3.1 Statistical analysis

This study consisted of 30 patients of whom 15 were male and 15 were female. The group of patients had a mean age of 59.2 years with a standard deviation of 14.1 years.

Mean values for statistical analysis were obtained from raw data to allow for differences in the numbers of tissue blocks available for individual patients.

Statistical analysis was undertaken using SPIDA (Statistical package for interactive data analysis 1994) provided by the statistical laboratory at Macquarie University.

3.2 Definitions

Tumour or clinically normal epithelium was regarded as p53 positive if 5% or more of the cells had a positive reaction (Kaur et al., 1994).

A tumour was regarded as recurrent, if it had recurred in the same site as that of the primary tumour within twelve months of the surgical or subsequent radiotherapy treatment.
### 3.3 P53 expression in the marginal tissue adjacent to the primary tumour.

Table 3.1 The association between the immunohistochemical detection of p53 at resection margins and subsequent recurrence of the tumour at the primary site within four years in 30 patients with primary oral squamous cell carcinoma.

<table>
<thead>
<tr>
<th>p53</th>
<th>No recurrence</th>
<th>Recurrence</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>8</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>Negative</td>
<td>11</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>11</td>
<td>30</td>
</tr>
</tbody>
</table>

The Chi square with one degree of freedom is equal to 0.43, with a p value of 0.5. There is no statistically significant difference between the primary site recurrence rates at four years in tumours with p53 positive margins compared to those with p53 negative margins ($X^2_1=0.43$, $p=0.5$).
Table 3.2 The association between the immunohistochemical detection of p53 at resection margins and the recurrence of tumour at the primary and distant sites, in 30 patients with primary oral squamous cell carcinoma.

<table>
<thead>
<tr>
<th>p53</th>
<th>No recurrence</th>
<th>Recurrence</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>5</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>Negative</td>
<td>8</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>17</td>
<td>30</td>
</tr>
</tbody>
</table>

Using the Chi square value with one degree of freedom, there was no statistically significant difference between the recurrence at any site (local and distant), at four years in cases which were p53 positive and those which were p53 negative at the resection margin. ($X^2 = 0.62, p = 0.4$).
Table 3.3 The association between the immunohistochemical detection of p53 at resection margins and survival in 30 patients with primary oral squamous cell carcinoma.

<table>
<thead>
<tr>
<th></th>
<th>&lt;2yrs</th>
<th>2-4yrs</th>
<th>≥4yrs</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>3</td>
<td>1</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
<td>1</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>2</td>
<td>21</td>
<td>30</td>
</tr>
</tbody>
</table>

Using the Chi square value with one degree of freedom, there is no statistically significant association between the proportion of patients surviving for at least 2yrs ($\chi^2_1 = 0.05$, p = 0.8), or for at least 4yrs ($\chi^2_1 = 0.03$, p = 0.9), and the p53 positivity status at the margin. This is demonstrated graphically in figure 3.1.
Figure 3.1 Patient survival and immunohistochemical detection of p53 at the margin.
Figures 3.2 and 3.3  Immunohistochemical detection of p53 protein at the resection margins of a primary oral squamous cell carcinoma of the lower lip (stained with Pab1801) (400x).
Figure 3.4 The association of immunohistochemical detection of p53 at the margin of resection and the presence or absence of epithelial dysplasia.

Percentage of dysplasia at the margin

<table>
<thead>
<tr>
<th>p53-ve</th>
<th>*</th>
<th>x</th>
<th>0</th>
<th>0</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53+ve</td>
<td></td>
<td>*</td>
<td>x</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* denotes the median value.

x denotes the maximum and minimum percentage of dysplasia for each group.

Of the thirty patients, twenty had p53 negative resection margins and ten had p53 positive resection margins. Of the ten immunohistochemically positive margins, the median percentage of dysplasia (all grades) was 10.5%, compared with a median of 2.0% for those with no p53 positivity at the resection margin. It would appear that dysplasia was less frequent at p53 negative margins and commoner in p53 positive margins. However using the Mann Whitney rank sum test, there was no statistical significance between p53 expression and dysplasia at the margin in this study (p = 0.16).
Table 3.4 The association of immunohistochemically detectable p53 at the tumour resection margins and the site of the primary tumour.

<table>
<thead>
<tr>
<th>Marginal tissue</th>
<th>site1*</th>
<th>site2*</th>
<th>site3*</th>
<th>site4*</th>
<th>site5*</th>
<th>site6*</th>
<th>site7*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 negative</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>5</td>
<td>-</td>
<td>5</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>p53 positive</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>3</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>8</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>30</td>
</tr>
</tbody>
</table>

* Specific oral sites are coded as follows: 1 = tongue  
2 = floor of mouth  
3 = buccal mucosa  
4 = retromolar trigone  
5 = upper and lower alveolus  
6 = hard and soft palate  
7 = vermilion of the lip

From the table there does not appear to be an association between intraoral sites and p53 immunoreactivity at the margin of the tumour. No conclusions can be drawn from this data due to the small number of individuals studied.
Table 3.5 The association of immunohistochemically detectable p53 at the marginal tissue with the oral habit of tobacco smoking.

<table>
<thead>
<tr>
<th>Marginal tissue</th>
<th>Non smoker</th>
<th>Smoker</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 negative</td>
<td>3</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>p53 positive</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>22</td>
<td>30</td>
</tr>
</tbody>
</table>

Non smokers in this study tended to present with more p53 positivity in the margin adjacent the primary tumour than smokers who tended to be more p53 negative at the margin. The Chi squared value with one degree of freedom was 4.18 where p equals 0.04. A p53 negative margin was statistically significantly more frequently found in tobacco smokers than in non smokers.

3.4 The association between the immunohistochemical detection of p53 at the margin and in the tumour.

There was a reasonably strong association between the p53 immunodetection in both the margin and tumour (Spearman rank correlation coefficient = 0.56, p=0.002). Using the Chi squared value with one degree of freedom, the possibility of finding any correlation between the presence of any detectable p53 in the margin with p53 positivity in the tumour was strongly significant (χ² = 8.2, p=0.004). Thus p53 positive carcinomas were statistically more likely to also have p53 positive resection margins than
were p53 negative tumours. Of the thirteen positive p53 tumours, eight had positive margins, whereas of the 17 p53 negative tumours, only four had positive margins.

Table 3.6 The association of immunohistochemically detectable p53 at the margin and in the tumour, in 30 patients with primary resected oral squamous cell carcinoma.

<table>
<thead>
<tr>
<th>p53 immunoreactivity</th>
<th>At the margin</th>
<th>In the tumour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Negative</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

It was noted that the length of the normal epithelial margin (NEM) measured histologically adjacent to the tumour, was often undermined by tumour to a considerable extent, sometimes with overlapping of up to 8 mm in length (see figures 3.5 and 3.6). An excision margin might therefore appear to be clinically well clear of the tumour but in fact only a margin of 2mm or 3mm may actually remain as clearance. On two occasions the primary tumour was at the SM of the tumour.
Results

Figure 3.5  Primary oral squamous cell carcinoma negative for p53 protein underlying a normal epithelial margin (immunohistochemically stained with PabDO-7) (40x).

Figure 3.6  Primary oral squamous cell carcinoma negative for p53 protein underlying a normal epithelial margin (immunohistochemically stained with PabDO-7) (100x)
3.5 P53 expression in the primary tumour

Staining was mostly localised to nuclei of cells in the primary tumours and adjacent resection margins. There were considerable differences between the staining patterns of different primary oral squamous cell carcinomas from the same site and differing sites, as well as within the same tumour. The p53 staining distribution in the epithelium varied from individual sporadic or scattered cells (see figure 3.7) to positive clusters. The intensities of the cellular staining between primary tumours and within the same tumour varied greatly. Apart from the areas of epithelial dysplasia adjacent to the tumour tissue, non dysplastic epithelium, tumour stroma, lymphoid cells and normal adjacent connective tissue present in the same tumour sections were uniformly stained with DO-7 and 1801 antibodies negatively for p53. The distribution of positive nuclei among carcinomas also remained identical irrespective of the antibody used. In those primary tumours where p53 was immunohistochemically detected, positive staining reside predominantly towards the deeper tumour layers and infiltrative margin (see figure 3.9). P53 positivity was never seen in the more superficial layers of the tumour.
Figure 3.7 Scattered appearance of p53 positive cells in a primary oral squamous cell carcinoma stained with Pab1801 (100x).
Figure 3.8 The association of immunohistochemically detected p53 in the tumour, with the Bryne index score.

Bryne index score

4  6  8  10  12  14

p53-ve  x---*---x

p53+ve  x---*---x

* denotes the median value.

x denotes the maximum and minimum Bryne scores for each group

Of the thirty patients, seventeen had p53 negative tumours and thirteen had p53 positive tumours. The median Bryne index score for both groups was nine, indicating an intermediate range of malignancy grading. There is no statistically significant difference between the Bryne index for those with or without p53 positivity in the tumour (Mann Whitney rank sum test showed a p value equal to 0.5).
Table 3.7 The association between the immunohistochemical detection of p53 in the tumour and patient survival.

<table>
<thead>
<tr>
<th>Expression in the tumour</th>
<th>Survival less than 4 yrs</th>
<th>Survival greater than or equal to 4 yrs</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 negative</td>
<td>4</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>p53 positive</td>
<td>6</td>
<td>7</td>
<td>13</td>
</tr>
</tbody>
</table>

Using Chi squared value with two degrees of freedom, there was no statistically significant difference in patient survival associated with the presence of p53 positivity in the tumour ($X^2_2 = 4.49$, $p = 0.106$) although it would appear that patients with p53 negative tumours tended to survive longer. A study with a larger patient cohort is required to decide whether this is a definite association.
Table 3.8 The association between the immunohistochemical detection of p53 in the tumour and the histological grade of the tumour.

<table>
<thead>
<tr>
<th>Expression in the tumour</th>
<th>well differentiated</th>
<th>moderately differentiated</th>
<th>poorly differentiated</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 negative</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>p53 positive</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>9</strong></td>
<td><strong>12</strong></td>
<td><strong>9</strong></td>
<td><strong>30</strong></td>
</tr>
</tbody>
</table>

There is no statistical significance between the positive presentation of detectable p53 within the tumour and the histological grade of the tumour ($X^2 = 1.8$, $p = 0.4$).
Table 3.9 The association of the immunohistochemical presentation of p53 within the tumour and the site of occurrence of the tumour.

<table>
<thead>
<tr>
<th></th>
<th>site 1*</th>
<th>site 2*</th>
<th>site 3*</th>
<th>site 4*</th>
<th>site 5*</th>
<th>site 6*</th>
<th>site 7*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 negative</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>p53 positive</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>8</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>30</td>
</tr>
</tbody>
</table>

* Specific oral sites are coded as follows: 1 = tongue
2 = floor of mouth
3 = buccal mucosa
4 = retromolar trigone
5 = upper and lower alveolus
6 = hard and soft palate
7 = vermilion of the lip

From the data, tumour p53 expression does not appear to be associated with any particular intra-oral site. An increase in the total number of cases would be required in order to test this more rigorously.
Table 3.10 The association between the immunohistochemical presentation of p53 detected in the tumour, with and without a clinical history of smoking.

<table>
<thead>
<tr>
<th>Detection in the tumour</th>
<th>non smoker</th>
<th>smoker</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 negative</td>
<td>3</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>p53 positive</td>
<td>5</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>22</td>
<td>30</td>
</tr>
</tbody>
</table>

Using the Chi square value with one degree of freedom, there is no statistically significant association between the detection of p53 expression in the primary oral tumour and smoking ($X^2_1 = 1.6$, $p = 0.2$). Although the results do not quite reach significance, there was a tendency for more tobacco smokers to have p53 negative tumours than did non smokers. This contrasts with the results on table 3.16 where a tendency for p53 positive presentation in the normal epithelium of clinically normal subjects were in smokers.
Table 3.11 The association between the immunohistochemical detection of p53 in the tumour of patients with and without a history of smoking and alcohol abuse.

<table>
<thead>
<tr>
<th>Detection within the tumour</th>
<th>no smoking and no alcohol</th>
<th>smoking and no alcohol</th>
<th>smoking and alcohol</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 negative</td>
<td>2</td>
<td>3</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>p53 positive</td>
<td>4</td>
<td>2</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>5</td>
<td>19</td>
<td>30</td>
</tr>
</tbody>
</table>

Using the Chi square value with two degrees of freedom, no statistical significance is shown between smoking and alcohol abuse, and the expression of p53 positivity in the tumour ($\chi^2 = 1.67$, p=0.43). There were no patients who abused alcohol who were non-smokers.
**Figure 3.9** Immunohistochemical detection of p53 protein predominantly in the deeper parts of the oral squamous cell carcinoma (Pab DO-7) (100x).
3.6 The Bryne index score, histologic and clinical correlations.

Figure 3.10 The association between the Bryne index and the tumour stage.

* denotes the median value.

x denotes the maximum and minimum Bryne index score for each group.

Although the association between the Bryne index and tumour stage did not quite reach statistical significance using the Kruskal Wallis test ($p = 0.10$), there appears to be a tendency for the malignancy grading to increase with the dimensions of the tumour at resection.
**Figure 3.11** The association of the Bryne index score with and without nodal involvement.

<table>
<thead>
<tr>
<th>Node status</th>
<th>n=No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>20</td>
</tr>
<tr>
<td>N1</td>
<td>4</td>
</tr>
<tr>
<td>N2</td>
<td>6</td>
</tr>
</tbody>
</table>

* denotes the median value.

x denotes the maximum and minimum Bryne index score for each group.

The Bryne index is significantly less in those with no lymph node involvement, than in those with either one or two nodes involved (histologically confirmed) (Kruskal Wallis test p=0.043). Higher Bryne scores were significantly associated with a higher risk of metastases.
**Figure 3.12** The association of the Bryne index malignancy score and tumour depth in 29 cases (measured histologically in mm. from the surface using the ocular micrometer).

(Bryne vs Tumour depth (mm))

![Graph showing the association between Bryne index score and tumour depth]

(The figures 4 and 2 in the graph denote the number of stars at that particular value.)

The Bryne index score and tumour depth showed a strongly significant positive correlation (rank correlation = 0.46, p=0.02). As tumour depth increased, so too did the Bryne index score.

The association between the Bryne index score and survival did not quite show statistical significance (rank correlation = -0.32, p = 0.08), although there was a tendency towards lower survival in those with a higher Bryne score. Perhaps study of a larger group would have clarified this trend.
Figure 3.13 The association between the Bryne index score and the STNMP staging.

Bryne index score

<table>
<thead>
<tr>
<th>STNMP</th>
<th>n=no. of cases</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage IV</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* denotes the median value.

x denotes the maximum and minimum Bryne index score for each group.

There is a fairly strong association between the Bryne index score and the STNMP staging (Rapidis et al., 1977) of oral cancer (Spearman’s rank correlation coefficient Rho = 0.51, p=0.006).

From the box plot above, it can be seen that as the stage increases, so too does the Bryne index. The association is however limited in that the Bryne score is unable to clearly define and separately categorise the different staged groups.

When the relationships between the STNMP score, Bryne index and tumour depth were
investigated using multivariate regression analysis, only the Bryne index remained an independent predictor of STNMP stage. Although increasing tumour depth is positively associated with nodal involvement (see figure 3.14), the tumour depth adjusted for the Bryne index was not significantly correlated with the STNMP stage. The Bryne index is highly correlated with the STNMP staging.

3.7 Site of the primary tumour, recurrence and survival.

Table 3.12 The association between local tumour recurrence and the site of the primary tumour.

<table>
<thead>
<tr>
<th>Recurrence</th>
<th>sites 1* and 4*</th>
<th>other sites*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>2</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>Yes</td>
<td>9</td>
<td>2</td>
<td>11</td>
</tr>
</tbody>
</table>

* specific oral sites are coded as follows: 1 = tongue 2 = floor of mouth 3 = buccal mucosa 4 = retromolar trigone 5 = upper and lower alveolus 6 = hard and soft palate 7 = vermillion of the lip

Nine out of eleven recurrences occurred at the tongue and retromolar trigone sites. Using
the Chi squared test with one degree of freedom, a highly significant association was found between local tumour recurrence and origin on the tongue and retromolar trigone sites ($X^2 = 15.2, p<0.01$). There may be other variables at work that have led to this apparent association, but of these the tumour size does not seem to have an influential role. This is illustrated in Table 3.13.

**Table 3.13** The association of tumour size (T stage) of the recurrent tumour group, affecting both the tongue and retromolar sites with the numbers of patients in each group.

<table>
<thead>
<tr>
<th>Tumour size</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 3.14 The association between the site of the primary oral squamous cell carcinoma and survival.

<table>
<thead>
<tr>
<th>Survival</th>
<th>sites 1 and 4</th>
<th>other sites</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>less than 2yrs</td>
<td>1</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>2 to 4 yrs</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>greater than or equal to 4yrs</td>
<td>9</td>
<td>11</td>
<td>20</td>
</tr>
</tbody>
</table>

*specific oral sites are coded as follows: 1= tongue
2= floor of mouth
3= buccal mucosa
4= retromolar trigone
5= upper and lower alveolus
6= hard and soft palate
7= vermillion of the lip

Due to wide spread data over the seven oral sites in this series, it was not possible to obtain any meaningful result. No statistical significance for survival was found using the Chi squared test with two degrees of freedom between sites 1 and 4 together and the other sites in the mouth ($\chi^2 = 2.1, p=0.3$). Survival therefore was not apparently adversely affected by the higher local recurrence at these sites as seen in table 3.12. This may reflect successful management of the recurrent tumour.
Table 3.15 The association between distant tumour recurrence and site of the primary tumour.

<table>
<thead>
<tr>
<th>Distant recurrence</th>
<th>site1*</th>
<th>site2*</th>
<th>site3*</th>
<th>site4*</th>
<th>site5*</th>
<th>site6*</th>
<th>site7*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>6</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Yes</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

* specific oral sites are coded as follows: 1= tongue
2= floor of mouth
3= buccal mucosa
4= retromolar trigone
5= upper and lower alveolus
6= hard and soft palate
7= vermilion of the lip

No particular intra-oral site for the primary tumour predisposed to distant metastases (table 3.15).

Tumour depth did not significantly affect patient survival, according to the rank correlation test (Rank correlation = -0.22, p= 0.2).

Although the association between the depth of the primary tumour and stage of the tumour using the TNM classification of the UICC (Smith & Pindborg, 1990) did not quite reach statistical significance (Rank correlation =0.31, p=0.1), a larger patient study may have shown it to be of value.
3.8 Tumour depth and nodal metastases.

Figure 3.14 The association between tumour depth and the presence of nodal metastases.

There was a statistically significant association between the tumour depth and the presence or absence of histologically confirmed nodal involvement (Spearman’s rank correlation coefficient $\text{Rho} = 0.42$, $p = 0.03$). The box graph illustrates that as tumour depth increases, so too does nodal involvement. The median depth identified without nodal involvement was 2mm (interquartile range of 1.7 to 3.8mm). Where one node was involved, the median depth of the primary tumour was 5mm (interquartile range of 3.4 to 6.7mm) and for two involved nodes, tumours had a median depth of 6.7mm (interquartile range of 5.5 to 13.0mm).
3.9 P53 Expression in clinically normal tissue.

Table 3.16 The association between the immunohistochemically detected p53 in normal epithelial tissue from normal subjects and tobacco smoking.

<table>
<thead>
<tr>
<th>Normal epithelium</th>
<th>Smoker</th>
<th>Non smoker</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 positive</td>
<td>4</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>p53 negative</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

Using Fisher’s exact test, there is a tendency for p53 positivity to be associated with smoking but this does not quite reach statistical significance in this small sample of ten patients (p= 0.12). A larger study may confirm the trend by statistical support.
3.10 Effect of the microwave technique for antigen retrieval compared with conventionally treated sections on p53 immunoreactivity in ten primary oral squamous cell carcinomas using Pab DO7.

From the series of thirty patients with primary oral squamous cell carcinomas, ten were randomly selected for assessment using the microwave antigen retrieval technique in immunostaining for p53 protein (see section 2.6).

Using the Wilcoxon test of differences (nonparametrics), there was a statistically significant difference in the detectable p53 within the tumour, in the proportion of positive cells in microwaved sections and those kept at room temperature (p=0.041). The microwaved sections tended to show more positive cells than those not microwaved. This difference however was not statistically significant in the detection of p53 at the margin of the tumour (p=0.21). The possible explanation for the differences which have resulted may be due to a change in the conformation of the p53 protein at higher temperatures using the microwave technique so that previously unexposed epitopes are now exposed. The contrast may also imply that the quality of the mutant protein with in the tumour may be different to the quality of the p53 protein at the adjacent epithelial resection margin. Further work using molecular studies would reveal the true status of the p53 mutation and aid in understanding these differences.
3.11 Reproducibility of investigation

Ten of the sections stained and previously assessed for p53 protein were selected at random by a laboratory officer and given coded labels. These sections were then re-examined by the author at least two weeks after the initial assessment and the presence of p53 protein re-evaluated in both the tumour and the resection margins (represented as a percentage positivity) and length of positive epithelium measurements (in millimeters). Using the Wilcoxon test of differences, there was no statistically significant difference between the results for each slide in this second study compared with the previous assessment of the same slides, indicating good reproducibility and low subjective error in the interpretation of results.
Chapter Four

DISCUSSION

4.1 p53 detection at margins of resection and within primary oral squamous cell carcinomas in relation to outcome, tobacco and alcohol abuse.

In this study, the immunohistochemical detection of p53 protein at the excision margin of resected primary oral squamous cell carcinomas did not show any statistical correlation with the clinical outcome assessed by tumour recurrence at the primary or distant sites (section 3.3, table 3.1 and 3.2) and survival of the patient (section 3.3, table 3.3, figure 3.1). There was however, a correlation between the activity of smoking and the absence of detectable p53 protein at the clinically normal epithelial margin (section 3.3, table 3.5). Field et al. (1991) also found no p53 oncoprotein staining in the adjacent normal tissues next to squamous cell carcinomas of the head and neck in patients who smoked heavily. They did however find a higher incidence of p53 staining in the tumours of heavy smokers compared with those of moderate smokers (<20 cigarettes/day). They concluded that the carcinogens in tobacco smoke cause a mutation in the p53 gene which may therefore be considered a probable initiation event in the development of cancer. In contrast, although no statistically significant result was obtained, this study showed a tendency for smokers to present with p53 negatively staining carcinomas (section 3.5, table 3.10). Absence of p53 expression in the tumours
of smokers compared with tumours of non-smokers was also demonstrated by Ranasinghe et al. (1993). The present study failed to find an association between the habits of smoking and alcohol abuse with the presence of p53 in the tumour (section 3.5, table 3.11). It is quite possible that other factors could be involved giving rise to a negative p53 staining pattern such as the deletion of both p53 alleles, or to premature truncated protein products because of nonsense mutations occurring, resulting in loss of antibody recognition sites and hence a negative p53 result.

Molecular biological techniques to sequence the gene are needed to more clearly define the genetic status of p53 in the tumour cells. Immunohistochemistry or other in-situ techniques can potentially detect small numbers of mutated cells in a tissue, whereas molecular biology techniques such as single stranded conformational polymorphism or direct DNA sequencing, even with refinements such as microdissection of tissue sections, may still be too insensitive to detect small copy numbers of DNA molecules of a mutated gene in an excess of wild type DNA.

4.2 The association between the detection of p53 at margins of resection and within the oral carcinoma.

The present data interestingly showed a strong association between p53 positivity in resection margins and in the tumour itself (p=0.004). Thirteen patients out of thirty (43%) had p53 positive tumours and ten patients of the same group (33%) had p53 positive margins. Since the detection of p53 protein is considered to be synonymous with mutation because the mutant form has a half life of up to 6 hours (Lane & Benchimol, 1990) and the wild type p53 protein is not expected to be identified immunologically in the nucleus of normal cells as its level is so low (Iggo et al., 1990)
the immunodetection of p53 protein has the potential to be used as a marker of malignancy, and allow the diagnosis of disease to be made at an earlier stage. Ogden et al. (1992) agrees that the detection of p53 positive cells may prove to be a useful marker in oral cancer screening since they can be identified in smears taken from positive tumours. Observations made during the present study support this concept. However the cells with positive p53 immunostaining tended to be located at the deep invasive margin of the invading tumour, rather than near its oral surface (see figure 4.1). This feature was also found by Ogden et al. (1992) where not all the neoplastic cells were found to be positive for p53 and the majority only positive at the advancing edge (or periphery) of the malignant cells. The p53 negative cell in the tumour could be in a resting state with little p53 protein expressed (Girod et al., 1993). It may be possible for large tumours therefore to give a false negative p53 staining pattern to sampling deficiencies in the cells obtained in an oral smear. Oral smears resulting in the positive immunodetection of p53 may be more frequent in the earlier stages of tumour progression because p53 positive cells would be positioned more superficially making them more readily accessible by exfoliative cytology.

4.3 p53 as a possible marker of malignancy.

It appears that most neoplasms constitute a heterogeneous population of cells. It is possible that the p53 positive cells in the advancing margins in some tumours and in selected regions of others, may represent cells which perhaps have a higher tendency to invade or are 'active cells'. Byrne et al. (1989), showed that the cells from the deeply invasive parts of the tumour have morphological appearances in common with those of the metastases. They perhaps mirror the true nature of the tumour better than do the cells situated elsewhere. Perhaps p53 positivity identified in this region of a tumour may signify greater intrinsic malignancy. Although this study found no statistical correlation between the Bryne index of histological grading and the presence of p53 in the tumour,
Figure 4.1  Positive immunohistochemical staining at the deep invasive margin of the invading tumour (stained with Pab 1801) (100x).
further research using a larger cohort of patients is needed to clarify this possibility. Hall et al. (1991) states that mutant p53 is a useful tumour-specific marker when used in diagnostic cytopathology from diverse sites and data indicate that the presence of p53 immunoreactivity can be used to infer that a cell is neoplastic. Although the absence of p53 immunoreactivity does not exclude neoplasia, approximately 50% - 65% of oral cancers may not express p53, emphasising the need for additional tumour markers besides p53 (Ogden et al., 1992) (Warnakulasuriya et al., 1992).

4.4 Dysplasia and p53 protein detected at the margin.

Much but not all of the literature on p53 claims that p53 mutation in oral malignancy may occur early in the multistep process of malignant transformation and well before the malignant phenotype is overt (Warnakulasuriya et al., 1992). This is supported by the evidence of the present investigation of the frequency of p53 immunopositivity in the progenitor compartment of dysplastic epithelial margins adjacent to the primary tumour (section 3.3, figure 3.4). Although a statistically significant association was not found between the two features due to small patient numbers, they did appear to be related. Wood et al. (1994) also noted a parallel increase in the number of p53 positive cells and the degree of dysplasia that was observed in oral leukoplakia. They concluded that if the severity of dysplasia is accepted as a reflection of transition from oral leukoplakia to cancer, the accumulation of p53 protein is seen as an early event in the oncogenic process (see figure 4.2).

It is generally believed that slight degrees of epithelial dysplasia do not carry a significantly increased risk of malignant transformation. Perhaps using a larger cohort, a statistical association between p53 detection and moderate or severe dysplasia (rather than all grades) might have become evident. Although it is accepted that the degree of dysplasia is linked to the probability of
Figure 4.2  p53 immunopositivity in the progenitor compartment of dysplastic epithelium at the resection margin (stained with Pab DO-7) (200x).
development of malignancy (WHO, 1978), dysplasia is unreliable as the only diagnostic indicator in predicting cancer development (Langdon et al., 1992). The immunodetection of p53 protein may hold therefore as a potential biomarker in the prediction of carcinomatous change.

4.5 The detection of p53 protein in the tumour and relationship with tumour grade.

The immunohistochemical expression of p53 positivity in the tumour in this study was shown not to correlate with tumour grade (section 3.5, table 3.8). Ogden et al. (1992) also found p53 expression not to be associated with any particular state of differentiation of the tumour. Girod et al. (1993) found that p53 positivity (reflective of mutation) is related to increasing dysplasia and loss of differentiation in carcinogenesis of the oral mucosa, but no correlation could be found between the number of positive cells and the grade of the lesions. Most data on oral, head and neck, oesophageal and laryngeal squamous cell carcinoma have shown no positive relationship between p53 expression and histological grading of tumours (Field et al., 1991) (Shintani et al., 1995). Other studies have demonstrated a positive correlation between p53 expression and high grades of malignancy (Langdon & Partridge, 1992) (Nishioka et al., 1993). In the present investigation the better differentiated carcinomas tended to form nests of cells with central keratinisation and peripheral 'basal like' cells at the periphery. p53 positive cells were observed predominantly in the periphery of these nests and not in the central areas where the cells are well differentiated (see figures 4.3 and 4.4). This was also noted by Gusterson et al. (1991). The relationship between p53 expression and TNM staging varies for different studies, with some showing increases in p53 positivity with T3 and T4 tumours (Nylander et al., 1995) and others an increase in p53 positivity with T1 and not T4 tumours (Nishioka et al., 1993). Though the histopathological grading of tumours including oral squamous cell carcinoma has been
Figure 4.3  p53 positive cells are predominantly at the periphery of the nest of cells (stained with Pab DO-7) (100x).

Figure 4.4  Nests of cells with central keratinisation and 'basal like' cells at the periphery (stained with Pab DO-7) (200x).
Discussion

4.6 p53 protein presentation in the tumour and the Bryne index.

Bryne et al. (1989) noted that cancers contained diverse cell populations with differing characteristics, some cells having the ability to metastasize and thus determine the biological characteristics of the tumour. It is still not possible to identify these metastatic cells although it is believed that the poorly differentiated cells demonstrate a higher probability to metastasize than highly differentiated cells. Histologically grading the least differentiated areas of the most invasive sites in the thirty primary oral squamous cell carcinomas were not always immunoreactive for p53 protein (section 3.5, figure 3.8). This was also found by Cox (1993). A possible reason why the present study showed no statistical correlation is that the sample size of patients used was not large enough or p53 protein is involved primarily in the earlier stages of carcinogenesis.

4.7 The immunohistochemical detection of p53 protein and site of the tumour.

The immunohistochemical detection of p53 was shown not to be associated with the site of the primary tumour. In the literature, discrepancies of the immunoreactivity for p53 was found, with the highest percentage positivity being in the oropharynx and oral cavity, and then decreasing in the regions of the larynx and hypopharynx (Nylander et al., 1995). When site was assessed by Ogden et al. (1992), no p53 positive tumours
were found in the buccal mucosa region. Field et al. (1991) also found no correlation with site. Koch et al. (1994) illustrated a schematic representation of molecular analysis of p53 mutations in two synchronous tumours of the oral cavity. A portion of the p53 gene was amplified, cloned and probed with a mutant - specific oligomer. A different missense point mutation was identified in the p53 gene in each of the two invasive carcinomas and a recurrent carcinoma in one site had the identical mutation seen in the original tumour. The potential for identifying powerful specificity and individuality using molecular techniques can lead to precise identifications of site of tumour. It may point to the tumour of origin when a positive lymph node is identified, particularly when there are multiple cancers in the one patient. As more and more mutations are identified, it may be possible to identify which OSCC’s have a more aggressive phenotype and allow greater predictability.

4.8 The Immunohistochemical detection of p53 and prognosis.

This study did show that patients who had a higher percentage of cells in their OSCC positively stained for p53 protein tended to have a worse prognosis. Although this did not quite reach statistical significance (p=0.10), a larger study may prove a definite correlation. Smaller studies (less than 200 cases) tend to find that p53 is of no statistically significant prognostic value, emphasising the need for larger study groups with higher statistical power (Dowell & Hall, 1995).
4.8.1 p53 immunodetection in the tumour and survival.

Since p53 overexpression is reflective of a genetic mutation, the change would be expected to be irreversible. In this study the tendency to find a relationship between p53 protein expression in the tumour and the survival of the patient supports the notion of the use of p53 overexpression as a prognostic marker for oral cancer (Regezi et al. 1995). There is a sufficiently large corpus of evidence to incriminate p53 in neoplasia, such as its presence in a high proportion of human neoplasms and good biological reasons for considering abnormalities of p53 as being indicative of a poor prognosis in neoplasia and to justify further study of p53 expression as such a marker.

4.8.2 p53 immunodetection in other human tumours and survival.

Large patient populations do provide interesting insights into the possible biological and clinical significance of p53 abnormalities and several authors have found a close correlation between p53 expression and survival time. Visakarpi et al. (1992) found that the accumulation of p53 positivity confers a proliferative advantage for prostatic cancer cells and defines a small subgroup of highly malignant adenocarcinomas. Patients with tumours showing strong p53 immunostaining had a significantly worse prognosis than those with p53 negative tumours (see figure 4.5) and that a high level of immunostaining was one of the powerful prognostic factors in prostatic cancer indicating a 12 fold elevated risk for death.

Shimaya et al. (1993) found that p53 expression in oesophageal SCC was correlated with a worse disease outcome in patients with positive node involvement and higher p53 expression in the primary tumour. Field et al. (1993) also demonstrated a positive correlation between p53 overexpression and a poor prognosis in patients at end-stage
Figure 4.5  Primary oral squamous cell carcinoma demonstrating negative immunostaining for p53 protein (stained with Pab DO-7) (100x).
While there is a clear weight of evidence in favour of p53 being a prognostic factor, not all studies agree. Dolcetti et al. (1992) report no differences in p53 positivity between tumours from patients with early relapse and those who had been disease-free for more than 5 years. p53 expression was also not found to be of clear prognostic value in a series of human breast cancer (Ostrowski et al., 1991).

**4.8.3 p53 as a tumour marker.**

When experimental mice lose p53 gene function, progression to the malignant state is greatly enhanced. However, a number of authors suggest, that an additional tumour marker is required since 50% of oral cancers may not express p53 (Ogden et al., 1992). In addition, some p53 negative tumours were found with a high S-phase fraction leading to the conclusion that p53 mutation was not the only mechanism by which prostatic carcinoma cells may acquire proliferative advantage.

The desire for a tumour marker in oral cancer as a diagnostic tool and indicator to monitor progress of disease or evaluate treatment effectiveness is well discussed in the literature, but many of the findings imply that no single genetic marker has been found, even though the exhibition of p53 overexpression in dysplasias and microinvasive OSCC’s raises the possibility that it may be of diagnostic value in oral cancer.

The usefulness of several tumour markers for diagnosis, monitoring and treatment evaluation could offer more of an advantage. Kurokawa et al. (1993) showed that a combination assay of three tumour markers proved to be more useful than those obtained with individual markers although this approach is more time consuming and expensive. The available data on p53 suggest that it may have a relatively small prognostic effect,
since the size of the effect is only apparent in studies of large sample size (Dowell & Hall, 1995). p53 is a weak prognostic factor which alone cannot identify a group with such a low risk of recurrence that treatment would not be indicated. More knowledge is needed about p53 mutations, their interactive and influential effects in cellular mechanisms before its true significance in prognosis can be determined.

4.9 Interpretation of the immunohistochemical expression of p53.

Important issues have been raised concerning the immunohistochemical assessment of the p53 protein. Mutation of the p53 gene is thought to stabilise and be synonymous with p53 protein detection in tumours but some evidence shows that this is not always the case. A study by Melhem et al. (1995), investigated the assessment of sensitivity and specificity of immunohistochemical staining of p53 in 17 head and neck cancers. p53 mutations were shown by sequencing to exist in 7 of them (41%) , but this only correlated with positive immunostaining in 3 cases and negative in the remaining 4 cases. Seven other head and neck tumours were positive when immunohistochemically stained despite no detectable mutation in exon 5-8. Mutations may not be detected by DNA sequencing of course for the reasons discussed above.

4.9.1 Variability in the immunodetection of p53 protein.

The immunohistochemical detection of an antigen is known to be influenced by many variables such as the absolute level of antigen, affinity of the antibody, concentration of the antibody, duration of incubations and sensitivity of the detection system, as well as the consequences that fixation methods may have. These factors may have influenced
the present results in regards to the minimal positivity of p53 protein detected in the tumours of smokers. Possible explanations of 'false negative' staining may be due to the presence of nonsense and frameshift mutations which result in deletion or truncation of the protein. Tumours with high proportions of non-missense mutations are less likely to be detected with immunohistochemical staining (Hall & Lane, 1994), and have been found in some p53 negative head and neck squamous cell carcinomas.

4.9.2 Negative immunodetection of p53 in OSCC.

A lack of p53 protein expression does not mean that the p53 gene is not mutated. Mutations involving the non-coding region of the gene may affect p53 transcription or translation (Burns et al., 1993). Scarpa et al. (1992) supports this view adding that the lack of p53 protein accumulation may reflect a specific effect of the particular mutation failing to stabilise the protein. Tumours with negative p53 expression may have lost both alleles for the p53 gene or contain a mutant p53 protein that cannot be detected by the antibodies used (Field et al., 1991). Other explanations may have involved the inactivation of p53 by other mechanisms. HPV types 16 and 18 are known to bind and degrade the p53 protein (Scheffner, 1990). The possible underestimation of actual p53 mutations and that seen by immunohistochemical detection should therefore be taken into account when examining the data. Sequencing the p53 gene following PCR may be more specific however even this method also has some limitations.
4.9.3 Excessive or false p53 positivity.

The present results did not reflect an excessive amount of positive p53 staining but reflected a pattern of staining in accordance with the literature for the oral cavity. It is possible for false positive staining to occur using immunohistochemical methods. Explanations that may account for this include the possibility that the formation of stable molecular complexes of p53 with a variety of viral proteins e.g. SV40 large T antigen, adenovirus E1B, or with peculiar host proteins such as heat shock (hsp 70) or MDM2 proteins (Scarpa et al., 1993). It is thought that the viral products may alter the ability of cells to process and breakdown the p53 at a normal rate. Others speculate that defects in p53 responsive cell cycle regulators leading to perpetual cell division are not inactivated by p53 produced at a normal level and the cell attempts to compensate by overexpression (Melham et al., 1995).

4.9.4 Complexities in interpretation of p53 positivity.

There are many complexities associated with the interpretation of p53 protein in immunohistochemistry (IHC). IHC does not appear to be a very specific method for detecting p53 mutations but it is a relatively rapid and simple method for the detection of abnormal p53 even in small numbers of cells in a tissue. Reported discrepancies of p53 positivity can be minimised by standardising antigen retrieval techniques, methods of quantitation and definitions of what represents a positive tumour. The clinical relevance of p53 detection immunohistochemically is already being claimed as an excellent biomarker for determining the predisposition of premalignant lesions (Raybaud-Diogene et al., 1996) and may continue to prove to be of real value in tumour pathology as more discoveries are brought to light.
4.10 Comparison of antigen retrieval using the microwave technique.

A concern in immunopathology is choosing the correct fixative and duration of fixation that will provide maximal preservation of tissue morphology with minimal loss of antigenicity. Retrieval of masked epitopes could significantly expand the range of antibodies that are useful in immunohistochemistry as well as reduce the incidence of false negative staining in over-fixed tissues. The frequency of detecting immunostained cells in tissues fixed in formalin with antibodies to p53 is significantly reduced compared to that of frozen tissue (Pavelic et al., 1993). Improving immunohistochemical procedures could provide greater diagnostic accuracy which is the intention of microwave oven heating of tissue sections in the presence of metal solutions.

4.10.1 Mechanism of antigen retrieval using the microwave technique.

Dramatic effects in enhancing antigen retrieval by this treatment have been claimed when testing for the recovery of many antigens in formalin-fixed paraffin-embedded sections (Shi et al., 1991). The mechanisms by which microwaves could recover antigen recognition is not clear, but it is possible that the cross-linking of proteins at the tertiary or quaternary structural level caused by formaldehyde may be altered by microwave heating. The heating of tissue sections in excess of 100°C by high energy microwaves breaks the crosslinkages thereby unmasking these epitopes. The use of heavy metal salts in the study by Shi et al. (1991) was based on the hypothesis that heavy metal salts act as protein precipitants forming insoluble complexes with polypeptides and improves the immunoreactivity above that achievable with treatment consisting of distilled water and heat. Gerdes et al. (1993) described similar findings but using a 0.01M citrate buffer
solution pH 6.0.

4.10.2 Enhancement of p53 retrieval using the microwave technique.

In the current investigation, ten of the thirty patients with primary OSCC were randomly chosen and sections immunohistochemically stained using the microwave method and then compared to the same sections stained for p53 protein at room temperature. Comparisons were made in regards to the intensity of staining, the numbers of cells stained in the tumour and at the margin. No statistical significance was found between the numbers of cells found positive for p53 at the margin using either method, although the cells that stained positive appeared to have much darker staining nuclei using the microwave method (see figures 4.6 and 4.7). A statistically significant difference was found between the methods when assessing for the staining intensity and numbers of cells that were positive for p53 protein within the tumour (p=0.04).

The microwave technique certainly enhanced p53 antigen retrieval significantly more than the conventional technique but no new tumors which were previously scored negative were converted to positive status by microwaving.

Other reports using the microwave method to retrieve p53 protein in head and neck tumours have demonstrated much success with a rise from 12% of tumour tissues staining positive for p53 when treated conventionally to 42% of tumours using the microwave technique (Pavelic et al., 1993).

Though there is an inability to stain formalin-fixed paraffin-embedded tissues reliably, the microwave method for immunohistochemical staining for p53 protein did not reveal any more useful information than that which was obtained by the conventional technique.
Discussion

**Figure 4.6** Immunohistochemically stained resection margin epithelium using the conventional technique (Pab DO-7) (100x).

**Figure 4.7** Immunohistochemically stained resection margin epithelium using the microwave technique (PabDO-7) (100x).
4.10.3 **Significance of microwave technique findings.**

The significance of more deeply staining cells in a tumour have not been definitively shown to be of any clinical significance compared with a weakly staining tumour. The numbers of positively reacting cells within a tumour may be more important than their intensity of staining (Hall & Lane, 1994). The microwave method tended to increase the number of cells within the tumour which appeared positive, but the present study examined only a small sample and would need to be explained to elucidate the findings. Increasing the intensity of stain using the microwave method improves visibility and detection of p53 protein, but controversy over the different antigen techniques has arisen, because of markedly altered detection thresholds due to different sensitivities. This has lead to confusion and caution in the interpretation of the significance of positivity in tumours.

4.11 **Assessment of other factors in prognosis.**

The prognosis of patients with OSCC depends considerably on the clinical staging, on the site and histology of the tumour. Clinical staging represents only part of the picture for any tumour and further investigations should be undertaken to link the clinical features with the histological parameters, indicators of biological activity of the tumour and other factors such as the patients nutritional and general health status. Studies on the mutations of the p53 gene emphasise their importance because of their high prevalence among human cancers. It is thought that they are an indication of malignant transformation and the possibility of tumour recurrence. Other features such as tumour depth and the Bryne index have been examined in this study in an attempt to find any significant correlation to improve predictability in OSCC behaviour.
4.11.1 Effect of intra-oral site, tumour behaviour and patient survival.

No correlations were found between the site of the primary tumour and the presence of distant metastases in any of the thirty patients. Carcinomas arising at the tongue and retromolar sites did show an increased rate of local recurrence independent of the size of the primary tumour at presentation (p<0.01). Difficulty in complete excision due to anatomical factors such as proximity to vital structures or limited access may explain this higher recurrence rate of the tumour and possible field changes in the region of the primary tumour giving rise perhaps to a second primary making it difficult to distinguish clinically. Advances in molecular biology enabling local recurrences to be distinguished from a second primary tumour by DNA sequencing (Koch et al., 1994) should be useful in this context.

There was no correlation found between the site of the tumour and survival even though 7 of 30 patients died with in 2 years of diagnosis. A larger study is needed to clearly determine the absence or presence of any correlation. It is interesting to note that ten of the thirty patients who presented with OSCC also had other malignant tumours affecting other organ systems. Five year survival rates have been reported to be better than 90% for lip cancer and under 40% for tongue and floor of mouth cancer (Partridge & Langdon, 1995).

4.11.2 Clinical staging, histological grade and prognosis.

The Bryne index score as a measure of histological grade for patients with oral SCC in this study did have a tendency to negatively correlate with survival, though not reaching statistical significance (p=0.08), the Bryne malignancy grading almost seemed to have
predictive value for assessing survival. In addition to this, the Bryne index had a strong correlation with both primary tumour depth (p=0.02) and lymph node metastases (p=0.04). With increasing tumour depth, increased Bryne scores were registered suggesting an increase in malignancy of the tumour as enlargement takes place. The Bryne index score did tend to increase as the clinical tumour (T stage) size increased although no statistical correlation was found. It is therefore possible that the longer a tumour remains untreated, the more poorly differentiated it may become, due possibly to the predominance of more rapidly proliferating intrinsically more malignant subpopulations of cells or due to further mutations conferring growth advantages. Alternatively rapidly growing tumours are larger by the time the patient becomes aware of them. Growth velocity assessed clinically is an independent prognostic factor for OSCC (Evans et al. 1982).

Patients with one or two nodes also showed a significantly higher Bryne score than those with no nodal involvement. It is therefore possible that grading the histologically most invasive areas of the tumours according to the Bryne malignancy grading system enables the prediction of the presence of lymph node involvement and when considered in conjunction with the tumour depth may be of even stronger predictive value in malignancy.

**4.11.3 Tumour depth, lymph node status, staging survival.**

This study showed that primary tumour depth had a definite correlation with lymph node status. Tumours with a depth up to 2 mm had no nodal involvement (IQ range of 1.7 - 3.8 mm), tumour depths up to 5 mm (IQ range 3.4 to 6.7 mm) had one node involved and a depth of 6.7 mm (IQ range 5.5 to 13.0 mm) had two nodes involved. This is consistent with data from other studies (Spiro et al., 1986) (Mohit-Tabatabai et al., 1986). Tumour depth unlike the Bryne index score did not have an association with survival. This
contrasts with the results found by Spiro et al. (1986) where survival rate was most significantly influenced by tumour thickness. In fact they believe that survival is more dependant on the tumour depth than on clinical staging alone which provides insufficient information to base a decision for elective neck treatment. From this study while the tumour depth gives information regarding the lymph node status of the patient, the Bryne score is more likely to provide insight regarding the survival of the patient even though the Bryne index and tumour depth show a high correlation with each other. Unlike the Bryne index, tumour depth cannot usually be assessed from a biopsy and therefore is less useful for planning treatment.

An increasing tumour depth did not quite correlate with the clinical staging (TNM) of the tumour ($p=0.1$) although there was a tendency towards a higher stage as the tumour depth increased. Using a multivariate regression analysis between the Bryne score, tumour depth and STNMP staging, only the Bryne index was seen to remain as an independant predictor of stage and that the tumour depth adjusted for the Bryne index is not statistically significant in association with the STNMP. Therefore the tumour depth furnished no more predictive information than available from the Bryne score and the Bryne index score when considered alone although valuable in conjunction with the STNMP, does not clearly distinguish between patients in different staging groups.

4.12 The detection of mutant p53 protein in clinically normal mucosa.

p53 protein was occasionally expressed in clinically normal oral mucosa and tended to be associated with a smoking habit ($p=0.12$), although this did not reach statistical significance possibly because of a small sample of patients. The p53 expression was in the form of single or isolated clusters of cells in the basal and suprabasal regions of the epithelium (see figure 4.8). This contrasts with the p53 positivity within the normal epithelium from the marginal tissue adjacent to the primary tumour in smokers. In any
Figure 4.8  p53 protein immunohistochemically detected in clinically normal mucosa (stained with Pab1801) (200x).
case, if p53 positivity immunohistochemical detection reflects a p53 gene mutation, the tendency to find positive cells in clinically normal mucosa of those who smoke is also consistent with the observations made by Burns et al. (1993). They go further to suggest that benzo-(a)-pyrene and nitrosamines which are the most abundant classes of carcinogens found in cigarette smoke were likely to cause p53 mutations predominantly G→A transitions or G→T transversions in squamous cell carcinomas of the upper aerodigestive tract.

The fact that p53 positivity is found in normal oral mucosa may suggest that the mutation of the p53 gene is possibly an early event in the development of oral carcinoma. Much of the published work agrees. However, much doubt persists as to the interpretation of the immunohistochemical technique in the detection of mutant p53 protein. It is generally accepted that many different p53 gene mutations give rise to mutant p53 proteins with prolonged half lives, the nuclear accumulation of which is immunohistochemically detectable (Bodner et al., 1992), but Cooper et al. (1993) found that basal cells in cervical epithelium showing the wart virus only contains increased p53 protein, representing further evidence that positive staining does not equate with p53 mutation.

Workers in Spain have also demonstrated p53 expression in occasional non-malignant cells in five out of fourteen negative lymph nodes or tonsils (Villuendas et al., 1992). A plausible explanation by Milner (1991) is the conformation model for p53 functioning and proposes that wt p53 is induced to change from suppressor to promoter form during cell growth response and that p53 is required in the nucleus early in the normal growth response, producing a positive promoter function for cell proliferation. According to this, positivity may represent either wild type promoter form or mutant p53 protein both sharing the same conformation. It is also possible as found by Scarpa et al. (1992) that mutations on the p53 gene were present in sites other than those analysed such as intronic sequences altering donor-acceptor splicing sites however mutations outside exons 5-8 are exceedingly rare (Hollstein et al. 1991). The fact that p53 positivity was found in clinically normal mucosa in some areas and not others requires explanation. Increased p53 protein expression may also be the cell response to DNA damage or nucleotide scarcity, halting the cell cycle in G1 until DNA repair can be
4.12.1 Immunodetection of p53 in the basal region of clinically normal epithelium.

Whether or not a true genetic mutation exists would require confirmation using sequencing. The proliferative organisation in oral epithelium from the early concept that the basal layer of cells were capable of division has changed. It is now known that not all basal cells can divide and that a number are already differentiating, and that there is a number of proliferative subpopulations of cells capable of division (Hume, 1991). The basal cells are organised in order of increasing maturity (decreasing proliferative potential and activity) with more superficial cell position (Hume & Potten, 1976). This could explain why p53 protein is not found in the more superficial layers of normal epithelium but rather in the basal and supra basal regions (transiently amplifying zone) (see figure 4.9). Wild type p53 is not normally detectable in cells due to its short half life, but due to other defects in the p53 responsive cell cycle regulators, proteins leading to perpetual cell division are not inactivated by p53 produced at a normal level and the cell attempts to compensate by overexpression (Melham et al., 1995).

4.13 Reproducibility and accuracy of this study.

The accuracy of this study has been confirmed using ten randomly selected sections which were re-examined with concealed identities. The data obtained was compared to that originally retrieved and was found to be compatible with low subjective error. The detection and interpretation of p53 positivity was therefore consistent throughout the study. Accuracy of the staining technique was consistently assessed using both positive (SW480) and negative (SAOS) control cell lines with each batch of slides, as well as having negative tissue controls for each section which were placed on the same slide.
Figure 4.9  p53 protein immunohistochemically detected in the basal and suprabasal regions of normal oral epithelium (stained with Pab1801) (200x).
SUMMARY

In this study various parameters have been investigated for their predictive value in oral squamous cell carcinoma. It is clear from the literature that other parameters need to be included in the assessment of patients. Mutations in the p53 gene are the commonest genetic errors so far identified in human malignancies including OSCC. Immunohistochemical detection of mutant p53 protein at the resection margin and in the tumour from 30 patients have been investigated showing the possibility of some value. Other prognostic factors were also considered and the conclusions are as follows:-

1. p53 immunostaining highlighted the detection of dysplastic areas of marginal epithelium where immunodetection of p53 protein occured in greater proportion than in the adjacent normal mucosa.

2. The presence of positive p53 protein at the resection margin may point to possible early genetic changes in keratinocytes within the epithelium, and the likelihood of an adjacent p53 positive tumour.

3. The presence of p53 at the resection margin or in the primary tumour was not associated with greater tumour recurrence at either local or distant sites.
4. Patients who had a higher percentage of cells in their OSCC positively stained for p53 protein tended to have a worse prognosis. Patients with p53 negative tumours tended to survive longer.

5. Patients with a history of tobacco and alcohol abuse had negative immunostaining for the p53 protein at the resection margin and in the tumour. This may result from the carcinogens in tobacco possibly inducing mutations involving both p53 alleles. Confirmation by molecular techniques is needed to confirm this.

6. The detection of mutant p53 protein in oral smears by immunostaining is more likely to be of value in the early stages of carcinogenesis since p53 positive cells were observed at the periphery of the tumour and deep invasive margins.

7. The immunohistological detection of p53 protein at the resection margins and in the primary tumour did not appear to be associated with any particular intra-oral site.

8. The immunohistochemical detection of p53 protein in the tumour had no association with the histological grade of the tumour or the Bryne index score.

9. There appeared to be a tendency for the malignancy grading (Bryne index score) to increase with the dimensions of the tumour at resection.

10. Higher Bryne scores were significantly associated with higher risk of metastases.
11. Increasing tumour depth is significantly associated with the presence of nodal metastases. Tumours up to 2mm (IQ range 1.7-3.8mm) had no nodal involvement, depths up to 5mm (IQ range 3.4-6.7mm) had one node involved and a depth of 6.7mm (IQ range 5.5-13 mm) had two nodes involved.

12. The Bryne index score increased as the tumour depth increased implying that the longer a tumour remains untreated the more poorly differentiated it may become.

13. There was no association between the primary tumour depth and the stage of the tumour.

14. Only the Bryne index score when compared with the tumour depth in a multivariate analysis remained an independent predictor of STNMP stage.

15. The tumour depth did not significantly affect patient survival, however a lower survival tended to be in those with a higher Bryne score.

16. No correlations were found between the intra-oral site of the primary tumour and survival.

17. A high association was found between local but not distant tumour recurrence and origin of the tumour on the tongue and retromolar trigone sites.
18. p53 tended to be immunohistochemically detected in clinically normal oral mucosa from subjects who tobacco smoke than in non-smokers.

19. The microwave technique for antigen retrieval showed more staining intensity and number of p53 positive cells in the primary tumour, but not the resection margins, than the conventionally treated sections.

20. Good reproducibility and low subjective error of the assessment of p53 positivity was shown by the evaluation of its variability in two assessments.
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