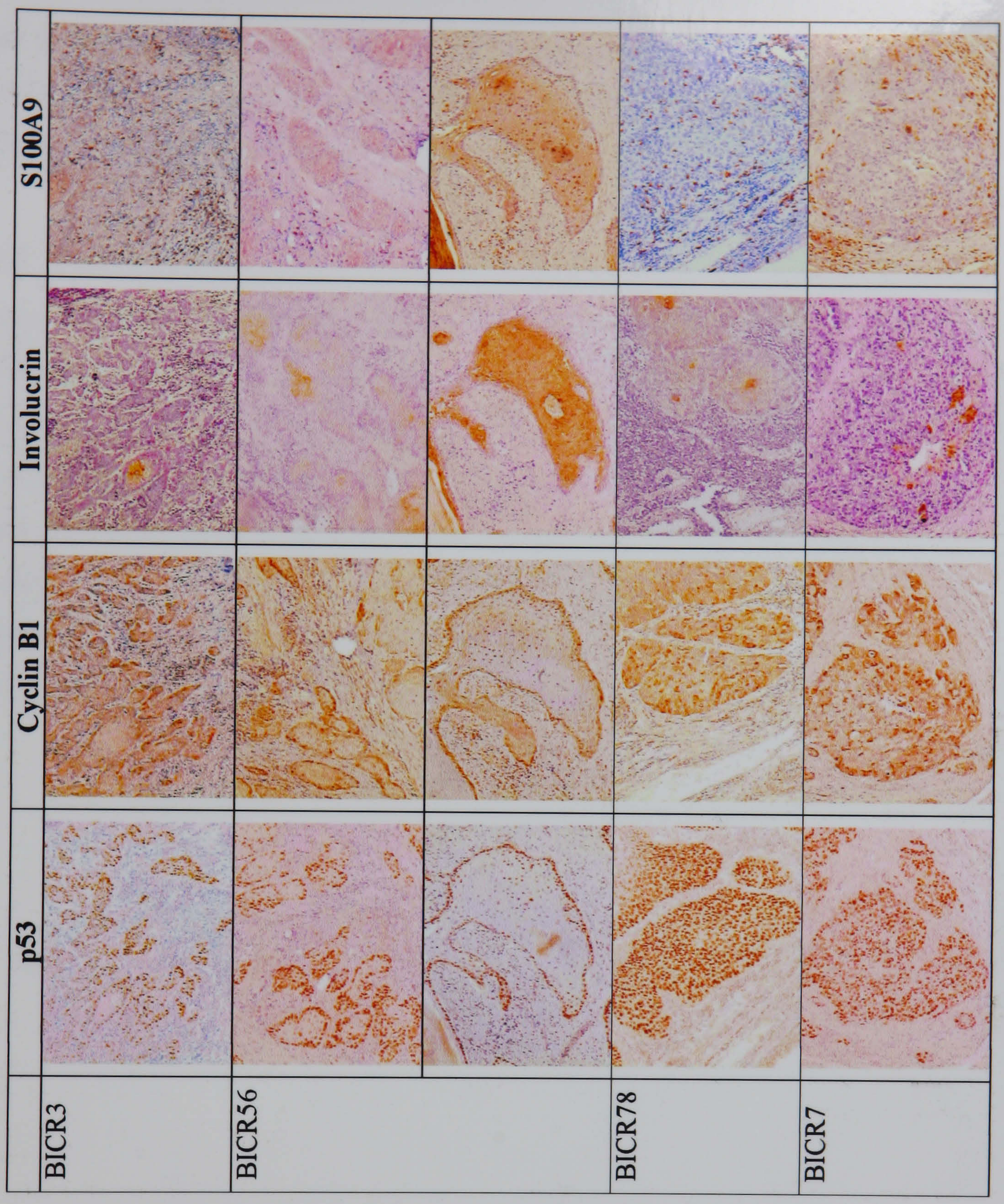


Figure 6.5 Validation of selected candidate immortality genes by immunohistochemistry in tumours which gave rise to immortal cultures and in BICR7 (crisis).

The panels show p53, Cyclin B1, Involucrin and S100A9 immunostaining in formalin-fixed biopsy tissue from which the cultures was derived. Negative controls (not shown), using IgG showed no non-specific staining. Whilst the pattern of staining of the markers of immortality was relatively consistent, there was still some level of heterogeneity, most evident in BICR56. The pattern of expression in BICR7 (in crisis in vitro) was similar to that of the other immortal tumours. This work was conducted on adjacent sections where possible and was completed by the author, Dr J Thurlow and Ms J Fleming.

patterns very similar to that found in most of the immortal carcinomas (see above), whilst others showed staining more characteristic of the mortal cells *in vitro* (very little p53 and Cyclin B1 staining, and high expression of involucrin and S100A9). One tumour, BICR 25, was composed almost entirely of nests of cells with a mortal phenotype.

6.3.1 Discussion

These IHC data indicate that the *in vitro* mortal and immortal phenotypes exist in HNSCCs *in vivo*. Also, it is clear that both may exist within the same tumour. Thus, the extensive gene expression differences associated with immortality *in vitro* also contribute to HNSCC heterogeneity *in vivo*. These findings have clear implications as to identification of the source cells within the tumours that grew in our cultures, as the immortal cells appear to be less differentiated, whilst the cells with a characteristic mortal phenotype are more differentiated. The factors that dictate which type of cell grows from a mixed cell population are not known.

Chapter Seven: General Discussion

7.1 The culture model

Whilst this study has largely centered round the use of microarray technology for the assessment of the GEPs of the various cell cultures, the ultimate clinical usefulness of the data is dependent upon the ability of the cell culture model to replicate the pattern of gene expression seen *in vivo*. The initial experiments outlined in this thesis indicated that the limited panel of molecular changes previously reported as having association with immortality (McGregor et al., 2002) was present to a large degree in the tissue from which the cultures were derived. In particular, this was true for changes in expression and activity of those genes that control the cell cycle and are involved in the replicative barriers of senescence and crisis. Generally, this is in keeping with the original observations of Rheinwald and coworkers, who indicated that serial cultivation with irradiated 3T3 fibroblasts, gave rise to a genetically stable cell population that corresponded well to the tissue of origin (Rheinwald & Beckett, 1981). The subsequent demonstration of good *in vivo/in vitro* correspondence of the novel markers of immortality further supports the culture system as a reasonable model of HNSCC development in vivo. This not only included proliferation markers but also markers of differentiation and motility, such as involucrin and S100A9.

The poor correlation of the expression of RAR-β in the cultures and tissue serves to indicate that the 3T3 culture system does not fully replicate the *in vivo* state. The observation that RAR-β was expressed in the immortal tissues requires explanation. It is likely that all of the dysplastic lesions, whether giving rise mortal or immortal cultures, allowed terminal differentiation to occur, but in the immortal cultures control of differentiation might have been either defective or occurring in a smaller proportion of cells. Additionally, the monolayer culture method does not allow the cells to establish an interaction with fibroblasts and other stromal cells. Such interactions have been shown to be of great importance in the development of neoplastic lesions as stromal cells have been shown to exert a significant effect on the gene expression profile of the neoplastic population (Tlsty, 2001; Tlsty & Hein, 2001). The further use of organotypic models, either as described earlier (Figure 4.10) or use of the more

sophisticated model recently described (Andriani et al., 2004) may improve this, but faithful reconstitution of the *in vivo* state may not be possible *in vitro*, due to its complexity.

To further explore the relationship between the cultures and tissues, a fuller exploration of the gene expression patterns in some of the matched tissue and culture samples has now been conducted using microarray analysis, as funding for this has become available since the completion of the laboratory work for this thesis. Analysis of the data may give further insight into the changes in gene expression on establishment in culture.

In terms of the pattern of gene expression related to proliferation and growth, the good agreement of *in vivo* and *in vitro* gene expression and the continued clear separation of the mortal and immortal samples laid a basis upon which the microarray study could proceed. There was a reasonable expectation that at least some of the changes in gene expression in culture would be identified in the tissue of origin. The identification of the atypical gene expression profiles of D17 and D38 in their related tissues was further evidence of the appropriateness of this tissue culture model, as is the extensive heterogeneity in gene expression in D4. This was initially a mortal culture, from which emerged an immortal cell line (McGregor et al., 1997).

The *in vivo* validation of the novel markers of immortality utilised immunohistochemistry, which allowed the variability of expression of the markers to be assessed. Heterogeneity in gene expression was noted in all the carcinomas, but particularly in the tumours that gave rise to mortal cultures. A possible alternative method of *in vivo* validation would have been microarray analysis of the original tumours. As there was no fresh frozen tissue from the original tumours, the only material available was formalin fixed. Whilst the advances in microarray technology now allow microarray analysis of RNA extracted from formalin fixed material, the interpretation of such data is difficult. In many ways, immunohistochemistry is superior to this, as it allows assessment of the final product i.e. the protein, together with visualisation of any variability in expression within the tumour. In the context of microarray analysis, this could only be achieved by the examination of morphologically different areas within the tumour, each captured by LCM. The variation in

staining seen in the primary tumours in this study indicated that "bulk" analysis of tumours might obscure important patterns of heterogeneity of gene expression within the tumour. Microarray analysis of HNSCC in many other publications is often based on the bulk analysis of tumours (Cromer et al., 2004; Kuriakose et al., 2004; Lemaire et al., 2003; Roepman et al., 2005).

7.2 The gene expression profiles

The most striking differences in the GEPs were the large number of differences upon comparison of the mortal and immortal carcinomas and also comparison of the mortal and immortal high risk dysplasia cultures (Table 5.2, upper and lower). The large number and pattern of these differences indicated that there may be two separate, perhaps divergent, pathways to the development of the mortal and immortal carcinomas, with intermediates much in keeping with the clinical history of the individual dysplastic lesion. Thus, it may be possible to identify low and high risk dysplastic lesions, with the high risk lesions having a very similar GEP to the SCC cultures. This was borne out by the relatively small number of differences when comparing the mortal and immortal dysplasia cultures with their respective carcinomas. However, the hierarchical clustering did not allow for the clear identification of these various groups in terms of their GEP. There were very few consistent differences in gene expression on comparison of the high risk immortal dysplastic lesions and the immortal carcinomas. This indicates that these cells had gained additional changes in their GEP that were not present in the low risk immortal dysplastic cultures. The fact that they clustered very closely with the immortal carcinomas indicated that they may have gained almost all of the immortal carcinoma related changes in gene expression, but had not yet acquired that ability to invade (Figures 5.8 and 5.9). In this respect, this is very similar to the findings of Ha and coworkers (Ha et al., 2002b), who found that most moderate/severely dysplastic lesions had a GEP very similar to HNSCCs.

Due to certain limitations of hierarchical clustering in its ability to demonstrate the differences in the GEP of the various groups we have recently employed more sophisticated clustering techniques, such as spectral clustering (Kluger et al., 2003) in collaboration with Dr G Kalna and Dr D Higham in the Department of Mathematics at the University of Strathclyde. This method of clustering is

based on singular value decomposition (SVD) which may be used to assign one or more numerical values to each gene and each sample. Spectral clustering has been demonstrated as a useful tool for analyzing microarray data from well characterized cancer types, for example, it correctly identifies known subtypes of leukemia, lymphoma, breast cancer and brain cancer (Higham et al., 2005; Kluger et al., 2003), which has proved difficult with other methods. Application of spectral clustering to the data allows for a clearer separation of the different groups of cultures and this can be demonstrated on several different dimensions, not merely the 2D relationship which is dictated by the use of hierarchical clustering algorithms (for example, Figure 5.8). The delineation of groups of cultures relates to the known clinical features the lesions (Figure 7.1).

The main groups of samples, based on both clinical and gene expression pattern, are shown in Figure 7.2. The number of gene expression differences for selected comparisons is also shown in this diagram and the pair-wise comparisons suggest that two pathways for the development of HNSCC exist– a mortal pathway and an immortal pathway. These can be observed in distinct patterns of gene expression. The number of changes in gene expression within each arm of the diagram was small when compared to the difference in gene expression between the mortal and immortal cultures. However, the interpretation of such a diagram must be treated with caution, as the cultures were derived from different individuals and not sequential biopsies taken during HNSCC development in a single patient. The demonstration of similar gene expression changes occurring in the development of an HNSCC within a dysplastic lesion from the same patient would allow such lineage relationships to be more surely established.

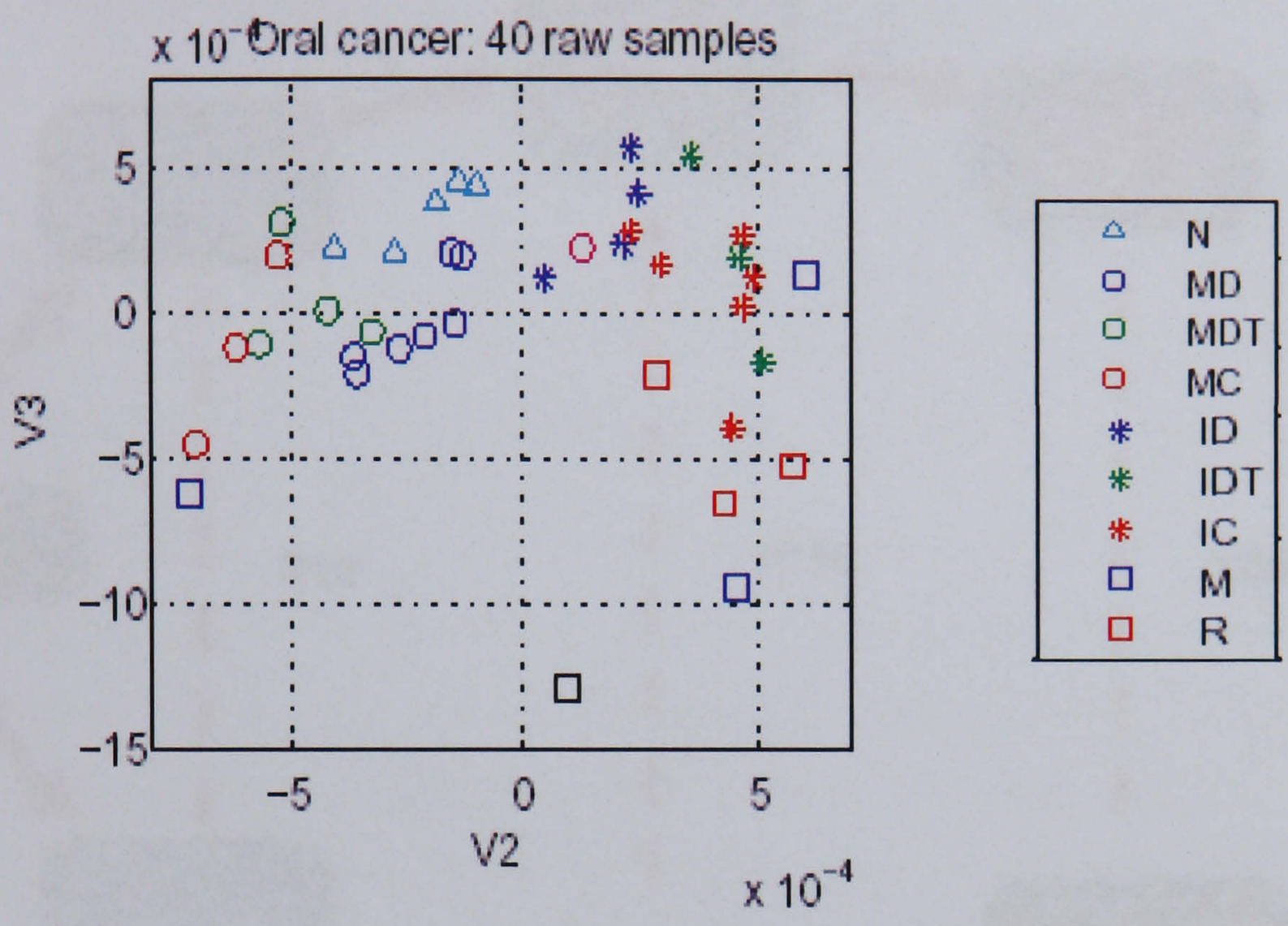


Figure 7.1 Analysis of the gene expression profiles by spectral clustering

Analysis of the GEPs of dysplasias by spectral clustering in relation to primary HNSCCs, SCC recurrences, metastases and normal mucosa. See discussion text for an explanation of the basis of spectral clustering. The diagram shows the components of vectors V2 and V3 for each sample as the horizontal and vertical coordinates, respectively. Samples that were close in this two-dimensional ordering can be viewed as similar in GEP. This method demonstrates an enhanced separation of the cultures utilising the different vector components. This data is reproduced courtesy of Dr G Kalna and Dr D Higham, Department of Mathematics, University of Strathclyde.

N = normal, MD = mortal dysplasia, MDT = mortal dysplasia which progressed to SCC MC mortal carcinoma, ID = immortal dysplasia, IDT = immortal dysplasia which progressed to carcinoma, IC = immortal carcinoma, M = metastases (mortal or immortal), R = recurrence (all immortal).

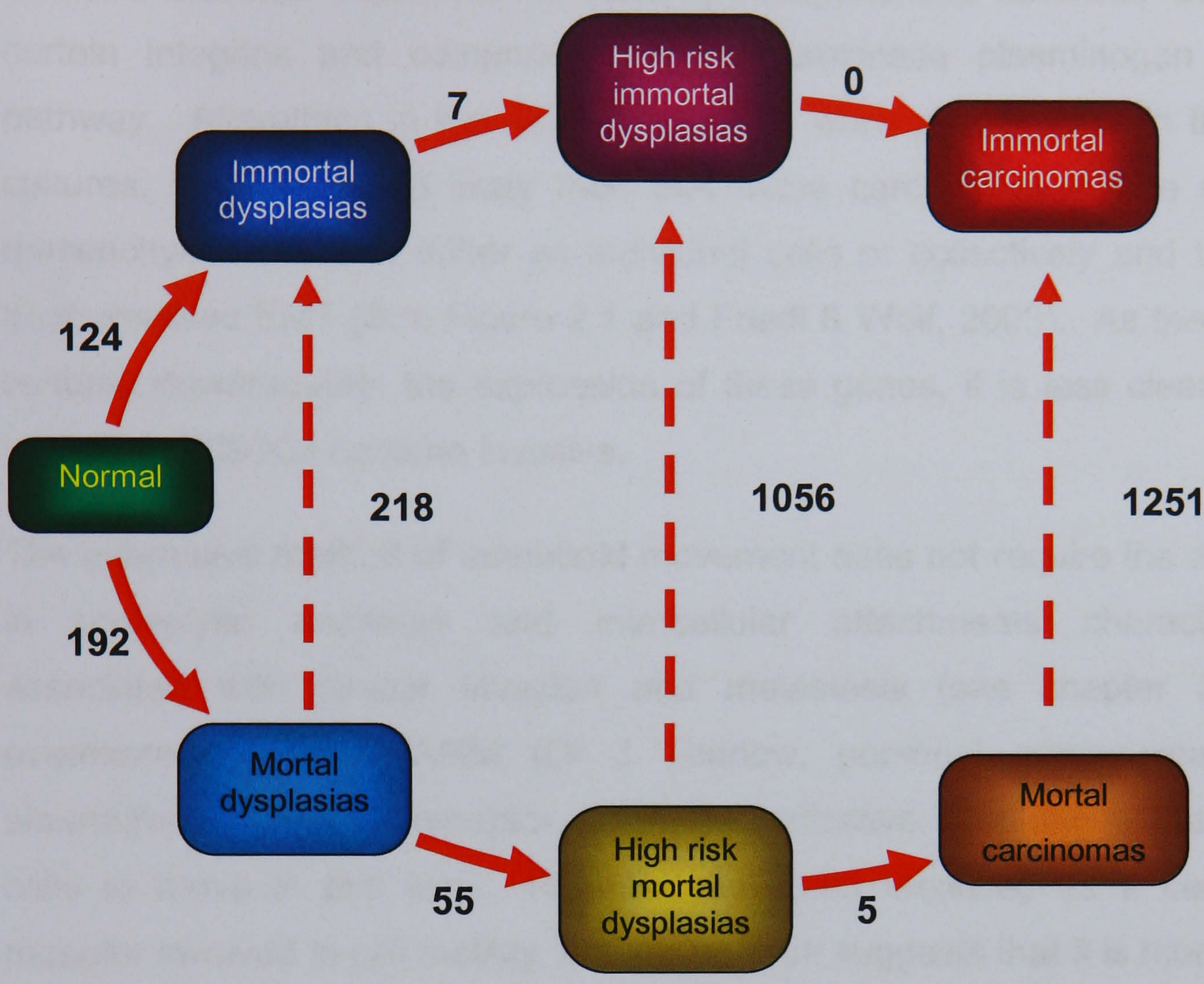


Figure 7.2 Possible pathways of oral cancer progression based on similarities in gene expression between normal oral mucosa, dysplasias and SCCs

Relationships of dysplasias to carcinomas revealed by gene expression profiling. The model demonstrates how the two types of HNSCC may be derived from intermediate dysplasias. Solid lines indicate likely routes of progression via mortal and immortal pathways, based on modest differences in gene expression between low and high risk dysplasias and SCCs. Dashed or dotted lines indicate possible alternative pathways; those indicated by dotted lines would involve much bigger gene expression changes. The numbers represent the number of statistically significant difference in gene expression, as also shown in Table 5.2 (upper and lower).

The possibility of two pathways in the development of HNSCC raises interesting questions in relation to the gene expression pattern of the two carcinomas. The classical markers of invasion and metastasis were predominantly found in the mortal carcinoma cultures and were absent from the immortal cultures. These included elevated expression of MMPs, collagens and laminins, CEACAM1, certain integrins and components of the urokinase plasminogen activator pathway. Alterations in the TGF-β pathways were also present in the mortal cultures. It would seem likely then that these carcinomas invade using the mesenchymal method, either as individual cells or collectively and this might have involved EMT (See Figure 2.1 and Friedl & Wolf, 2003). As the immortal cultures downregulate the expression of these genes, it is less clear how the immortal HNSCCs became invasive.

The alternative method of amoeboid movement does not require the alterations in proteolytic enzymes and intercellular attachments characteristically associated with cancer invasion and metastasis (see chapter 2). The overexpression of RHAMM (Dr J Thurlow, personal communication), an alternative hyaluronan receptor, might be indicative of changes that allowed cells to move in this way. RHAMM has been identified as a cell surface receptor involved in cell motility, but recent work suggests that it is more likely to act as an intracellular hyaluronan-binding protein interacting with both microtubules and actin filaments (Assmann et al., 1999). Investigation in other cancers has found overexpression of RHAMM at the invasive edge of lobular breast cancer and demonstrated a relationship between RHAMM expression and risk of nodal metastases in breast cancer and with clinical aggressiveness in pancreatic cancer (Abetamann et al., 1996; Wang et al., 1998). Thus, RHAMM over expression in immortal HNSCCs could be responsible for the invasiveness of immortal HNSCCs.

Amoeboid migration has also been related to increased signalling via Rho/ROCK (Croft et al., 2004; Sahai & Marshall, 2003). Although our immortal HNSCCs did not show significant changes in expression of Rho family members, my colleague Dr J Thurlow has recently shown that they did over express ECT2, a Rho GEF (Miki et al., 1993). ECT2 has been implicated in cytokinesis and in the activation of Cdc42 in mitosis (Oceguera-Yanez et al., 2005), but it also interacts with the polarity protein complex, Par6/Par3/PKCζ

and localises at sites of cell-cell contact as well as in the nucleus (Liu et al., 2004). ECT2 also interacts with the Kelch-like protein, KLEIP, which co-localises with actin at cell-cell contact sites (Hara et al., 2004). Thus, ECT2 could also play a role in immortal HNSCC motility. Further work is required to test these alternative hypotheses and to explain immortal HNSCC motility.

7.3 Gene expression profile and prognosis

Such profound differences in the GEPs of the mortal and immortal carcinomas might also have clinical importance. It was noted in the Chapter 2 that the ability to establish as a cell line in culture has been related to poor prognosis and lymph node metastases (Shimada et al., 2003). Although univariate analysis of immortality in culture showed correlation with nodal status, p53 pathway defects (elevated p53 or MDM2 expression), $p21^{WAF1}$ and $p27^{KIP1}$ expression and reduced FHIT expression, in multivariate analysis, p53 pathway defects were the main independent predictors of cell line establishment and of propensity to lymph node metastasis. This is compatible with the GEP seen in our immortal cells and suggested that acquisition of immortality was associated with future propensity of primary SCCs to metastasise to lymph nodes.

The link between immortality in cell culture and poor prognosis is borne out to some extent by our follow-up data. The small number of patients, the extensive co-morbidities in these patients and incomplete follow-up information - the clinical case notes for some of the patients have been lost or destroyed - places severe limitations upon this analysis. Another factor was stage at presentation, as those tumours that gave rise to immortal cultures tended to be more advanced. Of primary HNSCCs that gave rise to immortal cultures, five of seven were T4, whereas only three of eight primary mortal HNSCCs had a tumour of this size. In terms of the presence of nodal metastases, five of seven immortal tumours were node positive, whilst only three of eight mortal tumours had nodal metastases at diagnosis. Despite these caveats, none of the patients whose tumour gave rise to a mortal carcinoma has died in circumstances directly attributable to their HNSCC diagnosis. However, at least five of the patients who produced an immortal culture died from causes attributable to their HNSCC diagnosis, often after a protracted clinical course.

7.3.1 Other microarray studies

Interpretation of the results of microarray studies in the published scientific literature is greatly hindered by the variety of study designs and methodologies employed. In some studies, statistical analysis of the data is not ideal, as correction for multiple hypothesis testing has not been performed (Al Moustafa et al., 2002; Ha et al., 2003). In others, where appropriate corrections were made, the list of genes was very small, even allowing for a large FDR (Nagata et al., 2003). Interestingly (and perhaps not surprisingly in view of this), comparison of the GEPs of some of the early gene expression studies has produced agreement of around 7% in the lists of genes proposed as important in the development of HNSCC (see http://www.upstate.edu/microb/shillite/Microarray_Oral_Cancer_Genes.HTM).

Many recently published *in vivo* gene expression profiling studies have utilised various classification methods to subdivide HNSCCs into groups with GEPs that may be linked to different prognoses. Generally, these *in vivo* studies measure gene expression differences in the whole tumour cell population, not just the malignant epithelial component, as in our study. Whilst some studies have utilised LCM technology to select a pure epithelial tumour sample for analysis, none has attempted to select and analyse morphologically different areas, or to sample areas with differing patterns of differentiation. The merits of such bulk analysis of tumours are debatable, as the tumour heterogeneity alluded to above cannot be assessed. The presence of other types of cell in the sample analysed can vary extensively, particularly as the intensity of the immune response to the tumour differs between patients. One study has demonstrated that this can have a marked effect on the overall GEP, particularly with relation to immune system-related markers (Ginos et al., 2004). Others argue, however, that it does not matter if some of the markers are stromal or immunoregulatory elements (Roepman et al., 2005). That argument can only be valid if the sole aim is a clinically relevant set of molecular predictors – analysis of the GEP in terms of cellular and molecular biology of the tumours is compromised by inability to distinguish the relative contribution of the various cellular elements.

The study of 25 primary HNSCCs, 16 locally recurrent HNSCCs and 13 normal oral mucosa samples by Ginos and coworkers found a gene expression

signature correlating with local recurrence (Ginos et al., 2004). This included proteins involved in extracellular matrix interactions, such as certain laminins and integrins, proteins implicated in EMT, (such as SNAI2), and the MET oncoprotein. With the exception of MET, this molecular signature of recurrent disease strongly resembles the GEP of our mortal SCCs. A different study of 60 HNSCCs categorized the tumours into two main groups, one of which could be further subdivided (Chung et al., 2004). The main group, which was not further subdivided, was associated with increased local recurrence and displayed a gene expression signature which was both consistent with that demonstrated by Ginos and coworkers and resembled that of our mortal SCCs: e.g. LAMA3, B3 and C2, P-cadherin, desmocollin 2, keratin 14, kallikrein 10, SKALP and TGF-β.

Three recent *in vivo* studies have reported HNSCC GEPs associated with high risk of lymph node metastasis (Chung et al., 2004; Cromer et al., 2004) Roepman et al, 2005). The studies reported by Chung et al. and Cromer et al. also suggest that the gene expression changes associated with lymph node metastasis are different from those associated with local recurrence of HNSCCs. Despite the long lists of genes generated by these studies, there appears to be no overlap between the published profiles of high risk of metastasis of HNSCC. This may partly be explained by the use of different microarray platforms and variations in the design of the studies including the use of different reference samples. However, there is greater overlap between the GEPs predictive of lymph node metastasis in Chung et al and Cromer et al with our immortal HNSCC signature than with our mortal HNSCC signature. In contrast, the metastasis associated GEP identified by Roepman and coworkers which was based on 82 heterogeneous HNSCCs does not seem to have any particular bias in relation to our HNSCC immortality markers (Roepman et al., 2005).

The study by Chung and coworkers identified a gene expression signature of primary HNSCCs that predicted future lymph node metastasis with 80% accuracy (Chung et al., 2004). The lymph node positive signature included known proliferation associated genes and many other genes that were more highly expressed in our immortal SCCs, for example ECT2, UBE2C and STK6. The lymph node positive predictor signature derived by Cromer et al. based on

34 hypopharyngeal HNSCCs also shows greater similarity with our immortal SCC gene expression profile than our mortal signature, whereas the lymph node negative signature resembles our mortal SCC profile much more closely (Cromer et al., 2004). Overall, these various *in vivo*/*in vitro* comparisons and the observations of Shimada and coworkers cited above (Shimada et al., 2003), suggest that the HNSCCs that only recur locally may have a GEP similar to our mortal SCCs; whereas HNSCCs that metastasise to lymph nodes tend to have an immortal phenotype.

It is clear that, despite the efforts in standardization (including MIAME) the variation between microarray studies is still large. At present, it is difficult to see clinical utility of such data if these disparate studies, purporting to assess the same biological event, arrive at completely exclusive lists of genes (Chung et al., 2004; Cromer et al., 2004; Roepman et al., 2005). As the number of suggested useful markers grows, it is not going to be possible to test all of the candidate genes in properly constituted clinical studies. Perhaps a more rigorous validation of the markers in such studies is required, for example by immunohistochemistry in the original tumours. Obviously, this also has its limitations.

7.4 The possible origins of HNSCC

The establishment of cultures from HNSCCs with different replicative characteristics and the distinctive gene expression patterns seen in our analysis may reflect differences in origin. Two theories have been proposed regarding the derivation of such neoplastic cells. The selection hypothesis proposes that squamous neoplasia arises from pre-existing telomerase proficient stem cells (Owens & Watt, 2003). Stem cells are responsible for the continual replacement of cells within a tissue and in squamous epithelium under steady state conditions, it is likely that these cells are relatively quiescent and do not express telomerase. In contrast, the remainder of the cells in the proliferative compartment of squamous epithelia do express telomerase at a low level, but this is rapidly lost upon establishment in cell culture, as was demonstrated in Figure 4.6. However, when stem cells enter the cell cycle, it is likely that they too will express telomerase. These cells are obvious candidates for the origin of squamous neoplasia, as they are present within the tissue over a long period

and as such would have the opportunity to accumulate the required number of mutations for the establishment of neoplastic disease. As they are telomerase proficient in vivo, they should possess long telomeres and be genetically stable.

In some respects, the mortal HNSCC phenotype bears such features. These cells lack telomerase activity when cultured *in vitro*, have WT p53 and are relatively genetically stable. Tumours bearing these features have been shown to have a better prognosis *in vivo*. However, comparison with the microarray analysis of epithelial stem cells by Blanpain and coworkers, shows there is no consistent relationship of a suggested stem cell gene expression pattern to any of our groups of keratinocytes (Blanpain et al., 2004). Admittedly, the stem cells in this study were from hair follicle, and as such, analysis of stem cells from oral mucosa must be compared with our data before the hypothesis is dismissed. Additionally, the fact that the mortal tumours in our study appear to be more highly differentiated than the immortal tumours may also mitigate against this hypothesis. Recent telomere length studies indicate that, in general, the immortal cultures have short telomeres whilst the telomere length of mortal cancers is more variable (data not shown). This may indicate that both arose from telomerase deficient cells. Therefore, mortal HNSCC may simply be tumours that for stochastic reasons have not completed a sufficient number of cell divisions to precipitate senescence and telomeric dysfunction, and as such have retained the ability to differentiate.

An alternative viewpoint suggests that epithelial neoplasms may arise from differentiated, telomerase negative cells. The observation that keratinocytes lacking stem cell properties can be immortalised by adenovirus E1A supports this (Barrandon et al., 1989). Such telomerase deficient cells should demonstrate shorter telomeres with telomeric fusions, deregulated telomerase and mutation of p53, together with appreciable genomic instability. These cells would be expected to demonstrate aneuploidy, extensive LoH and unbalanced chromosomal translocations. The immortal HNSCCs (and dysplasias) display these characteristics. If this is so, the telomerase deregulation hypothesis predicts that cells which have bypassed senescence, but not crisis, should also be identifiable in developing lesions. One of our cell cultures, BICR7, displayed the features of crisis *in vitro* and *in vivo* analysis of HNSCC tumours has demonstrated several of these features (Gordon et al., 2003). Similar findings

have also been reported in other cancer models. In our study, these cells had also lost molecular markers of terminal differentiation.

The presence of intermediate features *in vivo* (D17, D38) and *in vitro* (Gordon et al., 2003) raises the possibility that conversion of mortal tumours to immortal may be possible. However, the data presented here indicates that such an effect requires extensive changes in gene expression. The observation that most of the tumours show heterogeneity of expression of mortal and immortal markers, may reflect such interconversion. The variation was present in all of the tumours which gave rise to the mortal cell cultures. Thus, in the carcinomas which had a uniformly immortal phenotype *in vivo*, the development of immortality may have been an early effect, perhaps at the dysplasia stage. The existence of atypical transitional forms of dysplasia cell culture (D17 and D38) is evidence which supports this. Alternatively, these tumours may have been at a more advanced stage of conversion from a predominantly mortal HNSCC phenotype to a predominantly immortal one.

The tumours which gave rise to the mortal carcinoma cultures have been shown to be very heterogeneous, containing areas with both the mortal and the immortal HNSCC phenotype (Figure 6.4). This may indicate that invasion predated the development of immortal clones within the tumour. As the immortal phenotype has been associated with poor prognosis, this presents a plausible explanation for the better prognosis if tumours have a population of mortal cells at the time of diagnosis i.e. late development of the immortal cell population. However, this advantage may be time-limited if the tumour undergoes conversion from a mortal to an immortal phenotype. Thus, there is no implication that the mortal HNSCCs are indolent lesions, as it is likely that given time they too will spread and kill the patient.

The establishment of both a retrospective and prospective analysis of dysplastic lesions may help in answering these questions. Both of these studies have received ethical approval from the relevant LRECs in Glasgow (KH in retrospective study as co-investigator, prospective study as main investigator). The retrospective study will utilise the issue archives in the Department of Oral Pathology at Glasgow Dental Hospital and School. The pathology database contains 27 patients with a biopsy-confirmed dysplastic lesion who have

subsequently developed a SCC at the same site. The prospective study, designed to collect a patient's first diagnosed dysplastic lesion, has been recruiting for two years. Whilst the recruitment rate has been disappointing, around 50 biopsies have been stored, with the availability of both fresh and formalin fixed material.

7.5 Consistent changes unrelated to proliferative capacity

There are surprisingly few consistent gene expression changes common to all HNSCC cultures, regardless of proliferative fate. Genes such as this may be good candidates for therapeutic intervention, as they are altered in the carcinoma cultures. Gene such as Creatine kinase B, SIX and FOXQ1 are upregulated in the HNSCC cultures when compared with normal cultures; whilst ZNF185, CUGBP2, PLCD4, HOP and CYP27B1 are all downregulated. All these gene expression changes still require to be validated by another method.

There is evidence for the involvement of some of these genes in other cancers, but how these changes might contribute to HNSCC development is unclear. For example, FOX members are known to be deregulated in various cancers (Katoh, 2004). SIX1, is believed to stimulate proliferation via activation of Cyclin A1 (Coletta et al., 2004). It is overexpressed in breast cancers, particularly metastases and is also involved in determination of the metastatic phenotype of rhabdomyosarcomas (Yu et al., 2004). CUGBP2/ETR-3 is a regulator of nuclear and cytoplasmic RNA processing events (Ladd & Cooper, 2004) and has been implicated in translational silencing of COX-2 during radiation induced apoptosis (Mukhopadhyay et al., 2003). CYP27B1 is increased in skin SCCs (Reichrath et al., 2004) and colon cancers (Reichrath et al., 2004), whilst HOP is a potential transcription cofactor, downregulated in hypopharyngeal carcinoma (Lemaire et al., 2004).

7.6 Markers of immortality

Our previous work has shown that p53 mutations are closely associated with immortality in the dysplasia and carcinoma cultures. Some of the dysplasia/HNSCC immortality markers are known p53 target genes, for example, ALD1A3 (Okamura et al., 1999). Other known p53 target genes also differ in expression level between mortal and immortal HNSCCs or dysplasias.

The pattern of these differences in gene expression varies, but in general, p53 target genes that show differences between mortal and immortal HNSCCs are involved in the control of the cell cycle, replication or cell cycle arrest, whereas those that show little change are often those genes whose function may be related to apoptosis. Loss of expression of RAR-β is also strongly associated with immortality, although its level of expression is barely detectable in the microarray experiments (Figure 6.1).

Many of the novel markers of the immortal phenotype seen in our cultures have been related to cancer biology in some way in the literature. What is most striking is the extent of over-representation of cell cycle associated genes amongst those more highly expressed in the immortal cells. These cells are proliferating faster, and this is reflected in a shorter doubling time *in vitro* (data not shown). However, there are some genes which have been relatively under-investigated, including G0S2, Keratin 23 and RHCG, amongst others.

Microarray analysis of mortal cells as they approach senescence has been conducted in many cell types, including normal oral keratinocytes (NHOKs) (Kang et al., 2003:Baek, 2003 #365). These analyses have shown that the gene expression profile of NHOKs as they approach senescence does in some respects mirror that seen in our mortal cultures when compared with the immortal cultures. In the paper by Kang et al, there is profound downregulation of cell cycle genes, and upregulation of some MMPs and collagens, amongst others (Kang et al., 2003). The analysis by Baek et al shows a similar pattern of changes, but with less overlap of the gene expression profile in our mortal cells (Baek et al., 2003). This raises the question of the extent to which genes associated with senescence influence the overall GEPs.

Microarray analysis of luminal mammary epithelial cells before and after immortalisation with SV40, demonstrated a spectrum of changes in gene expression with some overlap with our immortality markers (Park et al., 2004). Indeed many of the over expressed genes in the immortalised cells are related to cell cycle and mitosis, as in the immortal cultures. Similarly, those genes whose expression falls on SV40 immortalisation include genes related to differentiation and some which also are downregulated in our immortal cells, such as ALDH1A3. Although the particular genes altered only overlap to some

extent, presumably as the cells are of different tissue type and the process of SV40 immortalisation is different from that occurring in our cultures, it appears that very similar pathways have been affected. This indicates that the distinctive GEP of the immortal cultures is not solely due to the presence of senescent cells in the mortal cultures.

The profound downregulation of many markers related to differentiation may be of clinical note as it is known that poorly differentiated tumours tend to behave more aggressively. If the basis for this is closely linked with immortalisation, this analysis provides an underlying biological explanation for how tumours may become poorly differentiated, and for the link between loss of differentiation and poor prognosis. Rheinwald and co-workers showed that all cell lines derived from HNSCC have a subnormal rate of commitment to terminal differentiation in suspension culture when compared to normal cells (Rheinwald, 1982). Whilst all the cell lines in the study by Rheinwald and co-workers did eventually enter terminal differentiation, it is clear that their ability to do so was greatly compromised.

The capability of some of our immortal cultures to differentiate was tested in growth as xenografts in nude mice (data not shown). This demonstrated a variation in the capability of these cultures (mostly immortal carcinomas) to undergo terminal differentiation; BICR56 and BICR31 formed large keratin filled cysts whereas BICR22 grew as solid nests with no keratinisation evident. In addition, the fact that the expression of RAR-β in the immortal dysplasia cultures (either in monolayer or in organotypic culture) did not match that in the corresponding tissue, suggests that the link between immortality and a lack of differentiation may neither be strong nor causal and may be profoundly affected by the microenvironment and/or time. This requires further work, perhaps utilising more sophisticated organotypic culture systems, for example with tumour fibroblasts and other stromal elements, to attempt a more complete recapitulation of the microenvironment, as indicated earlier.

7.7 Markers of high risk in dysplastic lesions

A GEP of dysplastic lesions in relation to progression to carcinoma has not been reported in the literature. The first published data on the gene expression profile of PMLs in comparison to HNSCCs was that by Ha and coworkers (Ha et

al., 2003). However, there was no follow-up data given in this study to allow for assessment of "high risk" markers of transformation to HNSCC. This analysis suggested that the gene expression pattern of the dysplastic lesions became progressively more like that of HNSCCS with increasing grade of dysplasia. Indeed, the number of significant differences in gene expression between the dysplastic and HNSCC samples was very small. Additionally, the GEP of clinically normal tissue adjacent to dysplastic lesions was very different for true normal tissue. However, in both cases, the number of samples was small. There are two possible explanations for these patterns of gene expression: heterogeneity due to large variation in the expression profiles of PMLs or little real difference. Despite the authors' emphasis on the latter, it is clear that there was appreciable heterogeneity in the dysplasia samples reported in the study. Despite the existence of outliers in the cluster analysis, it appears that all the dysplasia samples were used in the comparison with HNSCCs, presumably, as the numbers were too small to give any statistical significance without the inclusion of the outliers. Thus, the size of the final gene list, and the genes contained within it, may be a little misleading. Despite this, we have also found that the difference between the dysplastic and invasive samples (mortal or immortal) is much smaller than for comparisons of the same samples with normal cells.

The completeness of follow-up data in the cases that progressed to carcinoma is vital in establishing a true relationship between each patient's dysplastic lesion and any subsequent malignant disease. In the case of the three immortal dysplastic cultures from patients who developed a carcinoma (D19, D20 and D35), the follow-up data clearly demonstrates that the original dysplastic lesion and the carcinoma were from the same anatomical site in the mouth. The provenance of these lesions as "progressors" is, therefore, good. However, it has not been possible to obtain follow-up data beyond one year for the mortal erythroplakia cultures and therefore the clinical history of the patients and the natural history of their lesions cannot be stated with any degree of certainly. This must be borne in mind when extrapolating from this data into the clinical situation.

Analysis of the gene expression profiles of the cultures derived from the dysplastic lesions that progressed to HNSCC is ongoing. This work, presently

being continued by Dr Paul Drake, is attempting to identify a genetic pattern that is a signature of high risk of progression to carcinoma in a dysplastic lesion. This is complicated by the identification of the two possible pathways to HNSCC development, as described earlier. The small number of changes seen within the two pathways and the large number of differences between the pathways, as shown in Figure 7.2, means that consistent markers of high risk of dysplasia progression, regardless of proliferative lifespan, are unlikely to be identified. Thus, two different panels may emerge - one specific to the mortal lesions and another to immortal lesions. In practical terms, this would result in lesions first being screened for markers of the mortal or immortal phenotype, before the use of the appropriate high risk panel of markers.

The small number of samples at our disposal is an obvious limitation of such analysis. However, the retrospective and prospective studies will be useful in the validation of any potential markers. The establishment of new dysplasia cultures may also be beneficial, but this is dependent on careful follow-up of lesions with no guarantee that the patient from whom the culture was established will develop a carcinoma, as only a small proportion of the lesions will progress to HNSCC. Thus, establishing whether high risk lesions are mortal or immortal (or mixed) may be important as a first stage in analysis of dysplastic lesions, with markers of high risk used subsequent to this.

7.8 Future work

7.8.1 Completion of validation steps

The future direction of the project depends largely on the completion of validation of the gene expression profiles and exploration of many of the possible hypotheses and molecular mechanisms outlined above. Validation of the selected novel immortality markers in dysplastic tissue related to the cultures is a vital step. Where commercial antibodies are not available, other gene expression changes of interest must be validated by quantitative RT-PCR and a decision made as to the necessity of raising antibodies for future immunoblotting and immunohistochemistry. The completion of this element will allow decisions to be made as to the future direction of the research. Whilst some of the basic functional experiments, such as knockout of RARβ from mortal cultures have also yet to be completed, the advent of RNAi in the interim period may allow such interventions to be accomplished more easily.

7.8.2 Mechanism of invasion

The possibility that different mechanisms of invasion are used by mortal and immortal cells requires further investigation. However, this will require the derivation of more mortal carcinoma cultures, as our current stocks are almost exhausted. New ethical approval for the collection of material for this purpose still requires to be obtained. It may be possible to immortalise some of the remaining stocks of mortal carcinomas and indeed, Ken Parkinson plans to attempt this by ectopic expression of hTERT in these cultures. However, it is not clear what effect this may have on the invasive properties of these cells.

The use of Matrigel Invasion assays and scratch wound assays initially to assess the movement of the immortal and mortal (where possible) cells would be a useful starting point in the assessment of the different types of migration in these cells

7.8.3 Tumour microenvironment

Further organotypic culture experimentation with a more concerted attempt at reconstitution of the microenvironment is required. This is particularly important

as the mortal cultures do not appear to have the ability grow as xenografts in nude mice. It may also be possible to utilise the new dysplasia associated fibroblast cultures collected in the prospective study. This may help demonstrate the influence that the microenvironment has on the cultures. It may also be possible to gain permission for the derivation of new tumour associated fibroblast cultures when the prospective study for SCC samples is set up.

In relation to the poor agreement of RAR-β expression in the tissues related to immortal cultures, the role of RAR-β in immortalisation requires further investigation. It may now be possible to knock out the expression of RAR-β in mortal cultures, for example by use of RNAi, or ectopically express it in immortal cultures.

7.8.4 Clinical studies

As indicated above, retrospective and prospective studies have been commenced for the analysis of both dysplastic and invasive oral lesions. These will allow us to test many of these hypotheses regarding the different pathways and cells of origin in development of HNSCC. This material also forms an important part of the ongoing work of the group in terms of markers of high disk of dysplasia progression to carcinoma.

Retrospective study

Markers validated at RNA and protein level in the cultures will be tested in a retrospective study. All patients since 1990 who have developed a squamous cell carcinoma at the same intraoral site as a previous dysplastic biopsy will be included. At present 26 such patients have been identified for inclusion. These patients will be assigned three control dysplastic lesions that have not developed into HNSCC. This will be matched for:

- age
- sex
- risk factors (smoking and alcohol intake).

The markers will be assessed in formalin fixed, paraffin embedded tissue sections by immunohistochemistry, with attention paid to staining location,

intensity and proportions of cells stained, using the Quickscore method (Detre et al., 1995). This will form part of Dr Paul Drake's project.

Prospective study

Markers which look promising in the retrospective study will then be forwarded into the prospective study. This removes the unavoidable bias of a retrospective study, can allow for the effect of clinical intervention and will also allow a large enough study sample to be collected to allow conclusions to be drawn despite the presence of clinical variables. Ethical approval for this project has been obtained. Both fresh and formalin-fixed material will be collected and consent for access to medical records (including ISD cancer registration data) will be obtained. Funding will be sought for a new microarray study on this material. Patients recruited must:

- have a clinical indication for an biopsy of a white or red patch.
- be 20-70 years of age
- have no history of previous HNSCC
- a confirmed diagnosis of dysplasia and no other concurrent oral pathology (e.g. candidosis and immunological diseases are excluded).

Assuming 10% progression over 10 years, the study requires to recruit 250 patients to detect a significant ($p<0.05$) difference in a single marker with 80% power. The patients will be followed for at least 5 (hopefully 10 years) and approached for donation of another sample if a biopsy is clinically indicated. For the purposes of the study, follow-up will be censored once a patient develops a SCCHN at the same site as the original biopsy. Results will be analysed using the Cox's proportional hazards model and Kaplan Meier survival curves.

Chapter Eight: References

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Chapter Nine: Appendix

Publications arising from this work are included in this section.

Muntoni, A., J. Fleming, K. Gordon, K. Hunter, F. McGregor, E. K. Parkinson and P. R. Harrison. (2003) Immortalization of senescing oral dysplasias by ectopic expression of hTERT requires loss of INK4A and/or retinoic acid receptor β, but not p53 mutation. Oncogene, 22, 49, 7804-8.

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