4.1.2 Clonal development and field cancerisation in oral cancer

Chapter 1 (sections 1.1 and 1.2 Tumourigenesis, progressive genetic mutation and clonality) described the growing evidence that the development of human cancers involves the accumulation of multiple genetic alterations leading to the evolution of clonal cell populations that possess growth advantages over other cells. Many common clonal molecular genetic alterations have also been identified in head and neck cancer as well as premalignant head and neck lesions (Field et al., 1995; Kim et al., 1993; Latif et al., 1992; Nawroz et al., 1994; Ostwald et al., 2000). This supports the concept that head and neck cancer develops in a series of steps similar to that reported for colon cancer.

The concept of field cancerisation was introduced in 1953 by Slaughter. It was based on the fact that most of the epithelial surface of the aerodigestive tract is likely to be exposed to many of the common carcinogens such as tobacco and alcohol and thus has an increased risk of oral cancer development (Slaughter, 1953). Clinical histopathological and molecular biological evidence supports this concept in head and neck cancer. Clinically, patients with head and neck squamous cell carcinoma (HNSCC) may present with multiple synchronous primary tumours or develop secondary metachronous primary tumours (Cooper et al., 1989). Histological abnormalities, some of which may have malignant potential, can be observed in the respiratory tract epithelium of almost all smokers and patients with lung cancer, the extent of these changes in the oral cavity, however, is unknown. Multiple distinct genetic and cytogenetic alterations are identified in epithelium from different regions of the mouth obtained from patients with oral premalignant lesions (Califano et al., 1996; Lee et al., 1993a). This suggest that all oral mucosa exposed to carcinogens
may have a significant risk of cancer development and that multiple tumours may arise independently as a result of wide field defects.

This concept has been challenged from the evidence suggesting that multiple oral tumours in individual patients can be derived from a single progenitor cell. The expanding clone of cancer cells may spread through the mucosa by an unknown path and mechanism. This would give rise to geographically distinct but genetically identical cancers. Subsequent genetic modifications, due to spontaneous mutation or continued exposure to exogenous mutagens may mask the clonal origin of tumours at different sites (Ogden, 1998). In head and neck cancer, studies provided cytogenetic evidence indicating that multiple tumours share identical chromosomal abnormalities (Carey, 1996). In analysis of primary tumours from eight female patients with head and neck cancer, identical break points on chromosome 9p or 3p by microsatellite analysis as well as identical X-chromosome inactivation were demonstrated (Bedi et al., 1996). Worsham and colleagues (1995) studied two synchronously arising primary squamous cell carcinomas from separate sites in the anterior floor of the mouth and the pyriform sinus from a 42-year-old man. They found by karyotyping and fluorescence *in situ* hybridisation, that all tumour samples were of monoclonal origin. Studies have previously demonstrated this phenomenon in bladder, breast and ovarian cancer (Kupryjanczyk et al., 1996; Noguchi et al., 1994; Sidransky et al., 1992a). However Scholes and co-workers (1998) compared the pattern of allelic imbalance in paired tumours from five male patients with two synchronous oral squamous cell carcinomas and in peripheral dysplasia using microsatellite markers on chromosomes 3p, 9p and 17p. Discordant results were seen in two patients, while three patients had identical molecular alterations in their tumours (Scholes et al., 1998). In a case report, Zhang et al. (1999) found similar results. Using microsatellite
analysis for loss of heterozygosity of chromosomes 3p, 9p, 17p as well as 4q, 8p, 11q and 13q, one recurrence of an oral squamous cell carcinoma in a young woman was clonogenically similar to the primary but the second recurrence was not.

4.1.3 Carcinogenic agents related to oral cancer

Environmental factors contribute to the variable frequency of p53 mutations found in human cancers. The vast majority of head and neck cancers and lung cancers of smokers harbour a p53 mutation, whereas in cancers of non-smokers the mutation frequency is around 20% (Brennan et al., 1995; Hernandez-Boussard et al., 1999; Kondo et al., 1996). It is considered that tobacco use, heavy alcohol consumption and poor diet could explain over 90% of cases of head and neck cancer. Among men in industrialised countries, smoking is estimated to be the cause of over 85% of oral cancer deaths (Johnson, 2001). The synergistic action of alcohol with tobacco as a risk factor is well recognised (Brennan et al., 1995; Franceschi et al., 1990; La Vecchia et al., 1997) and recent studies confirm these effects (Hsieh et al., 2001).

It was shown for lung cancer that the occurrence of G:C to T:A transversions correlates with tobacco consumption (Nilsson, 2001), and that it is the benzo(a)pyrene diol epoxide that induces this mutation in vitro (Denissenko et al., 1998), but it is not known whether carcinogenic substances of the tobacco smoke play a comparable role in p53 mutational events in oral carcinomas. Some studies demonstrated an association of a history of tobacco use with high incidence of p53 mutations (Brennan et al., 1995), while others did not (Franceschi et al., 1995; Nylander et al., 1995). Ostwald et al. (2000) did not find a higher incidence of G to T transversions in intra-oral cancers.
4.1.4 p53 alterations in oral cancer

Immunohistochemical studies have shown accumulation of p53 in 50 to 100% of cell lines derived from head and neck tumours (Burns et al., 1993; Ogden et al., 1992). Eleven to 79% of primary tumours have been observed to have detectable levels of p53 protein (reviewed in (Raybaud-Diogene et al., 1996)). Although mutations of p53 could be identified in many tumours with positive p53 protein staining, cells with mutant p53 may react negatively. Moreover, tumours with positive p53 protein staining but that do not contain a mutant p53 gene have been reported to occur with various frequency (Melhem et al., 1995; Nylander et al., 1995).

Molecular studies show that approximately 50% of head and neck tumours harbour p53 mutations (Ahomadegbe et al., 1995; Boyle et al., 1993). In the upper aerodigestive tract, p53 mutations are only second in frequency to p16 gene alterations, p16 being the most common altered gene in head and neck squamous cell carcinoma (Reed et al., 1996; Shahnavaz et al., 2001). Mutations are located predominantly in exons 4 to 9, with “hot spots” ranging from codons 205-248 (Pavelic & Gluckman, 1997; Prime et al., 1997). Mutation data of p53 mutations in head and neck cancers were collected from other recent studies (Boyle et al., 1993; Burns et al., 1993; Chung et al., 1993; Matsuda et al., 1996; Olshan et al., 1997; van Heerden et al., 1998; Weber et al., 2002) showing relatively similar mutation spectra, with “hot spots” at codon 220 and 245 (Fig. 4.1). Data collated from these recent publications found that from a total of 312 cases of head and neck cancers, 121 cases presented with mutations. Only 2 studies evaluated mutations outside exons 5-9 (Kropveld et al., 1999; Weber et al., 2002) and Kropveld et al. (1999) found 33% of cases were outside the core domain. Twenty four percent of mutations affected exon
7, while 22% affected both exon 5 and 8. The majority of the mutations were found within the region between exon 5 to 9 (90%).

The exact role of the p53 genetic alteration in different stages of the tumourigenic process has not been completely established. The timing of p53 changes in the development of SCC has mainly been investigated by immunohistochemical analysis of p53 protein overexpression in oral mucosal dysplasia found adjacent to invasive carcinoma or in dysplastic lesions. These data have come from studies that infer the derangement of p53 function early in cancer which leads to overexpression of p53 protein, changes which are not necessarily synonymous with each other (Gallo et al., 1995; Lavieille et al., 1995; Pavelic et al., 1992; Slootweg et al., 1994). Various studies have found that positive immunoreaction in dysplasia might reflect nuclear accumulation of wildtype p53 protein (Nakanishi et al., 1995). However, molecular studies have shown p53 mutations in dysplasia (Boyle et al., 1993; Lazarus et al., 1995). p53 mutations have also been revealed in histologically innocuous epithelia at a significant distance from the primary tumour (Nees et al., 1993).

Few studies suggest that the development of p53 mutations is a late event. Lee et al (1993b) were only able to demonstrate p53 mutations in invasive tumours but not in adjacent tissue, but dysplasias were not investigated. Another group (Li et al., 1995) examined eight oral premalignant lesions that progressed to oral cancer and found no p53 mutations in the precursor lesions. The majority of the current data suggests that p53 is an early event in the progression of head and neck cancer.

### 4.1.5 p53 as clonal marker

p53 has been suggested to be a good molecular marker of clonality because it could be associated with the development of cancer. Alteration in a clonal marker, such as mutations, should occur prior to or at the time of the development of invasive cancer,
and these mutations would be clonally preserved (Chung et al., 1993). However, it has been argued that p53 mutations follow other events in the genetic progression of head and neck tumours and the identifications of p53 could be misleading if tumours arose from a same clone but migrated before the p53 mutation event. Where malignant cells may leave the primary carcinoma at an early stage of tumour development and p53 gene is not yet mutated, targeting p53 as a clonal marker would not be appropriate (Offner et al., 1999).

### 4.1.6 Summary

The clonal evolution of head and neck cancer is still under intense investigation, and recently models for the genetic progression of head and neck cancer have been suggested (Califano et al., 1996; Califano et al., 2000; Nagai, 1999). To date, all molecular studies using markers to identify the genetic profiles and clones of abnormal or malignant epithelium have used extractive methods to demonstrate and define a specific mutation, but the destruction of the tissue precludes specific identification of cells containing the mutation of interest. DNA extraction followed by PCR does not provide information on the proportion, location or clonal distribution or nature of the mutated cells.

Various groups have used p53 mutations as markers of clonality (Chung et al., 1993) and in the current models proposed for the progression of head and neck squamous cell carcinomas, p53 mutations occur prior to development of invasive cancer.

In Chapter 3, it was shown that p53 point mutations in whole cells could be detected in situ. From these results it appeared that ARMAS-PCR might have the added potential for investigating the clonal distribution of cells with predetermined p53 mutations in preserved tissues from malignant or premalignant lesions.
Figure 4.1 Mutation data of p53 mutations in head and neck cancers collected from more recent studies. (Boyle et al., 1993; Burns et al., 1993; Chung et al., 1993; Matsuda et al., 1996; Olshan et al., 1997; van Heerden et al., 1998, Kropveld et al 1999) Chart shows the tally of different p53 mutations identified in the above studies.

4.2 ARMAS-PCR for in situ identification of p53 mutations in oral squamous cell carcinomas

The aim of this study was to use ARMAS-PCR in situ followed by in situ hybridisation to identify epithelial cells harbouring the same p53 mutations in oral squamous cell carcinomas and adjacent normal epithelia.

4.2.1 Materials and methods

4.2.1.1 Tissue and sample collection

Tissue samples of the tumours and adjacent mucosa were collected from 24 patients (Table 4.III) who had undergone surgical excision of histologically confirmed primary
oral squamous cell carcinoma (as defined in 4.1.1.1) at the Westmead Hospital, Westmead, NSW, Australia between 1996 to 1998. One portion of the sample collected was taken at the time of surgery and frozen in liquid nitrogen and stored at -70°C, and a second portion was fixed and embedded for cryosection (see below section 4.2.8.1 for details). Archival paraffin blocks were also obtained. Archival tissues were fixed in 10% buffered formal saline and paraffin embedded in the Department of Anatomical Pathology, Westmead Hospital, NSW, Australia.

Control non-neoplastic specimens of gingiva were collected from the Department of Periodontology, Westmead Centre for Oral Health, from patients without neoplastic disease undergoing surgery for periodontal disease. These gingival tissue specimens were frozen in liquid nitrogen and stored at -70°C.

Tissue from fifteen patients numbers 1-15 was also analysed by PCR-SSCP for p53 mutations in extracted DNA. Analysis by PCR-SSCP of remaining 9 patients (numbers 16-24) was not possible due to insufficient tissue and time constraints for the PCR-SSCP (Figure 4.2).

In 4 of the first 15 patients (patients numbers 7,10, 11,13), a band shift indicative of a p53 mutation was obtained in the extracted DNA by PCR-SSCP. In 2 patients (numbers 7 and 10), point mutations were confirmed and defined by DNA sequencing. DNA sequencing was not possible for the tumours from patients 11 and 13 due to insufficient tissue and time constraints for the analysis of these tissues by in situ ARMAS-PCR. Primer pairs were then designed for the tumours in patients 7 and 10 to detect each mutation by ARMAS-PCR in extracted DNA and then to localise mutant cells by in situ ARMAS-PCR in tissue sections. The analysis and subsequent optimisation of the in situ ARMAS-PCR took 12 months for these samples from patients 7 and 10.
Tissue from each of the 24 cases of oral squamous cell carcinomas was tested by immunohistochemistry for p53 protein expression (Figure 4.2) for comparison with the results of testing for p53 mutations by PCR-SSCP in extracted DNA and the tissue distribution of p53 positive cells by immunohistochemistry with those positive by in situ ARMAS-PCR.
Fig 4.2 Selection of samples for analysis for in situ ARMAS-PCR

Tissue samples chosen from 24 patients, most suitable for in situ ARMAS-PCR

9 tissue samples were omitted as tissue size were inadequate

10 tissue samples were positive for PCR-SSCP

4 tissue samples were positive for PCR-SSCP

2 of the tissue samples could be properly optimised for in situ ARMAS-PCR

10 tissue samples were negative for PCR-SSCP

2 tissue samples were not able to be optimised adequately for in situ ARMAS-PCR

10 samples positive by immunohistochemistry

15 samples negative by immunohistochemistry
4.2.2 Tobacco and alcohol history

The tobacco and alcohol history was obtained from the medical records for each patient. According to the National Health and Medical Research Council, Australian Alcohol Guidelines (NHMRC 2001), low risk levels are defined as a level of drinking at which there is only a minimal risk of harm and for some, the likelihood of health benefits. A standard drink is defined as containing 10 g or 12.5 ml of alcohol. Risky levels are those at which risk of harm is significantly increased beyond any possible benefits. High-risk drinking levels are those at which there is substantial risk of serious harm and above which risk continues to increase rapidly. Risks were also divided into long-term and short-term. Long-term risk was the level of long-term risk associated with regular daily patterns of drinking defined by the total amount of alcohol typically consumed per week, while short term risks are the risk of harm in the short-term that is associated with given levels of drinking on a single day. These levels assume that overall drinking patterns remain within the levels set for long-term risk and that these heavier drinking days occur infrequently and never more than three times per week. Guidelines for low-risk drinking in the long term are listed in Table 4.I. Categories for defining cigarette consumption and the definition of an ex-smoker were based on previous studies (Table 4.II)
Table 4.1 Guidelines for low risk drinking (NHMRC, 2001)

<table>
<thead>
<tr>
<th></th>
<th>Low-risk Standard drinks</th>
<th>Risky Standard drinks</th>
<th>High-risk Standard drinks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>Up to 4 per day</td>
<td>5-6 per day</td>
<td>7 or more</td>
</tr>
<tr>
<td>On an average day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall weekly level</td>
<td>Up to 28 per week</td>
<td>29 to 42 per week</td>
<td>43 or more per week</td>
</tr>
<tr>
<td>Females</td>
<td>Up to 2 per day</td>
<td>3 to 4 per day</td>
<td>5 or more per day</td>
</tr>
<tr>
<td>On an average day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall weekly level</td>
<td>Up to 14 per week</td>
<td>15 to 28 per week</td>
<td>29 or more per week</td>
</tr>
</tbody>
</table>

Table 4.2 Categories for tobacco intake (Moore, 1971; Wynder et al., 1969)

<table>
<thead>
<tr>
<th>Category</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smoker</td>
<td>Never smoked</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>Cessation &gt; 6 months</td>
</tr>
<tr>
<td>Occasional</td>
<td>&lt;1 cigarette/day</td>
</tr>
<tr>
<td>Light</td>
<td>1-9 cigarettes/day</td>
</tr>
<tr>
<td>Moderate</td>
<td>10-19 cigarettes/day</td>
</tr>
<tr>
<td>Heavy</td>
<td>20-39 cigarettes/day</td>
</tr>
<tr>
<td>Excessive</td>
<td>&gt;40 cigarettes/day</td>
</tr>
</tbody>
</table>

4.2.2.1 DNA preparation

DNA was extracted from 15 patient samples chosen as the most suitable tissue samples according to size and availability of tissue, for molecular analysis. The remaining 9 samples did not have sufficient tissue material for the subsequent part of the study which involved in situ PCR analysis. From each archival specimen, 4-5 μm thick serial sections were prepared. One section was stained with haematoxylin and eosin to identify the tumour and normal tissue and reviewed by a pathologist (Prof. D M Walker). Serial sections were dewaxed by two washes in xylene and hydrated through graded washes of ethanol mounted on glass slides.

Haematoxylin- and eosin-stained sections 4-5μm thick were inspected by a pathologist and the tumour area marked. The area was identified in sequential slides stained only with haematoxylin, examined when placed under a dissecting microscope and the area of interest was removed mechanically using the tip of a 27-gauge needle.
The tissue was carried in 50:50 glycerol: egg albumin and collected in an Eppendorf
tube and DNA extracted as previously described in Chapter 2 with prolonged
proteinase K (Boehringer Mannheim cat#1092766) digestion time of 5 days at 55°C.

4.2.3 Immunohistochemical detection of p53

4.2.3.1 Detection of stabilised p53 protein: background
Mutant p53 gene product is characterised by conformational changes in the protein
that may prolong its half-life and stability (Finlay et al., 1988). As a result, mutant p53
protein is immunohistochemically detectable, while wildtype is not. Studies have
previously shown good correlation between overexpression of p53 protein and
mutation (Kropveld et al., 1999). However, as mentioned above, staining of p53
protein is an imperfect indicator of molecular-genetic alterations of the p53 gene.
Many technical factors have now been identified that affect the results of detection by
antibodies against p53 protein.

Various antibodies are now available commercially, that bind to different
epitopes of the mutant and of wildtype p53 protein. Studies using monoclonal
antibodies have shown that mutant p53 protein has an exposed epitope near amino
acid 25 which is present but not exposed in wildtype p53 (Stephen & Lane, 1992). In
particular, an epitope recognised by PAb 240 monoclonal antibody is localised in the
central region of the normal protein and is hidden in the folded structure, but is
exposed at the surface of the mutant proteins (Stephen & Lane, 1992). Antibodies
PAb 421, BP53-12, BP53-24, DO-1, DO-7 and CM-1 recognise both mutant and
wildtype protein. PB 53-12, PB53-24, PAb 1801, DO-1 and DO-7 are very efficient
on formalin-fixed paraffin-embedded tissue materials, whereas PAb 240, PAb 421,
PAb 162 and PAb 246 are not suited for use on formalin-fixed paraffin-embedded
tissues (Furihata et al., 1995). DO-7 with Target Unmasking Fluid for antigen retrieval was found to be the most sensitive by Baas et al. (1994).

Antigen retrieval techniques have improved the results of p53 protein detection, and have made archival tissue specimens accessible for p53 immunostaining. However, lowering the detection threshold for p53 protein has potentially led to the detection of both wildtype as well as mutant p53 protein (Hall & Lane, 1994). Bass et al. (1996) questioned whether a false positive reaction might result from antigen retrieval. Careful interpretation of data is needed when comparing studies that use antigen retrieval and those that do not.

Biological mechanisms also affect the detection of p53 protein. Functional expression of p53 protein can be found in response to cellular stresses. Possibly due to its genotoxic effect, irradiation has been shown to produce immunohistochemically detectable levels of wildtype p53 in normal cells without mutations (Kuerbitz et al., 1992). Conversely, certain molecular alterations of the gene can abrogate or abolish the production of p53 protein.

Stabilisation of the p53 protein by other proteins allows the p53 protein to be detected immunohistochemically. Viral gene products can stabilise the protein after cellular infection (Garcia et al., 1997; Kovacs et al., 1996). It has been shown that p53 protein is stabilised by the interaction with cellular proteins including the mdm-2 protein and heat shock proteins (Gandour-Edwards et al., 1998). Recent evidence has described a greater role of mdm-2 in the stabilisation of p53. mdm-2 is transcriptionally activated by p53 and then targets it for destruction (described in Chapter 1). It is now believed that mutant p53 cannot activate the transcription of mdm-2 and it is for this reason that p53 accumulates (Oren et al., 2002).
4.2.3.2 Positive and negative controls

p53 mutant SW480 cells were used as positive controls and SAO 2 cells ATCC-HTB 85 a p53 null allele cell line were used as a negative control. Cells were harvested, cytospun onto poly-L-lysine (Sigma, Missouri, USA cat # P8920) coated slides and fixed in acetone for 5 min. Slides were stored at -70°C. Omission of the primary antibody was also used as an internal control and was used to compare staining when the primary antibody was used. (An irrelevant antibody of the same isotype should ideally have been also used as a negative control.)

4.2.3.3 Oral tissue sample preparation

Four micron sections were cut from the formalin-fixed paraffin-embedded tissue and placed on poly-L-lysine coated slides.

4.2.3.4 Antibodies

Mab DO7 was used. It is an IgG2b class of antibodies and recognises both wildtype and mutant forms of the p53 protein. It binds the N-terminal 45 amino acids of the p53 protein. It is effective on both frozen sections and paraffin-embedded sections (Vojtesek et al., 1992).

4.2.3.5 Microwave antigen retrieval

Sections were collected onto poly-L-lysine coated slides. Sections were fixed on to slides by baking at 60°C for one hour. Before immunohistochemistry, sections were dewaxed, rehydrated and rinsed in running water.

0.01 M sodium citrate buffer (pH 6.0) was used as retrieval solution. Rehydrated sections were immersed in retrieval solution in a glass trough covered with a loose-fitting cap and processed in a microwave oven with power set to high
(Samsung model #M8145, 850W), for a total of 15 min, in 3 cycles of 5 minutes each. The level of fluid was constantly maintained during the procedure by replenishing with pre-warmed retrieval solution following each cycle. After the completion of the third cycle, sections were left undisturbed in the microwave oven for a further 20 min and then rinsed twice in phosphate buffered saline (PBS) pH 7.3.

Primary and secondary antibody was diluted from the commercial stock solution into PBS (pH 7.3) containing 5% goat serum, 1% bovine serum albumin and preservative thiomersal. The sections were incubated with the primary antibody for 1 hour at 25°C in 50 μl of a 1:100 dilution of monoclonal MAb DO7 (Novacastra, Laboratory Ltd. UK cat# NCL-p53-DO7).

Slides were then washed in tap water, rinsed twice in PBS and endogenous peroxidase activity blocked by incubating slides in a 3% aqueous solution of hydrogen peroxide for 5 min. Sections were then washed in tap water for 2 min and rinsed in PBS.

Approximately 50 μl of a 1:250 dilution of the biotinylated secondary antibody (goat anti-mouse) IgG and IgM (Biotin Targo Immunologicals cat# AM 13709) were applied to the sections and incubated for 1 hour at 25°C. Sections were washed in tap water and rinsed twice in PBS. 100 μl of a 1:400 dilution of peroxidase conjugated streptavidin (Tago Immunologicals cat# SNN1004) were applied to the sections and incubated for 1 hour at 25°C. The sections were then washed in tap water and rinsed twice in PBS. 0.05% diaminobenzidine tetra hydrochloride (DAB) (Sigma cat # D-5637) in PBS and 0.1% hydrogen peroxide were used as the chromogen, producing a dark brown stain in SW480 cells within 5 mins. The sections were washed in tap water, counter-stained with haematoxylin and mounted.
4.2.4 Molecular analysis of tissue samples for p53

mutations

p53 mutations in head and neck cancer have been found predominantly between exons 5 to 9 (reviewed in section 4.1.4). Hence extracted DNA from tissue samples was screened for p53 point mutations in the regions of exons 5 to 9 of the p53 gene by PCR–SSCP.

4.2.4.1 Primers

Primers and information on the optimal conditions for the amplification of exons 5 to 9 of the p53 gene were as described by Jones (1998), and as described in section 2.1.4. Primers illustrated in Figure 2.1 were used.

4.2.4.2 PCR-SSCP

Amplification of exons 5 to 9 of the p53 gene for PCR-SSCP analysis was performed using a total PCR volume of 40μl using primers illustrated in Figure 2.1 (Table 2.1) and according to the parameters given in Table 2.1I.

The PCR products were electrophoresed on 2% agarose gel and visualised by ethidium bromide staining.

SSCP was carried out as described in section 2.5.1.1

4.2.5 Selection of suitable samples for in situ ARMAS-PCR

PCR fragments with a band shift revealed by SSCP were sequenced using automated DNA sequencing as described in section 2.13. PCR products showing band shifts were initially re-electrophoresed on a 0.5% MDE\textsuperscript{TM} gel using the same protocols described in section 2.5.1.1 but stained with ethidium bromide rather than silver. The DNA of interest was then reamplified by PCR. The PCR products were selectively
picked up from the desired shifted band with mutated p53 sequence on the ethidium bromide stained gel, using a sterile pipette tip.

Samples showing positive immunohistochemical tissue staining as well as demonstrating a shift in banding pattern in any of the amplified PCR products of the extracted DNA were chosen for in situ ARMAS-PCR.

Mutations identified by sequencing were confirmed by sequencing both sense and antisense strands. Products were also sequenced twice to confirm the mutation.

4.2.6 ARMAS-PCR for detection of defined mutation in oral squamous cell carcinoma

4.2.6.1 Primers for ARMAS-PCR analysis of oral squamous cell carcinoma (OSCC) specimens

(Refer to Table 4. I for specimen numbers)

Primers were designed according to results obtained in Chapter 2.

Subject number 7 mutant-specific sense primer:

(3888MS): AACAGCTTTTGGTGCCTTTATT

The underlined letters represent deliberate mismatches designed into the primer to increase specificity.

Antisense primer: E9AS (refer to Table 2.I).

A wildtype p53 primer (3888NS) complementary to the wildtype p53 gene at the base pairs corresponding to those of the mutant primer (3888MS) was used as a control primer for amplification of DNA.

Normal control primer (3888NS): AACAGCTTTTGGTGCCTTTTG (Fig. 4.3).
Figure 4.3 Positions of primers for ARMAS-PCR for p53 mutations in patient number 7. Boxes represent exons of the p53 gene. Black arrows depict wildtype primers. Red arrows depict mutant-specific primers.

For OSCC patient number 10 the specific primer was

(14793MS): GACCCCCGGTCCGCAGCATGTCTATCC (underlined letters represent deliberate mismatches designed into the primer to increase specificity) and antisense primer: A5NAS (refer to Figure 2.4).

A wildtype p53 primer (14793NS) complementary to the wildtype p53 gene at the base pairs corresponding to those of the mutant primer (14793MS) was used as a control primer for amplification of DNA.

Wildtype control primer (14793NS):
GACCCCCGGTCCGCAGCATGGCCATCC (Fig. 4.4).

Figure 4.4 Primer positions for ARMAS-PCR for mutations in the p53 gene of patient number 10.

The box represents exon 5 of the p53 gene. Black arrows depict wildtype primers. Red arrows depict mutant-specific primers.
4.2.6.2 ARMAS-PCR on DNA extracted from OSCC patient number 7

A total PCR volume of 40 µl was used, containing 150-250 ng of genomic DNA, 50 mM KCl, 10 mM TrisHCl, 2.5 mM MgCl₂, 0.2 mM dATP, dGTP, dCTP, dTTP and 0.2 µM primers (3888MS and E9AS) with 1 unit Taq. Amplification was carried out in a Corbett thermocycler at 94°C for denaturation for 3 mins, followed by 35 cycles at 94°C for 20 sec, 60°C for 20 sec annealing time followed by an extension time of 72°C for 1 min. The PCR products were electrophoresed on 2% agarose gel and visualised by ethidium bromide staining.

4.2.6.3 ARMAS-PCR on DNA extracted from OSCC patient number 10

A total PCR volume of 40 µl was used, containing 150-250 ng of genomic DNA, 50 mM KCl, 10 mM TrisHCl, 5.0 mM MgCl₂, 0.2 mM dATP, dGTP, dCTP, dTTP and 0.2 µM primers (14793MS and A5NAS) with 1 unit Taq. Amplification was carried out in a Corbett thermocycler at 94°C for denaturation for 3 mins, followed by 35 cycles at 94°C for 20 sec, 64°C for 20 sec annealing time followed by an extension time of 72°C for 1 min. This was followed by reamplification of 2 µl of the PCR product under the same conditions using 1 unit Taq plus (Stratagene Cloning Systems, La Jolla, CA, USA cat#600203). The PCR products were electrophoresed on 2% agarose gel and visualised by ethidium bromide staining.
4.2.6.4 ARMAS-PCR analysis of p53 mutations of tumour tissue and adjacent normal epithelium using extracted DNA from microdissected tissue.

Tumour tissue and histologically normal tissue was identified in histological sections by a pathologist and DNA was extracted from microdissected malignant epithelium and normal epithelium separately as described in Section 4.3.1.3. A margin of tissue between normal and malignant tissue of 2-3 mm was left untouched to avoid contamination of tissue. This DNA was analysed separately by mutant-specific primers described above for each corresponding patient to detect mutant DNA in histologically normal epithelium.

4.2.7 Biopsy of clinically normal oral epithelium 2-3 years after surgery of 2 patients returning for recall

Only 2 patients selected with suitable oral cancer tissues for analysis were available at recall. Biopsies of clinically normal epithelium were taken from patients 7 and 13, three and two years respectively after the initial surgery for analysis of mutations. Biopsies of 1 cm in size were taken from the same site as the initial lesion. Biopsy taken from patient number 7 was taken from the base of the tongue, and from patient number 13, from the ventral side of the tongue. Tissues were collected and DNA extracted as described above.

4.2.8 In situ ARMAS-PCR in situ hybridisation

4.2.8.1 Tissue fixation and preparation

At the time of collection, both tumour tissue or normal gingival tissue were washed in cold PBS and then fixed in Karnovsky’s fixative (2% paraformaldehyde, 2.5%
glutaraldehyde in PBS) for 2 hours at 4°C. Each specimen was then washed in PBS, 3 times and then placed in 30% sucrose, 2 mM MgCl₂, in PBS at 4°C overnight. Specimens were embedded in M-1 Embedding Material (Lipshaw Pittsburg cat#1310) and slowly frozen in liquid nitrogen and stored at -70°C.

8 μm frozen sections were cut and placed onto 3-aminopropyltriethoxy silane (Sigma, cat # A3648) coated slides. A section of tumour tissue and a section of normal gingival tissue as internal control were placed onto each slide.

**4.2.8.1.1 ** *In situ* ARMAS-PCR: patient number 7

All steps of the *in situ* ARMAS-PCR methods were initially optimised to gain the best selective amplification signal with the least background following *in situ* hybridisation.

Sections were rehydrated in PBS for 15 min, permeabilised with 0.3% Triton X/PBS for 15 min, washed twice in PBS for 5 min. Samples were treated with 50 μg/ml of proteinase K for 10 min at 25°C. Proteinase K digestion was stopped with 0.2% glycine in PBS and post-fixed in 4% PFA for 3 min at 4°C. Sections were washed twice in PBS for 10 min and equilibrated with 1 X PCR buffer (50 mM KCl, 10 mM TrisHCl, 2.5 mM MgCl₂). A hot-start PCR was performed, using a 100 μl PCR solution, containing 50 mM KCl, 10 mM TrisHCl, 5.0 mM MgCl₂, 0.2 mM dATP, dGTP, dCTP, dTTP and 0.8 μM 3888S and E9AS primers preheated to 75°C, while tissue on slides was also pre-warmed to 75°C on a Omnislide thermocycler (Hybaid, Integrated Sciences, Sydney, Australia). Five units of Taq were added to the pre-warmed PCR solution and applied to the cells on slides. PCR solutions were confined on the slides with a Gene frame (Integrated Sciences, Sydney, Australia). PCR was performed on the Omnislide thermocycler, with an initial denaturation of
3min at 94°C, followed by 1 min at 94°C, 1 min 30 sec at 60°C (annealing) and 1 min 30 sec at 74°C (extension) for 35 cycles.

After *in situ* amplification, samples were post-fixed in 4% paraformaldehyde for 3min at 4°C and then washed in PBS twice for 5 min.

### 4.2.8.1.2 *In situ* ARMAS-PCR: patient number 10

The same protocol was applied for the tumour from patient number 10, with a number of modifications. Primers used were 14793MS and A5NAS (see section 4.3.5.1). The annealing temperature used during *in situ* amplification was raised to 64°C.

## 4.2.9 *In situ* hybridisation for ARMAS-PCR amplified products

### 4.2.9.1 Synthesis of digoxigenin-labelled single stranded genomic DNA and cDNA probes for use in situ ARMAS-PCR

Single stranded digoxigenin(DIG) labelled DNA probes were prepared by amplified primer extension labelling (Paine et al., 1995), using either 3888MS and E9AS or 14793MS and A5NAS primers (see section 4.3.5.1) and 250 ng DNA or cDNA template. In summary, 100 µl of the PCR solution was used containing 250-500 ng genomic DNA from corresponding tumour samples and the same protocol was used as that described in Sections 4.3.5.2 and 4.3.5.3. PCR products were purified by a commercially available purification kit according to the manufacturer’s guidelines (Qiagen PCR purification kit cat# 28106, Qiagen, Hilden, Germany) or by Polyethylene glycol (PEG) purification as described in section 2.13.

Asymmetric PCR was carried out for 50 cycles, using template, 5 µl 10 X dNTP/DIG-labelled dUTP and 20 pmole of either 3888MS, E9AS, 14793MS or A5NAS for each reaction. PCR products were then analysed on a 2% agarose gel to
confirm that the labelled products were heavier than unlabelled products. Probes were
dot blotted on nylon membranes (Hybond N+, Amersham, Buckinghamshire, UK)
and the labelling efficiency assessed using colorimetric detection with NBT and X-
Phosphate according to the manufacturer's (Boehringer Mannheim, Basle,
Switzerland) instructions.

4.2.9.2 Assessment of probe specificity by Southern analysis

PCR products were electrophoresed on 2% agarose gel and PCR products were
transferred by Southern blotting on to Hybond™ N+ nylon membrane (Amersham)
according to the manufacturer’s instructions. The specificity of probe hybridisation
was confirmed by membrane hybridisation as according to the manufacturer's
(Boehringer Mannheim, Basle, Switzerland) guidelines for filter hybridisation of
digoxigenin-labelled probes.

4.2.9.3 In situ hybridisation of in situ amplified ARMAS-PCR
products

After thermocycling, tissue sections were washed in 2 X SSC (1 X SSC =0.15M
NaCl, 0.015M Sodium citrate, pH7). A new Geneframe (Integrated Sciences, Sydney,
Australia) was attached to contain solutions applied. 100μl of prehybridisation
solution (50% deionised formamide, 6 X SSC, 5 X Denhardt’s solution, 0.1 mg/ml
salmon sperm DNA, 10% dextran sulfate, 5 mg/ml sodium pyrophosphate, 0.5% SDS
and 1 mM levamisol) were applied gently to each slide and incubated in a humidified
chamber at 37°C for 2 hours. Slides were removed from the hybridisation chamber,
drained and 100 μl hybridisation solution applied comprising the prehybridisation
solution with digoxigenin-labelled probe at 1 ng/μl. Slides were heated to 95°C for 10
minutes to denature amplified products and hybridisation was performed for 2 hours
at 37°C. This was followed by a digoxigenin detection system as previously described (Paine et al., 1995) to visualise amplified products.

4.2.9.4 Controls

As negative controls, Taq polymerase or primers were omitted from the PCR step. Normal gingival tissue was used as internal controls on the same glass slide as the test tumour tissue section.
4.3 Results

4.3.1 Clinical samples collected

Table 4.11 Clinical information relating to samples collected

<table>
<thead>
<tr>
<th>Subject number</th>
<th>Pathology accession number</th>
<th>DOB</th>
<th>Age at time of surgery</th>
<th>Gender</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>547</td>
<td>17.11.28</td>
<td>67</td>
<td>M</td>
<td>SCC of the piriform fossa</td>
</tr>
<tr>
<td>2</td>
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<td>19.9.50</td>
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<td>M</td>
<td>SCC of the tongue</td>
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<tr>
<td>3</td>
<td>2709</td>
<td>28.9.38</td>
<td>57</td>
<td>M</td>
<td>SCC of the tongue</td>
</tr>
<tr>
<td>4</td>
<td>1320</td>
<td>8.1.25</td>
<td>71</td>
<td>M</td>
<td>SCC of the piriform fossa</td>
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<tr>
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<td>1869</td>
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<td>68</td>
<td>F</td>
<td>SCC of the tongue</td>
</tr>
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<td>6</td>
<td>4650</td>
<td>6.2.37</td>
<td>59</td>
<td>M</td>
<td>SCC right mandible</td>
</tr>
<tr>
<td>7</td>
<td>3888</td>
<td>2.1.33</td>
<td>63</td>
<td>M</td>
<td>SCC base of the tongue</td>
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<td>5396</td>
<td>29.6.37</td>
<td>58</td>
<td>M</td>
<td>SCC of the tonsillar fossa</td>
</tr>
<tr>
<td>9</td>
<td>14398</td>
<td>30.3.38</td>
<td>58</td>
<td>M</td>
<td>SCC floor of the mouth</td>
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<tr>
<td>10*</td>
<td>14793</td>
<td>12.6.28</td>
<td>68</td>
<td>M</td>
<td>SCC of the tongue</td>
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<td>10.1*</td>
<td>14793.1</td>
<td>12.6.28</td>
<td>68</td>
<td>M</td>
<td>SCC of the tongue</td>
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<td>F</td>
<td>SCC of the tongue</td>
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<tr>
<td>12</td>
<td>1786</td>
<td>20.7.28</td>
<td>68</td>
<td>M</td>
<td>SCC lateral surface of tongue</td>
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<td>13</td>
<td>3643</td>
<td>4.7.42</td>
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<td>M</td>
<td>SCC ventral surface of tongue</td>
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<td>14</td>
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<td>68</td>
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<td>15</td>
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<td>16</td>
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<td>F</td>
<td>SCC of the gingiva</td>
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<td>17</td>
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<td>11558</td>
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<td>M</td>
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<td>SCC of the floor of the mouth</td>
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<td>M</td>
<td>SCC of the gingiva</td>
</tr>
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<tr>
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<td>10922</td>
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<td>M</td>
<td>SCC alveolar mucosa</td>
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<td>M</td>
<td>SCC of the left upper lip</td>
</tr>
</tbody>
</table>

* 10 and 10.1 were different blocks from the same tumour, counted as one sample
# This patient had a previous history of lichen planus and subsequently developed SCC of the lip. This patient provided 2 separate samples
Abbreviations: Squamous cell carcinoma (SCC), M (Male), F (Female).

Table 4.1V Tobacco and Alcohol history

<table>
<thead>
<tr>
<th>Subject number</th>
<th>Age at time of study</th>
<th>Gender</th>
<th>Tobacco Intake</th>
<th>Alcohol Intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>71</td>
<td>M</td>
<td>Moderate</td>
<td>Low-risk</td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>M</td>
<td>Never smoked</td>
<td>Non-drinker</td>
</tr>
<tr>
<td>3</td>
<td>61</td>
<td>M</td>
<td>Moderate</td>
<td>High-risk</td>
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<tr>
<td>4</td>
<td>74</td>
<td>M</td>
<td>Excessive</td>
<td>Low-risk</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>F</td>
<td>Non smoker</td>
<td>Low-risk</td>
</tr>
</tbody>
</table>
4.3.1.1 Samples collected from 24 patients

Table 4.III summarises the clinical information for each of the samples collected. Of the 24 patients studied, 21 patients were males and 3 were females. According to the diagnosis by the pathologist (Associate Prof. M Bilous, Prof. D M Walker), all 24 samples were squamous cell carcinomas. Table 4.IV summarises the tobacco and alcohol history for each patient.

4.3.2 p53 overexpression in oral squamous cell carcinoma

Of the samples collected from 24 patients and analysed by immunohistochemistry for overexpression of p53 protein, samples from 11 (46%) were positive were positive. Specimens presenting with clusters of cells (5 cells and more) were considered positive. Generally most of the tumour epithelial cells were intensely stained in positive specimens. These included basal cells as well as cells in the spinous layer. Figure 4.5 illustrates immunohistochemical staining obtained in the samples from patient numbers 9 and 13. All other positive samples had similar staining intensity,
anatomical and distribution of immunoreactive epithelial cells. Table 4.V and 4.VI, summarises the results obtained from the 24 patients.
Figure 4.5 p53 overexpression (arrows) detected by immunohistochemistry in tumour specimens of patient numbers 9 and 13 (200X magnification).

Fig. 4.5a Patient number 9

Fig. 4.5b Patient number 13
Table 4.V p53 detection in oral squamous cell carcinomas

<table>
<thead>
<tr>
<th>Subject number</th>
<th>Immunohistochemical detection of p53</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>-ve</td>
</tr>
<tr>
<td>2</td>
<td>-ve</td>
</tr>
<tr>
<td>3</td>
<td>-ve</td>
</tr>
<tr>
<td>4</td>
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<td>+ve</td>
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<td>8</td>
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</tr>
<tr>
<td>9</td>
<td>-ve</td>
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<tr>
<td>10* and 10.1*</td>
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</tr>
<tr>
<td>11</td>
<td>+ve</td>
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<tr>
<td>12</td>
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<td>+ve</td>
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<td>+ve</td>
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<tr>
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</tr>
<tr>
<td>21</td>
<td>+ve</td>
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<tr>
<td>22</td>
<td>+ve</td>
</tr>
<tr>
<td>23</td>
<td>-ve</td>
</tr>
<tr>
<td>24#</td>
<td>+ve</td>
</tr>
<tr>
<td>24.1#</td>
<td>+ve</td>
</tr>
</tbody>
</table>

* 10 and 10.1 were different blocks from the same tumour, counted as one sample

# This patient had a previous history of lichen planus and subsequently developed SCC of the lip. Two separate samples were available, 24 and 24.1
Table 4.VI Summary of p53 immunopositive samples.

<table>
<thead>
<tr>
<th>Subject numbers</th>
<th>Proportion of immunopositive p53 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>7,10,11,12,13,15,17,19,21,22,24,</td>
<td>11/24 (46%)</td>
</tr>
</tbody>
</table>

4.3.3 Molecular analysis for p53 mutations of p53 immunopositive samples by PCR-SSCP.

4.3.3.1 PCR amplification of tumour samples

PCR products were analysed initially by electrophoresis on 2% agarose gel and stained by ethidium bromide. Normal leukocyte and fibroblast DNA were used as normal positive PCR amplification controls. Fifteen patient samples from (patients 1-15) were analysed for p53 mutations in exons 5 to 9 using PCR-SSCP. (Analysis by ARMAS-PCR was not possible for all patients due to time constraints and tissue availability, therefore the most suitable samples of tissue were selected with priority for analysis.) Figures 4.6a to c illustrate PCR products of exons 5 to 9 amplified from DNA of subject numbers 3, 4, 6, 7, 8 and 9.
Figure 4.6 a to e Analysis of PCR products from amplification of the p53 gene prior to SSCP.

Examples of PCR products from amplification of the p53 gene of exons 5-9 from DNA of patient numbers 3, 4, 6, 7 and 8 Agarose gels stained with ethidium bromide. Normal DNA (HUVEC and fibroblast) as positive controls and omission of template DNA as negative (-ve) controls.

4.6a
Lane 1: mw IX (Boehringer Mannheim)
Lane 2: Fibroblast DNA as template, exon 5 PCR product
Lane 3: HUVEC DNA as template, exon 5 PCR product
Lane 4: Leukocyte DNA as template, exon 5 PCR product
Lane 5: Subject # 7 DNA as template, exon 5 PCR product
Lane 6: Subject # 8 DNA as template, exon 5 PCR product
Lane 7: -ve control with omission of DNA template
Lane 8: Fibroblast DNA as template, exon 6 PCR product
Lane 9: HUVEC DNA as template, exon 6 PCR product
Lane 10: Leukocyte DNA as template, exon 6 PCR product
Lane 11: Subject # 7 DNA as template, exon 6 PCR product
Lane 12: Subject # 8 DNA as template, exon 6 PCR product
Lane 13: -ve control with omission of DNA template
Lane 14: Fibroblast DNA as template, exon 7 PCR product
Lane 15: HUVEC DNA as template, exon 7 PCR product

4.6b
Lane 1: mw IX (Boehringer Mannheim)
Lane 2: Leukocyte DNA as template, exon 7 PCR product
Lane 3: Subject # 7 DNA as template, exon 7 PCR product
Lane 4: Subject # 8 DNA as template, exon 7 PCR product
Lane 5: -ve control with omission of DNA template
Lane 6: Fibroblast DNA as template, exon 8 PCR product
Lane 7: HUVEC DNA as template, exon 8 PCR product
Lane 8: Leukocyte DNA as template, exon 8 PCR product
Lane 9: Subject # 7 DNA as template, exon 8 PCR product
Lane 10: Subject # 8 DNA as template, exon 8 PCR product
Lane 11: Fibroblast DNA as template, exon 8 PCR product
Lane 12: HUVEC DNA as template, exon 8 PCR product
Lane 13: Leukocyte DNA as template, exon 8 PCR product
Lane 14: Subject # 7 DNA as template, exon 8 PCR product
Lane 15: Subject # 8 DNA as template, exon 8 PCR product
Figure 4.6c
Lane 1: mw IX (Boehringer Mannheim)
Lane 2: Leukocyte DNA as template, exon 5 PCR product
Lane 3: Subject #4 DNA as template, exon 5 PCR product
Lane 4: Subject #3 DNA as template, exon 5 PCR product
Lane 5: Subject #6 DNA as template, exon 5 PCR product
Lane 6: -ve control
Lane 7: Leukocyte DNA as template, exon 6 PCR product
Lane 8: Subject #4 DNA as template, exon 6 PCR product
Lane 9: Subject #3 DNA as template, exon 6 PCR product
Lane 10: Subject #6 DNA as template, exon 6 PCR product
Lane 11: -ve control
Lane 12: Leukocyte DNA as template, exon 6 PCR product
Lane 13: Subject #4 DNA as template, exon 7 PCR product
Lane 14: Subject #3 DNA as template, exon 7 PCR product
Lane 15: Subject #6 DNA as template, exon 7 PCR product
Lane 16: mw IX (Boehringer Mannheim)
Lane 17: -ve control
Lane 18: Leukocyte DNA as template, exon 8 PCR product
Lane 19: Subject #4 DNA as template, exon 8 PCR product
Lane 20: Subject #3 DNA as template, exon 8 PCR product
Lane 21: Subject #6 DNA as template, exon 8 PCR product
Lane 22: -ve control
Lane 23: Leukocyte DNA as template, exon 9 PCR product
Lane 24: Subject #4 DNA as template, exon 9 PCR product
Lane 25: Subject #3 DNA as template, exon 9 PCR product
Lane 26: Subject #6 DNA as template, exon 9 PCR product
Lanes 27-29: -ve control
4.3.3.2  **SSCP analysis of PCR products**

Four out of 15 (27%) tumours had a detectable p53 mutation; with a majority in exon 5 (Table 4.VII). All tumours detected with a p53 mutation were also positive by immunohistochemistry (see Table 4.V). One sample (subject 12) was positive by immunohistochemistry, however no shift in banding pattern was detected by PCR-SSCP.

*Figure 4.7a* illustrates the normal bands obtained from normal fibroblast, HUVEC and leukocyte DNA. *Figure 4.7c* also demonstrates the band shifts detected by SSCP of PCR products amplified from patient 7. *Figure 4.8* illustrates band shifts in PCR products analysed by SSCP for patient numbers 10, 11 and 13. For patient number 10, where two samples were analysed from the one subject, both samples showed the same banding pattern on SSCP analysis.

4.3.3.3  **Comparison of results from p53 immunohistochemical and molecular analysis**

Of the 15 cases analysed both by immunohistochemistry and by molecular sequencing, 4 had band shifts by PCR-SSCP suggesting point mutations. Six out of 15 cases were positive by immunohistochemistry for expression of the p53 protein, while 4 of the 6 cases that were immunopositive had band shifts indicative of point mutations, although the mutations were confirmed and defined by DNA sequencing only for patients 7 and 10, due to constraints of available tissue, sequencing was not subsequently performed in the cases of patients 11 and 13. No mutations were identified in the immunonegative cases (Table 4.VIII).
Table 4.VII Summary of subject samples that showed abnormal band shifts by PCR-SSCP analysis.

<table>
<thead>
<tr>
<th>Subject number</th>
<th>PCR-SSCP analysis for p53 mutations</th>
<th>Immunohistochemical detection of p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Band shift in exon 8 PCR product</td>
<td>+ve</td>
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<tr>
<td>10*</td>
<td>Band shift in exon 5 PCR product</td>
<td>+ve</td>
</tr>
<tr>
<td>10.1*</td>
<td>Band shift in exon 5 PCR product</td>
<td>+ve</td>
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<tr>
<td>11</td>
<td>Band shift in exon 5 PCR product</td>
<td>+ve</td>
</tr>
<tr>
<td>13</td>
<td>Band shift in exon 5 PCR product</td>
<td>+ve</td>
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</table>

* samples from different sections through the same tumour

Table 4.VIIa Immunohistochemical detection of p53 and PCR-SSCP band shifts

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<th>PCR-SSCP band shifts detected</th>
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<td>10* and 10.1*</td>
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<table>
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<td>Total</td>
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<td>15</td>
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</table>

Table 4.VIIb Comparison of results from immunohistochemical and molecular analysis
Figure 4.7a to d SSCP mobility patterns for PCR products of exons 5 to 9.

SSCP mobility patterns for each exon fragment generated by PCR from fibroblast DNA (lanes 1), HUVEC DNA (lanes 2), leukocyte DNA (lanes 3), subject 7 (lanes 4) and 8 (lanes 5). Arrow indicates the additional band detected in subject number 7. (0.5% MDE™ gel silver stained)

**Figure 4.7a Exon 6**

Lane 1: Fibroblast DNA as wildtype control  
Lane 2: HUVEC DNA as wildtype control  
Lane 3: Leukocyte DNA as wildtype control  
Lane 4: Subject number 7 DNA  
Lane 5: Subject number 8 DNA

**Figure 4.7b Exon 7**

Lane 1: Fibroblast DNA as wildtype control  
Lane 2: HUVEC DNA as wildtype control  
Lane 3: Leukocyte DNA as wildtype control  
Lane 4: Subject number 7 DNA  
Lane 5: Subject number 8 DNA

**Figure 4.7c Exon 8**

Lane 1: Fibroblast DNA as wildtype control  
Lane 2: HUVEC DNA as wildtype control  
Lane 3: Leukocyte DNA as wildtype control  
Lane 4: Subject number 7 DNA. Arrow indicates the additional band detected compared with controls  
Lane 5: Subject number 8 DNA

**Figure 4.7d Exon 9**

Lane 1: Fibroblast DNA as wildtype control  
Lane 2: HUVEC DNA as wildtype control  
Lane 3: Leukocyte DNA as wildtype control  
Lane 4: Subject number 7 DNA  
Lane 5: Subject number 8 DNA
Figure 4.8a and b SSCP analysis of PCR products of exon 5 from subjects 10, 11 and 13.
(0.5% MDE™ gel silver stained)

Figure 4.8a

Lane 1: Subject 13 DNA. Blue arrow indicates the shifted band and additional band detected.
Lane 2: Subject 11 DNA. Red arrow indicates the additional band detected.
Lane 3: Normal banding pattern of SSCP analysis for exon 5
Lane 4: Normal banding pattern of SSCP analysis for exon 5

Figure 4.8b

Lane 1: Subject 10 DNA. Black arrow indicates the additional band detected
Lane 2: Normal banding pattern of SSCP analysis for exon 5
Lane 3: Subject 10.1 DNA. Arrow indicates the additional band detected.
4.3.4 Sequencing of samples to identify mutations

The tumour samples from patient subject numbers 7 and 10 showing band shifts by PCR-SSCP were confirmed to have point mutations in the p53.

The mutant band demonstrated in the SSCP analysis of exon 8 of the p53 gene in the tumour sample from subject number 7 was reamplified. A missense mutation in codon 275 causing a TGT to TTT transversion was seen in exon 8.

Figure 4.9 illustrates the automated sequencing data obtained. A dTTP is detected showing a strong signal depicted by the red peak; a background dGTP signal is seen and depicted by a black peak.

Sequencing the exon 5 reamplified PCR product from DNA the tumour sample from subject number 10, a missense mutation at codon 164 in exon 5 was detected, causing a TAC to CAC transition. Figures 4.10a and b illustrate the automated sequence data obtained, again a background normal dATP/dTTP is seen superimposed by the dCTP/dGTP. Both samples 10 and 10.1 showed the same mutation point.

Due to time constraints and tumour sample portion availability, the tumour samples patients 11 and 13 showing and shifts for PCR-SSCP were not submitted to DNA sequencing to confirm and define the mutation. Analysis by in situ ARMAR-PCR was not carried out therefore.

7 and 10 were the only samples suitable for analysis by in situ ARMAS-PCR.

4.3.5 ARMAS-PCR analysis of mutation defined for patient number 7

Primers were designed according to Newton et al. (1989) and according to literature described in Chapter 2.
For the TGT to TTT transition, the primer: 3888MS (AACAGCTTTGAGGTGCGTTTATT) was designed. The mutant-specific primer was designed with two deliberate mismatches upstream from the 3’ end. These deliberate mismatches were A: A mismatch which has a medium destabilising action and a T: C mismatch which has a strong destabilising action (refer to Table 2.XII). 3888MS and E9AS were chosen according to the Amplify program, to give a good amplification product.

4.3.5.1 ARMAS-PCR analysis of extracted DNA

Using primers specific for the mutation previously identified in exon 8 of the p53 gene, the mutant fragment of the gene was selectively amplified. Figure 4.11 illustrates the specificity of amplification of the ARMAS. A PCR product was produced only upon amplification of DNA from tumour tissue of patient 7 (pathology accession number 3888). Normal fibroblast DNA and leukocyte DNA were refractory to amplification (Fig. 4.11, lanes 6 and 7).

PCR product was amplified from DNA obtained from frozen tumour tissue of patient subject number 7. Paraffin-embedded archival tissue in the Department of Anatomical Pathology, for this particular patient, had been pretreated with Fast-Cal Decalcifier (Histolabs, Riverstone, Australia), a decalcifying solution used prior to embedding bony material into paraffin for sectioning and analysis by haematoxylin and eosin. This solution contained HCl (10%) and may have caused degradation of DNA within the sample. Therefore fresh frozen material was used in its place.
Figure 4.9 Automated sequence of exon 8 PCR product (sense strand) showing mutation in codon 275 in subject number 7. Black arrow indicates the mutation detected. A wildtype background signal was demonstrated showing the presence of dGTP nucleotides (blue arrow).
Figure 4.10a to c Mutation detected in tumour samples number 10 and 10.1.

Figure 4.10a Sense and antisense of mutation detected in codon 164 of the p53 gene in sample number 10 and 10.1 (Table 4.1).

Figure 4.10b Automated sequence of exon 5 PCR product (sense strand) showing a mutation in codon 164 in sample number 10.1. Black arrow indicates the mutation detected. A wildtype dTTP is also detected in the background (pink arrow).

Figure 4.10c Automated sequence of exon 5 PCR product (antisense strand) showing a mutation in codon 164 in sample number 10. Black arrow indicates the mutation detected. A wildtype dATP is also detected in the background (pink arrow).
Fig. 4.10a

- TAC
- CAC
- GTG
- ATG

Wildtype sense strand
Mutant sense strand
Mutant antisense strand
Wildtype antisense strand

Fig. 4.10b

ATGGCCATCNAACAGCAC

Fig. 4.10c

CTGCTTGTNGATGGCCA
Figure 4.11 ARMAS-PCR amplification products (328 bp lanes 2-4) using mutant-specific primers for mutation detected in exon 8 of patient number 7.

EB2%

Lane 1: mw IX (Boehringer Mannheim)
Lane 2: Subject #7 DNA (200ng/μl) used as template
Lane 3: Subject #7 DNA (20ng/μl) used as template.
Lane 4: Subject #7 DNA used as template, positive control using wild type primers
Lane 5: (no samples loaded)
Lane 6: Fibroblast DNA used as template
Lane 7: Leukocyte DNA used as template

Fig. 4.10
4.3.5.2 Detection of mutation in histologically normal epithelium

Fresh tissue collected at the time of surgery was fixed and embedded as described above ready for in situ ARMAS-PCR. The 8 μm section was stained with haematoxylin and eosin and examined by a pathologist (Prof. D M Walker), normal and tumour tissue were microdissected as described in Section 4.2.6.4 and a band of histologically normal tissue was left on the slide to prevent cross-contamination of tumour tissue DNA into histologically normal epithelial DNA at the time of DNA extraction.

The mutation detected in the tumour tissue was also detected in the histologically normal epithelium adjacent to the tumour of patient number 7, using ARMAS-PCR. A PCR product was produced when mutant-specific primers were used to amplify DNA extracted from the histologically normal epithelium (Fig. 4.12). Mutant-specific primers were tested on DNA extracted from tumour tissue of other patients. No PCR product was seen (Fig. 4.12, lane 6).

4.3.5.3 Controls

DNA from both tumour and normal epithelium were amplified initially using non-mutant-specific primers 3888NS and E9AS.
Figure 4.12 ARMAS-PCR analysis of surrounding epithelium.

2% agarose gel electrophoresis of ARMAS-PCR analysis of tumour tissue and histologically normal epithelium from subject number 7

Lane 1: mw IX (Boehringer Mannheim)
Lane 2: DNA from histologically normal epithelium, a 328bp product was produced indicating the presence of mutant DNA
Lane 3: DNA from tumour tissue producing a 328 bp product
Lane 4: –ve control, with omission of template
Lane 5: DNA from tumour tissue producing a 328bp product
Lane 6: DNA from tumour tissue of subject number 9, no product was seen
4.3.5.4 Use of ARMAS-PCR to detect presence of mutations in regional lymph nodes of patient number 7

Facial and supra-hyoid nodes dissected from the patients and examined by a pathologist (Associate. Prof. M Bilous), were available for analysis. Nodes were reported to be histologically uninvolved.

The sensitivity of ARMAS-PCR to detect mutations was shown previously in Chapter 2. ARMAS-PCR was used in an attempt to detect histologically undetected metastatic tumour cells. DNA was extracted from archival lymph nodes embedded in paraffin blocks from patient number 7 as described above. Mutant-specific primers for the previously identified mutation were used to amplify DNA from lymph nodes.
No mutations were detected in DNA extracted from any of the lymph.

4.3.6 ARMAS-PCR analysis of mutation defined for patient number 10

Primers were designed according to Newton et al. (1989) and according to literature described in Chapter 2. A mutant-specific primer was designed with two deliberate mismatches up stream from the 3' end. The deliberate mismatches were a T: G mismatch which has a weak destabilising strength but also a T: C mismatch which gives maximum destabilising action (refer to Table 2.XII).
4.3.6.1 ARMAS-PCR analysis of extracted DNA from paraffin embedded material from patient number 10

DNA from microdissected tissue of paraffin-embedded material was used for ARMAS-PCR analysis of the previously defined mutation in exon 5 of subject number 10. Amplification of DNA from paraffin-embedded material was more difficult than from fresh frozen tissue, and fainter bands were produced.

Mutated DNA in exon 5 of the p53 gene was successfully amplified using the mutant-specific primers, but there was an indication that the proportion of mutant cells was small. The use of Taq plus (Stratagene Cloning Systems, La Jolla, CA, USA cat#600203) which contains a high fidelity enzyme, as well as an increase in the number of amplification cycles, were needed to generate more intensely stained PCR products (Figure 4.13 lanes 2 and 3).

4.3.6.2 Detection of mutation in histologically normal epithelium of patient number 10

Histologically normal epithelium adjacent to the tumour confirmed by a pathologist (Prof. D M Walker) was microdissected out as described for patient number 7; DNA was extracted and purified from it. Figure 4.14a illustrates a haematoxylin- and eosin-stained section of tissue from patient number 10 and outlines the area microdissected for ARMAS-PCR amplification. Figure 4.14b shows a high-power magnification of the microdissected tumour tissue area from Figure 4.14a (boxed area). A mutation was not detected in the adjacent normal epithelium, even with the use of Taq plus.

4.3.6.3 Controls

DNA from both tumour and normal epithelium were amplified initially using wildtype specific primers 14793NS and A5NAS.
4.3.7 Detection of mutations in clinically normal epithelium by ARMAS-PCR 3 years after surgery of patient 7

Only 2 out of the 4 subjects identified with p53 mutations in the primary tumour specimens returned for review at 2 to 3 years after surgery of the initial primary tumour. Subject number 11 was deceased at the time of recall and subject number 10 did not attend the review clinic.

DNA from biopsy of patient number 7 was the only suitable subject for analysis by ARMAS-PCR. Patient 7 had a period of radiotherapy without chemotherapy postoperatively in 1996. Using mutant-specific primers for the previously identified mutation, no mutations were observed using ARMAS-PCR (results not shown).
Figure 4.13 ARMAS-PCR amplification of tumour mutant DNA using mutant specific primers for previously determined mutation in patient (number 10) with oral squamous cell carcinoma.

Lane 1: mw IX
Lane 2: Subject number 10 DNA amplified with increased cycle number and with the use of Taq plus
Lane 3: Subject number 10.1 DNA amplified with increased cycle number (50) and with the use of Taq plus
Lane 4: Subject number 10 DNA amplified with increased cycle number (50) and with the use of Taq only
Lane 5: Subject number 10.1 DNA amplified with increased cycle number (50) and with the use of Taq only
Lane 6: -ve control with omission of template
Lane 7: Subject number 10 DNA amplified with 35 cycles of amplification
Lane 8: Subject number 10.1 DNA amplified with 35 cycles of amplification
Figure 4.14 Haematoxylin-and eosin-stained paraffin section of tumour sample from patient number 10.

The microdissected area has been demarcated by the solid black line. The boxed area in Figure 4.14a is shown at a higher (200 X) magnification in Figure 4.14b illustrating nuclear polymorphism, hyperchromatism and lack of stratification of epithelial cells.
4.3.8 In situ ARMAS-PCR analysis of p53 mutations in oral squamous cell carcinomas

After extensive optimisation of both the in situ PCR and in situ hybridisation, p53 mutant cells were selectively amplified in situ for both subjects number 7 and 10 (Fig. 4.15 to 19).

Figure 4.14a is a haematoxylin- stained section of the tumour tissue in subject number 7.

Figure 4.16b illustrates the DNA amplification in p53 mutant cells in a serial section of the malignant epithelium in this tumour tissue from subject number 7. The positively staining cells demonstrate the presence of the specific TGT to TTT transition in the p53 gene.

Figure 4.16c is a haematoxylin and eosin stained section of tumour tissue from patient number 10, while Figure. 4.16 d and e are higher power (100 and 400 X) views of the malignant lesion.

Figure 4.16e is a duplicate of the illustration in Fig. 4.16c. Figures 4.16f and g illustrate the amplification of p53 mutant cells in malignant and superficial epithelium of tissue derived from subject number 10, corresponding to the areas marked by boxes. Boxes in Figure 4.16e illustrate each of these areas. Tumour and histologically normal epithelium in Figure 4.16e correspond to those indicated in Figures 4.16f and g respectively. The positively staining cells indicate the presence of the specific TAC to CCC transversion.

No signal was detected in the internal controls using normal gingiva (Fig. 4.16g) and with omission of Taq or primers (Fig. 4.16h).
Figure 4.15 A haematoxylin and eosin-stained section of paraffin embedded tumour tissue from patient number 7.

Section shows the surface epithelium invading into the underlying tissue (tissue was previously decalcified prior to paraffin embedding resulting in faint haematoxylin staining).
Figure 4.16a to h *In situ* ARMS-PCR amplification of p53 mutations previously identified using allele-specific primers in subjects number 7 and 10.

Figure 4.16a A haematoxylin and eosin stained frozen serial section of tumour tissue from patient number 7. Figure 4.16b shows the amplification of cells positive by ARMAS-PCR for p53 mutations in exon 8 of patient number 7.
Fig 4.16 c to e

C: H and E of frozen section from patient 10 (40X)
D: H and E section of frozen section at higher magnification (100X) of Fig 14.16 a of the area marked with red box. It demonstrates the dysplastic epithelium of the specimen
E: H and E section of frozen section at higher magnification (400X) of Fig 14.16a of the area marked with blue box.
Figure 4.16 f to h. Fig 4.16A haematoxylin-stained section of tumour tissue from patient number 10. (duplicate of Fig. 4.16 f) Figures 4.16g and h show detection of cells positive by ARMAS-PCR for p53 mutations in exon 5 of patient number 10. Figures 4.16 g and h correspond to boxes illustrated in Figure 4.16f.
Figure 4.16 i and j
Negative controls using normal gingival tissue (Fig. 4.16i) and a sample processed with the omission of primers in the PCR mixture during in situ amplification (Fig. 4.16j) (note negative nuclear staining in comparison with the reaction of nuclei in Fig. 4.16b).
4.3.9 Comparison of in situ ARMAS-PCR amplified cells
with immunohistochemical detection of overexpression of
p53 protein

Tumours were analysed by (a) in situ ARMAS-PCR followed by in situ hybridisation
on one section followed by (b) immunohistochemical detection of overexpression of
p53 protein on a consecutive section. When these serial sections were compared, it
was observed that for both subjects number 7 and 10, there was some but not
complete correlation in the distribution of immunohistochemically positive cells with
in situ ARMAS-PCR amplified cells.

For subject number 7 (Fig. 4.17a and b) islands of p53 mutant cells positive by
in situ ARMAS-PCR corresponded topographically with those by
immunohistochemistry. In situ ARMAS-PCR positive islands of cells for mutant p53
involved more suprabasal cells when compared to immunohistochemically stained
sections, spanning a larger area of the tumour.

For subject number 10 (Fig. 4.18a and b) some superficial epithelial cells were
positive both by immunohistochemical detection of p53 protein as well as in situ
amplification of mutant p53 by ARMAS-PCR. Cells were positive both within the
malignant tissue as well as the adjacent normal epithelium. A larger proportion of the
histologically malignant epithelium was positive by in situ ARMAS-PCR when
compared with immunohistochemistry (Fig. 4.18c and d). Similar to subject number
7, the cells positive by immunohistochemistry in one serial section did not correspond
to all cells positive by in situ ARMAS-PCR amplification (Fig. 4.18a to d).

Interestingly, however, immunohistochemical analysis of archival tissue that
was fixed (10% buffered formalin) and embedded in paraffin produced more intense
and a larger number of cells with positive nuclear and cytoplasmic reaction for p53 protein expression.
Figure 4.17 a and b Comparison of in situ ARMAS-PCR amplification of mutation in exon 8 of the p53 gene in subject number 7 (Fig. 4.16a) with immunohistochemical detection of p53 protein (Fig.4.16b) in serial frozen sections (200X magnification).

(a) Red arrows indicate the basal cells with positive nuclei by in situ amplification by ARMAS-PCR. White arrows show the suprabasal cells with positive nuclei by the same method.
(b) Black arrows show the basal cells positive by immunohistochemistry.

Fig. 4.17a

Fig. 4.17b
Figure 4.18a to d Comparison of in situ ARMAS-PCR amplification and overexpression of p53 in subject number 10 in serial frozen sections (200 X magnification).

Mutant-specific primers for the mutation previously detected in exon 5 of subject number 10 were used for ARMAS-PCR amplification.

Figure 4.18a illustrates the basal and suprabasal cells positive by in situ ARMAS-PCR amplification (red arrows) in histologically normal peripheral epithelium (refer to Fig. 4.16).

Figure 4.18b illustrates the basal cells positive by p53 immunohistochemistry (black arrow).

Figure 4.18c illustrates histologically malignant cells positive by in situ ARMAS-PCR in tumour epithelium.

Figure 4.18d Immunohistochemical detection of p53 protein in serial frozen sections showing lack of staining in corresponding malignant cells (Fig 4.18c) positive by in situ ARMAS-PCR. (Section was lightly counterstained with haematoxylin.)
4.4 Discussion

Consistent with various studies (Partridge et al., 1999; Rowley et al., 1998; Taylor et al., 1999), this study showed that the detection of p53 protein was not synonymous with the presence of a mutation in the gene. Of the 15 cases analysed both by immunohistochemistry and by molecular sequencing, 4 had band shifts by PCR-SSCP suggesting point mutations. Six out of 15 cases were positive by immunohistochemistry for expression of the p53 protein, while 4 of the 6 cases that were immunopositive had band shifts indicative of point mutations, although the mutations were confirmed and defined by DNA sequencing only for patients 7 and 10, due to constraints of available tissue, sequencing was not subsequently performed in the cases of patients 11 and 13. No mutations were identified in the immunonegative cases (Table 4.VIII).

Since the early 1990s it has been apparent that although mutations of the p53 gene lead to the stabilisation of the protein, p53 positive immunostaining is not always accompanied by evidence of mutation and absence of p53 immunostaining does not necessarily mean absence of a mutation (Wynford-Thomas, 1992). Recent studies have strongly questioned the reliability of p53 immunostaining as a surrogate marker of p53 gene alterations. Studies by Calzolari et al. (1997) and Bradford et al. (1997) reported a discordance rate of 29% and 52% respectively. The relationship between p53 gene status and levels of p53 protein may be complex and a variety of technical and biological factors affect the correlation. Different antibodies have sensitivities and specificities that vary in their ability to detect p53 alterations. In this study D07 was selected because it had been previously found to be the most sensitive of a panel of antibodies for detection of p53 expression (Baas et al., 1994).
Technical and biological mechanisms have been proposed to account for the discrepancies. While DNA sequencing is currently the most reliable method of confirming the presence of a mutation, the mutation may not be detected if less than 20-30% of the tumour cells harbour the mutation (Ahrendt et al., 1999). Most studies so far concentrate on the sequencing of the exons 5 to 9. Approximately 5% of p53 mutations reside outside the exons 5 to 9 (Hollstein et al., 1991, Kropveld et al., 1999) and therefore could be missed. Gene alterations may not always stabilise the protein or they may even eliminate the protein, for example, where a gene alteration leads to the truncation of the protein, or deletion that may abolish all p53 protein production. Frame shifts and nonsense mutations may result in a p53 protein which is not detectable by immunohistochemistry because of the loss of critical epitopes.

Accumulation of the p53 protein may occur independently of gene mutation. Overexpression of the wildtype p53 gene product can be induced by certain types of genotoxic stress (Maltzman & Czyzyk, 1984). Stabilisation of the p53 gene product can be mediated by certain cellular and viral proteins. In cervical cancer, high p53 protein levels coexist with HPV 16 infection in the absence of p53 gene mutations (Mittal et al., 1995). Discordant p53 protein immunostaining was more commonly observed in tumours harbouring HPV 16 than in tumours without HPV 16 infection. However, recent studies suggest that HPV and p53 alterations may be independent events in some tumours (Badaracco et al., 2000). The cellular protein mdm-2 has been shown to be important in the stabilisation of the p53 protein. Mdm-2, as described in Chapter 1, binds to p53 and not only blocks its biological activity but also targets p53 for destruction via the ubiquitin proteosome pathway (Momand et al., 1992), which then forms an autoregulatory mechanism. It is now believed that mutant p53 cannot activate the transcription of mdm-2 and it is for this reason that p53 accumulates.
(Midgley & Lane, 1997). Lack of glutathione-S-transferase (Sarhanis et al., 1996), an enzyme involved in detoxification of products of oxidative stress, may be an underlying mechanism, such that failure to destroy these products may result in expression of wildtype p53 in the absence of a mutation. In the 15 samples analysed both for p53 protein by immunohistochemistry and PCR-SSCP only two of the samples (subject 12 and 15) were positive by immunohistochemistry in the absence of band shifts detected by PCR-SSCP, suggesting that there was no genetic alteration that contributed to the immunohistochemical staining.

The percentage of mutations demonstrated in this study is relatively low when compared with that in other previous studies (Boyle et al., 1993; Rowley et al., 1998). The main aim of the current study was to find suitable samples for the in situ analysis of p53 mutations, therefore PCR-SSCP was used as a quick and efficient way to screen samples for mutations. PCR-SSCP does not consistently detect 100% of all mutations. It had been previously shown that PCR-SSCP with silver staining of the gels could detect 1 mutant cell amongst 100 normal cells (Jones, 1998). It remains possible that mutations not detected by PCR-SSCP may have been otherwise detected by direct sequencing.

Sequencing data in the present investigation revealed mutations in codons 275 and 164, in samples from patients 7 and 10 respectively. Detection of point mutations in these samples gave appropriate mutation sites that could be used in ARMAS-PCR. Although these do not fall within any known “hot spots” (Hollstein et al., 1996) for p53 mutations, they have been previously recorded in head and neck cancer (Fig. 4.1). Interestingly samples 10 and 10.1, which were tissue blocks through different parts of the tumour, displayed the same shift in banding pattern in the SSCP analysis (Fig 4.8). Consistent with this finding, the two samples displayed the same mutation in exon 5.
in codon 164 (Fig. 4.10). This suggested a dominance of a population of mutant cells with the same mutation throughout the tumour. This was later further investigated by in situ ARMAS-PCR.

Tobacco and alcohol have been previously shown to induce signature-like point mutations in the p53 gene. In the current study both subjects investigated by in situ ARMAS-PCR were moderate to heavy smokers, while subject number 10 was only a low-risk drinker. G to T transversion as was detected in subject 7 has previously been shown to be associated with tobacco smokers, while a T to C transition has not been seen commonly associated with carcinogenic induction.

Using specifically designed primers for the mutations detected, mutant DNA from each sample was selectively amplified from both samples 7 and 10. It was not possible to extract DNA from the microdissected paraffin-embedded sample 7 probably due to the degrading effect on DNA of decalcification of the specimen. Therefore a small 0.5 g sample of whole frozen tumour tissue was used initially. Microdissection was preferred to avoid the contamination of the tumour by normal tissue, which might have masked the presence of any mutations. However, it is often difficult to obtain good quality high molecular weight DNA from processed paraffin-embedded material. It was necessary to use Taq plus which contained a high-fidelity enzyme to amplify mutant DNA extracted from paraffin-embedded material from subject number 10. This was not required for fresh tissue from subject number 7. Taq Plus is a mixture of cloned Pfu and Taq2000™ (Stratagene Cloning Systems, La Jolla, CA, USA) DNA polymerases. If Taq polymerase, which is devoid of 3' to 5' exonucleolytic proof-reading activity, is used, mismatches especially A·T to G·C can be expected to occur once every $10^3$ to $10^4$ bases (Innis et al., 1995). Therefore, use of Taq plus should have resulted in a greater and more accurate yield of PCR products.
Fixed frozen sections and paraffin embedded material were used to obtain microdissected histologically normal tissue from patient numbers 7 and 10 respectively to probe for the presence of cells containing the same p53 mutations in these areas. Paraffin-embedded material from patient number 7 was treated with decalcifying solution prior to wax embedding and was unsuitable for obtaining DNA, therefore fixed frozen sections prepared for in situ analysis was used. DNA from histologically normal epithelium microdissected from the margins of the tumour specimen of sample number 7 tested positive by ARMAS-PCR for the same mutation found in the tumour, suggesting the presence of genetic abnormalities in histologically normal epithelium, and an expanded clonal population of cells with the same p53 mutations at the margins of the tumour. The presence of the mutation found in the substance of the primary tumour of sample number 10 was not found in the surrounding histologically normal epithelium. The “histologically normal tissue” as identified by a pathologist was collected from similar distances of approximately 2-3 mm away from the margin of the tumour in the two cases analysed. It is accepted there are limitations in analysis of these “histologically normal tissues” and it would be more informative if progressively distant tissue were to be obtained. Manual microdissection was also a serious methodological limitation. Recent advances in the use of laser microdissection offer a more accurate method of dissection.

These results support the results of studies demonstrating the genetic progression of head and neck cancer, showing a clonal relationship between adjacent epithelial areas of diverse histopathological appearance (Califano et al., 1996; Rosin et al., 1997). A recent study by Califano et al. (2000) found that progression to malignancy occurs over a period of years, and during this progression, a significant number of genetic events occur early in carcinogenesis, whereas clinical expression of
a malignant phenotype occurs later. Similar to what is suggested from the present investigation, histologically normal epithelium may contain genetic alterations, whose phenotype may not become manifest until after a prolonged period.

Considerable precautions were taken in the present study in order to eliminate any possibilities of contamination of dissected normal epithelial tissue with tumour tissue. Normal and tumour tissues from other subjects were processed at the same time as test samples. No contamination was detected in these samples as shown in Figure 4.12 (lane 6). More information may have been gained if tumour resections had been carried out with a wider margin, so that tissue progressively further away from the tumour margin could be analysed.

DNA from lymph nodes available from subject number 7 was analysed for the presence of mutant cells spreading to a regional lymph node, which histologically was diagnosed as an uninvolved lymph node. ARMAS-PCR amplification using allele-specific primers did not uncover the presence of mutant DNA. There was no molecular indication of the metastatic lymph node spread of mutant cells and, equally, this eliminated the possibility that the patient may have carried a germline mutation. Consistent with this, no mutations were found in biopsies taken 2 years after surgery, although patient 7 did have a postoperative course of radiotherapy. However, studies have indicated that patients with p53 positive tumours could be radioreistant (Couture et al., 2002; Kandioler et al., 2002). For patient 7 there was no suggestion of residual genetic disease 2 years post operatively, although other genetic changes such as the 9p21 and 3p chromosomal losses could have been examined. These have been previously shown to occur earlier in the progression of head and neck cancer (Califano et al., 2000) and have been shown to precede any alteration in chromosome 17p13 (Koch et al., 1999). Although it was previously shown (Chapter 2) that
ARMAS-PCR was capable of detecting at least 1 mutant cell diluted amongst 1,000 normal cells, this level of sensitivity may still be inadequate for detecting residual disease.

Following the analysis of extracted DNA for mutations, the present study aimed at correlating the genetic information with the in situ localisation of the cells with the mutated DNA. In situ ARMAS-PCR was used in an attempt to visualise the distribution of cells with the same p53 mutation.

Tumours from patients 7 and 10 proved positive by immunohistochemistry for expression of the p53 protein. In situ ARMAS-PCR analysis was then used to allow the correlation of immunoreactive cells with cells with mutant genomic p53.

Tissue sections examined by immunohistochemistry for p53 overexpression or stabilisation were compared with those analysed by in situ ARMAS-PCR. For sample number 7 immunoreactive cells did correspond topographically with those that were positive by in situ ARMAS-PCR analysis but, in addition, some epithelial cells located above the basal layer positive by in situ amplification were negative by immunohistochemistry. These results were interpreted as implying that immunohistochemical disclosure of p53 overexpression or stabilisation was not representative of p53 mutations. This in turn raises the question as to why the most suprabasal cells positive by molecular amplification reacted negatively for p53 protein stabilisation assessed by immunohistochemistry. The implication from more recent evidence is that the detection of p53 by immunohistochemistry occurs as a result of a decreased degradation of p53 by mdm-2 or other important regulatory proteins, resulting in an apparent increase in the half-life of p53 allowing its detection (Oren et al., 2002).
Technical insensitivity may account for the lack of staining of immunohistological cells in the suprabasal epithelium. However, immunohistochemical detection was repeated on multiple serial sections to confirm the findings and antigen retrieval methods were used to enhance detection.

When comparing the results of in situ ARMAS-PCR amplification with immunohistochemical detection of p53 in the carcinoma of patient number 10, the protein was expressed in some tumour epithelium (Fig. 4.18a and c), but reactive cells were also seen in peripheral histologically normal epithelium. Consistent with this, ARMAS-PCR amplification detected positive cells within this band of histologically normal epithelium, as well as cells within the tumour lesion. However, similar to those in patient number 7, not all cells positive by ARMAS-PCR were positive for overexpression of p53 protein.

A small portion of the surgical specimen was used for analysis by in situ amplification. The larger portion was sent for histopathological analysis and later collected from archival collections for molecular research in this study. The tissue analysed for in situ p53 mutations displayed less tumour epithelium and more dysplastic epithelium with a periphery of normal epithelium with a smaller population of p53 immunopositive cells. This portion of tumour may have been derived from the surgical margins of the original specimen. The larger portion of the surgical specimen, obtained from fixed paraffin-embedded material contained proportionally more tumour by volume and demonstrated a larger number of intense p53 immunopositive cells, compared with peripheral tissue used for in situ analysis.

Different fixation procedures may have affected the staining intensity of p53 protein. Serial sections analysed both for in situ ARMAS-PCR amplification and for p53 expression were fixed in Karnovsky's fixative (2% paraformaldehyde, 2.5%
glutaraldehyde in PBS), while archival samples were fixed in 10% buffered formalin. Previous studies have examined the effects of different fixatives on immunohistochemical demonstrations of specific antigens. Arnold et al. (1996) found that neutral buffered formalin (used in this study) was the poorest fixative and that dehydrating fixatives (ethanol and methanol) provided the best preservation of p53 for the immunorecognition. No studies to my knowledge have compared paraformaldehyde fixed frozen sections with those fixed with neutral buffered formalin and their effects on p53 protein detection.

In conclusion, the results for the tumours from patients 7 and 10 suggest some correlation of p53 mutation with immunohistological detection of an aberrant p53 protein, but there appear to be other mechanisms that affect the expression or half-life of the protein for it to be detectable by immunohistochemistry. The results of in situ amplification suggest that there are cells in histologically normal tissue at the periphery of the tumour which have the same p53 mutations and therefore are clonally related to cells in the substance of the tumour. Some of these cells were histologically normal and this supports the concept that clonal genetic alterations may precede the development of malignancy by a significant amount of time. Some p53 mutations may not impair the protein's function, at least without other essential mutations as yet undefined being present, and the cellular phenotype remains unchanged. This suggests that there may be a prolonged latency period during which clonal genetic populations may be detected, but invasive progression has not yet taken place (Califano et al., 2000). The invasive phenotype only follows the further accumulation of genetic events and after a prolonged interval.

Since the original submission of the current dissertation, similar techniques to examine the expression and mutational status of the p53 gene have been conducted
using very similar techniques. Ebina et al. (2001) looked at p53 expression in non-small cell lung cancers (NSCLC) and compared p53 immunohistochemical detection of the p53 protein with genetic mutation using \textit{in situ} RT-PCR. They had a sample of 3 NSCLCs. They had similar findings to those in this thesis. Subgroups of mutated cells demonstrated by \textit{in situ} RT-PCR over-expressed p53 protein. They found the distribution of mutant p53 mRNA coincided with that of immunohistochemical over-expression of p53 protein. They proposed "topographical genomapping" of p53 as an approach to viewing the molecular mechanism of lung cancer. Several hypotheses were proposed to explain this heterogeneity between mutation and expression of p53. A posttranslational mechanisms such as the interaction between Mdm2 was suggested. Other more recent research has added to the complexity of cell cycle regulation. ARF tumour suppressor protein is encoded by Alternate Reading Frame of the Ink4A tumour suppressor locus. It binds to Mdm2 and inhibits its activity, thereby allowing p53 to escape Mdm2-mediated repression. Several genes are coupled with p53 induction via ARF: Myc, Ras, adenovirus E1A and E2F1 (Lowe, 1999).

Coupling also involves another oncogene beta catenin. It is a cell-to-cell adhesion component; it can double as a transcription factor in the nucleus. This occurs normally in developmental contexts, and abnormally in cancer cells where beta catenin becomes deregulated owing to mutational events (Damalas et al., 2001). While interference with apoptotic pathways has been identified via the AKT/PKB protein kinase loop, Akt can bind to Mdm2 and phosphorylate it. This potentiates Mdm2 activity (Gottlieb et al., 2002). Yet another tumour suppressor, PTEN, encodes a phosphatidylinositol phosphatase, which counteracts the action of PI3-kinase. PTEN serves to prevent the activation of AKT, thereby facilitating apoptosis, P53 can
activate the PTEN gene and when p53 is activated it may cause elevation in cellular PTEN levels (Simpson & Parsons, 2001). The complexity of the balance that regulates p53 is great. Multitudes of events may occur that tip the balance of opposing forces to allow the emergence of cancer.

4.4.1 Limitations of present research

In situ ARMAS-PCR has been demonstrated to be an extremely sensitive technique and labour intensive in its use. Adequate optimisation of the assays at their present stage of development requires an enormous amount of time and clinical material. Tissue preservation can often be inadequate, as tissue is required to be subjected to gruelling conditions and for extended periods. In situ amplification is a relatively new technique that is still currently under development and which has certain technical limitations at its present stage of development. False positives and false negatives listed in Chapter 3 are still a technical challenge to eliminate. All possible precautions were taken in order to control these by using internal controls with negative control tissue sections mounted on the same slide as the test tissue sections. Other negative controls included the omission of Taq polymerase and omission of primers. As with ARMAS-PCR using extracted DNA, in situ ARMAS-PCR amplification requires a predetermined knowledge of the mutation of interest to design suitable primers needed for selective in situ amplifications and is not a tool for screening cells or tissue for unknown mutations. The leakage of PCR product from the nucleus or cytoplasm of mutant cells in in situ ARMAS-PCR and in situ RT-ARMAS-PCR, respectively, further investigated in Chapter 3 in the cultured malignant cells is probably one factor in the problem encountered of achieving intense staining of mutant cells and minimising background non-specific staining. The negative controls were not non-specifically stained however. The prolonged in situ ARMAS-PCR amplification
procedure was associated with adverse effects on the preservation of the histological appearances of cells and tissues making it essential to have conventionally stained serial sections to assist in interpretation of the in situ ARMAS-PCR.

Case selection for analysis has also proved to be important. In the present study, cases were limited by the tumour tissue available for analysis and appropriate point mutations that were amenable to analysis by ARMAS-PCR. Lengthy optimisation steps required the availability of large amounts of tissue. Cases with larger biopsies that involved an area of histologically normal epithelium, continuous with dysplasia and to carcinoma in the same section would have provided greater insights into the progressive nature of genetic lesions during carcinogenesis. As mentioned in section 1.3, the point at which clonal mutational analysis is performed in the life history of the development of the tumour is significant. Analysis of mature lesions may not be appropriate, since tumours of polyclonal origin may become clonal because of clonal evolution (Nowell, 1986).

Various studies have used microdissected tissue to assess for the alteration of the p53 gene (Bedi et al., 1996; Califano et al., 1996; Califano et al., 2000; Chung et al., 1993; Koch et al., 1999). The only study so far to our knowledge that has identified the molecular lesion in situ is the study by Ebina et al (2001) mentioned previously. As Garcia et al. (2000) alluded to in their review article, the methods of analysis which involve the disaggregation of tissues, albeit microdissected, are far from ideal, and more effort should be invested in techniques allowing clonal architecture of normal tissues, preneoplastic and pre-invasive lesions and tumours to be directly visualised in situ.

Because of the technical problems discussed above, the few cases in which sufficient suitably fixed tissue was available, the fact that p53 mutations were only
detected in a proportion of the carcinomas and only some of which had point
mutations suitable for PCR amplification, and the constraints of time, only two cases
were successfully analysed by ARMAS-PCR using extracted DNA and in situ for p53
mutations. These difficulties have also limited other researchers attempting similar in
situ analysis of p53 mutations and only one other paralleled study is available, on non-
small cell lung cancers by Ebina et al 2001 who reported on p53 mutations localised
by in situ RT-PCR and by immunohistochemistry in only 3 tumours, with similar
findings to those of the present investigation of head and neck cancer.

Obviously general conclusions cannot be made from such small samples but
nevertheless in situ ARMAS-PCR analysis supplies information on the distribution of
the mutated cells in intact tissues in tumours and adjacent tissue which is not available
from microdissected or extracted DNA samples.
Chapter 5: Final Discussion

Multiple genomic errors accumulate, probably in no fixed order, during the transformation of normal to tumour cells. They mark steps on a pathway to malignancy that is characteristic for each tumour type. Biomarkers are indicators of changes or events in human biological systems. Biomarkers for cancer development reveal the genetic and molecular changes related to early, intermediate and late points in the process of carcinogenesis. They have the potential or are already in use in monitoring prevention, the detection of the earliest stages of malignant transformation or, in the future, possibly for treatment of cancer. Biomarkers will refine our ability to enhance the prognosis, diagnosis and treatment of cancers. In this study the use of an allele-specific mutation detection system was analysed for its sensitivity and extended application of in situ localisation of point mutations in the biomarker p53 gene in oral squamous cell carcinoma. This biomarker was used as a marker to determine cells of the same lineage with identical genetic mutations.

Greater understanding of the natural history of oral cancer will enable better management of the disease. However, the natural history of oral cancer has yet to be elucidated. Only in recent years have molecular models of the progression and clonal relationship of oral cancers been developed (Califano et al., 1996; Koch et al., 1999; Califano et al., 2000). Studies suggest that a clinically apparent potentially malignant oral epithelial lesion occurs in a variable proportion of cases, preceding the development of cancer. Leukoplakia is the most commonly encountered clinical lesion, with various clinical types including nodular, speckled and exophytic forms and erythroplakia carrying a higher risk (van der Waal & Axell, 2002). However, the annual incidence of malignant transformation varies in different parts of the world. So although 6% of all leukoplakias will transform to cancer over a 10-year period, among

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histologically dysplastic lesions, this figure has been suggested to rise to 16-36% depending on severity of the dysplasia (Lumerman et al., 1995). Sudbo et al (2001) provided evidence that ploidy analysis of leukoplakias may be a better indicator than dysplasia of the risk of oral squamous cell carcinoma (Sudbo et al., 2001). It appears that the histopathology and genetic/molecular changes for oral squamous cell carcinoma are still unclear. Figure 5.1 summarises molecular and genetic changes found in biochemical and cytogenetic studies and compares them to the histopathological changes during transformation. It has also been proposed that environmental as well as individual host responses determine individual genetic progression profiles. Koch and co-workers (1999) found that clinical and genetic features of head and neck squamous cell carcinomas were distinct between groups defined by tobacco use. Tumours of non-smokers contained a lower frequency of common genetic alterations. Other factors include lack of intake of fruit and vegetables (Gridley et al., 1992), poor dentition (Day et al., 1993) and lower socioeconomic status (Macfarlane et al., 1994b).
Time course

Months (variable) → Years 20-30 ave

Genetic/Molecular Changes

Genomic Stability Changes → Genomic Instability increases

Differentiation → Dedifferentiation

Polymorphisms → Polysomes

(Chr. 7 and 17)
Oncogene/Tumour Suppressor [p53(17p), Rb(13q), p21 waf-1(6p)]
Growth Factors
Proliferation-Cyclin-Kinase Alteration
Oxidative-Anti-oxidant Regulation
Programmed Cell death Immunologic Modifications

Loss of Genetic Material (3p, 4, 5p, 6p, 8, 11q, 13q, 14, 15, 19p, 19q)

Gain of Genetic Material (1q, 2, 3q, 5p, 7, 8q, 9, 10p, 13q, 14q, 16, 17, 20p, 21q, 22q)

Sequence amplification (3q26, 11q13, 12p, 2p33-36, 7q21-22, 7q33, 9p, 13q32)

Chromosome Band Abnormalities (8q11-q11, 1p11, 3p11-q11, 11q13, 13p11-q11, 1p13, 5p11-q11, 7p11-q11, 15p11-q11, 14p11-q11)

Figure 5.1 The relationship between histopathology and genetic/molecular changes in oral squamous cell carcinoma.

The figure depicts the time relationship between histopathologic changes during the transformation process and the genetic and molecular changes found in biochemical and cytogenetic studies (Schwartz, 2000).

A greater understanding of the progression of head and neck squamous cell carcinoma should also generate more information for the management of the most significant cause of mortality and morbidity of head and neck cancer, which is the development of second primary tumours and recurrences. These have a profound impact on long-term survival, particularly of patients with early disease. Our current understanding of the origin of the second primary tumours is limited. The two
possibilities are that either 1) the tumours either arise independently after transforming events in separate cells or 2) the tumours develop from a single clone, with cells migrating to different sites, or 3), both events might even occur in the same patient. Elucidation of whether tumours are of independent or common clonal origin requires determination of whether separate tumours have developed along similar or disparate genetic pathways. Analysis should focus on early genetic events, which conceivably occur before the migration of cells forming tumours of clonal origin. Analysis of later events may result in discordance due to molecular evolution of tumours.

Various methods have been developed to identify genetic mutations, as described in Chapter 1. Mutation detection methods in the analysis of clinical tumour pathology must be sensitive and specific because surgical biopsies collected for molecular analysis contain a mixture of non-tumour cells that depends on the tumour type and location of the biopsy within the tumour. As yet there is not one perfect method for mutation diagnosis or mutation scanning. Some methods are difficult to perform while others, although technically easier, prove less sensitive. The choice of technique will depend on the type of alteration of interest. The use of the amplification refractory mutation system, allele-specific-PCR on DNA extracted from tissue, proved to be well suited for the detection of previously identified p53 mutations. This approach proved to be both sensitive and specific and a relatively simple method to use, needing little specialised equipment and a result could be gained in a relatively short time. The extension of its use to in situ was also an advantage.

In situ localisation of amplified PCR products is a potentially valuable advance in molecular techniques for analysis of genetic alterations. The advantages
and disadvantages have been described in Chapter 3. It has the potential to overcome the drawback of PCR solution phase amplification, which does not allow the visualisation and localisation of the amplified products within cells and tissue specimens. The amalgamation of ARMAS-PCR with in situ PCR was used in cultured malignant cells to identify cells in situ with a specific p53 mutation. In situ detection of mRNA by in situ RT-ARMS-PCR proved to be a potentially useful technique to identify cultured malignant cells actively expressing a mutant genome.

Further application of ARMAS-PCR to the localisation of tumour cells with the same p53 mutations was performed in oral squamous cell carcinoma specimens. It allowed a comparison of cells with a genomic p53 mutation localised by in situ ARMAS-PCR with cells positive by immunohistochemistry for stabilised p53 protein. The use of in situ ARMAS-PCR demonstrated in this study that immunohistochemical detection of the protein did not always signify a p53 mutation nor were cells reacting negatively by immunohistochemistry always free from mutations. These results confirm the complexity of the "p53 pathway" (Oren et al., 2002; Prives & Hall, 1999) and its interaction with various upstream and down stream events and its numerous roles in cell regulation (section 1.5).

The clonality of cells harbouring p53 mutations in oral squamous cell carcinoma specimens analysed raised some questions. Not all histologically malignant cells were positive by in situ ARMAS-PCR. The mutational status of these cells needs to be established. These cells may be derived from another clone of malignant cells with a different mutation profile. Alternatively problems with technical sensitivity may have limited the detection of some cells. What is the clonal relationship of the histologically normal cells positive for the same p53 mutation in the substance of the tumour? Was there spread of the mutated clone through the epithelium at an early
stage in tumour development, as was suggested in the study by Franklin et al. (1997), in which the same p53 mutation was found in both lungs of one patient? Another possible example is the lateral migration of morphologically abnormal cells seen in lesions such as Bowen's disease (Pinkus & Mehregan, 1986). Intravascular dissemination has been proposed for the finding of clonal cells in multiple deposits of Kaposi's sarcoma (Rabkin et al., 1997) and the identical point mutations in multiple synchronous and metachronous carcinomas in urothelium has been attributed to intraluminal seeding (Habuchi et al., 1993). Complex regulatory mechanisms or disarray of these mechanisms could lead to posttranslational modifications of expressed proteins in the control of cell proliferation and apoptosis; this has been described in section 4.4. These may be involved in the alteration of expression of p53 detected.

In conclusion, it has been demonstrated that ARMAS-PCR is a potentially useful technique that may be applied to the study of genetic alterations and genotyping as indicated by Ebina et al. (2001). However, we have shown that in situ ARMAS-PCR is technically difficult and interpretation of data must be made with care, using well-optimised controls.

5.1. Potential for future research

ARMAS-PCR has proved to be a powerful mutation detection system and its application in situ has been demonstrated. ARMAS-PCR has the potential to be used for any previously identified mutation. A modification of the ARMAS-PCR technique is currently being used in our laboratory for the analysis of HPV and its relationship to oral squamous cell carcinoma.
Following the analysis of p53 mutations in clinical oral squamous cell carcinoma specimens in this study, analysis of premalignant or dysplastic lesions may give greater insight into the earlier genetic changes that occur in the progression of the malignant lesion. Analysis of tissue at increasing distances away from the centre of malignant tissue could provide information on the progression pathway of adjacent epithelial tissues and whether they are also evolving along the same pathway of tumour development.

Long-term follow-up of patients with identified p53 mutations and analysis of recurrent tumours using ARMAS-PCR would be a method of addressing the issue as to whether recurrent tumours were second primary tumours or a recurrent lesion. Current studies propose that the presence of p53 mutations is associated with a higher risk of recurrence (Ma et al., 1998). Only a limited number of biopsies was analysed for recurrent p53 mutations in the current study. A greater number of patient samples would give greater information on the possibility of use of ARMAS-PCR detection of p53 mutations in clinically normal mucosa and its use as a molecular biomarker of potential for recurrence.

Analysis of metastatic spread may also be another potential application of ARMAS-PCR which could be used to detect malignant cells in distant sites such as lymph nodes or for the presence of blood-borne malignant cells. In situ ARMAS-PCR followed by in situ hybridisation could give valuable information on the genetic make-up of metastatic cells.

ARMAS-PCR may be further applied to the detection of other genetic alterations such as p16, which is the most commonly mutated gene in head and neck cancer and its role in the pathway of oral squamous cell carcinoma.
Recent evidence has shown that family members of p53, p63 and p73 have structural similarities to p53; however, there are significant differences between the family members. Most interesting is that data has implicated that p63 may be important in epithelial cell stem cell renewal and differentiation (Irwin & Kaelin, 2001). Compiling a complete picture of the molecular progression in oral carcinogenesis is an indispensable prerequisite for improving the clinical diagnosis, treatment and management and prevention of the disease. ARMAS-PCR and in situ ARMAS-PCR have been shown to be useful methods in advancing towards these goals.
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