Tumor progression: Role of apoptosis, its regulation, angiogenesis and proliferation during pathobiological progression.
PART IV
Tumor progression: Role of p53 protein, influence of apoptosis, its regulation, angiogenesis and proliferation during pathological progression.

INTRODUCTION
The process of pathological progression involves transformation of normal cells to pre-malignant cells by means of multiple accumulations of mutations, which then further progresses on to malignant cells. These stages show gross visible changes in phenotypic appearance observable under microscope following more or less a uniform pattern. There are very few reports regarding the nature of biological changes, specifically on apoptosis regulation and angiogenesis during the progression of oral cancer. It is unlikely that molecular changes will also show similar uniformity as that of phenotypic changes, because the tumors with identical phenotype show wide difference in biological behaviour. This is further evident since that phenotypic appearance is often of little value in predicting biological behaviour, such as treatment response, recurrence of disease or metastasis (Ensley et.al., 1987). Further in a given a tumor sample, analysis may show mixed phenotype i.e., different portions of the tissue might show different phenotypic appearances. Therefore it becomes difficult in applying phenotype as a potential prognostic parameter.

The biological behaviour of the tumor is often a reflection of molecular changes. Thus it is imperative to analyze for such molecular changes in order to understand
as to how the tumor behaves. Such a study is applicable in terms of oral cancer because of the involvement of multiple etiological factors involved in oral oncogenesis. Owing to high frequency of mutations detected in p53 gene, various chemical (ex. carcinogens present in tobacco) and biological agents in the oral cavity appear to converge on a common cellular target, namely the p53 tumor suppressor gene (Field et al., 1991). As discussed in the introductory part the p53 protein, play a major role in regulation of apoptosis, proliferation and angiogenesis. However the regulatory influences may or may not be synchronous, which could probably be responsible for the variations observed in tumor behaviour, an aspect that has not been described in any previous studies.

Hence this study was designed to analyze p53 protein, de novo apoptosis, apoptosis regulatory proteins such as bax and bcl-2, proliferative potential and neo-angiogenesis during disease progression from normal to invasive cancer. A total of 137 cases were included in the study, (87 malignant lesions (invasive cancer), 38 premalignant lesions (dysplastic and hyperplastic lesions) and 12 normal buccal mucosa).
RESULTS

Apoptosis

Apoptotic cells detected by TUNEL were visualized as cells with dark brown bodies in the nucleus.

Figure 7 (Magnification X 45x Apoptotic cell shown indicated by arrow head).

Negative controls run in the TUNEL assay showed no such reactivity. Normal oral mucosa showed insignificant apoptosis (Class 1 TUNEL reaction). Eighteen of the 22 hyperplastic oral mucosa samples had moderate to intense levels of TUNEL reactive nuclei (Class 3 and 4) while the remaining 4 had insignificant or mild AIs (Class 1 and 2). Of the 16 dysplastic epithelium samples, 14 showed moderate AI levels (Class 2 TUNEL reaction), 1 showed Class 3 reaction and 1 showed Class 4 reaction. The extent of apoptosis was generally low in invasive carcinomas. Of the 37 well differentiated tumors, 18 showed TUNEL reactivity and the remaining 15 samples had insignificant TUNEL reactions. Among the 26 moderately differentiated tumors, 17 showed evidence TUNEL reactivity and the remaining 9 had Class 1. The picture was similar in the 24 cases of poorly differentiated tumors where 12 samples had insignificant TUNEL reaction and 10 had Class 2. However, one sample each had Class 3 and Class 4 TUNEL reactions. (Plate 6)
Table 1. Histopathological distribution of patients in various grades of TUNEL reactivity.

<table>
<thead>
<tr>
<th>TUNEL</th>
<th>Normal (n=12)</th>
<th>Hyperplasia (n=22)</th>
<th>Dysplasia (n=16)</th>
<th>WDSCC (n=37)</th>
<th>MDSCC (n=26)</th>
<th>PDSCC (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>15</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>Mild</td>
<td>0</td>
<td>3</td>
<td>14</td>
<td>18</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>Moderate</td>
<td>0</td>
<td>12</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Intense</td>
<td>0</td>
<td>6</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**p53**

p53 expression was apparent from clear nuclear immunoreactivity in tumor cells as shown in the figure below.

Figure 8. (Magnification X 45x).

Expression of p53 was limited to tumor and dysplastic tissue and was absent in normal and hyperplastic epithelium except for one sample (Plate 7). These results
are summarized in Table 2.

Presence of mutant p53 was detected by ELISA showed positivity in 2 of the 16 dysplastic lesions, 24 of the 37 WDSCC, 19 of the 26 MDSCC and 12 of the 24 PDSCC. No mutant p53 could be detected in normal and hyperplastic epithelium.

Table 2. Histopathological distribution of patients under various grades of p53 expression.

<table>
<thead>
<tr>
<th>p53</th>
<th>Normal (n=12)</th>
<th>Hyperplasia (n=22)</th>
<th>Dysplasia (n=16)</th>
<th>WDSCC (n=37)</th>
<th>MDSCC (n=26)</th>
<th>PDSCC (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>12</td>
<td>21</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Moderate</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>29</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Intense</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>15</td>
<td>14</td>
</tr>
</tbody>
</table>

**Bcl-2**

Bcl-2 protein was predominantly expressed evenly throughout the cytoplasm and in the perinuclear areas.

Figure 9. (Magnification X 45x).
As in the case of p53, bcl-2 immunoreactivity was mostly limited to dysplastic and malignant tissue with only two samples of hyperplastic epithelium significantly expressing the protein (Plate 9). Bcl-2 was absent in normal tissue.

Table 3. Histopathological distribution of patients and extent of bcl-2 expression in various grades.

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=12)</th>
<th>Hyperplasia (n=22)</th>
<th>Dysplasia (n=16)</th>
<th>WDSCC (n=37)</th>
<th>MDSCC (n=26)</th>
<th>PDSCC (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>12</td>
<td>20</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>0</td>
<td>1</td>
<td>8</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Moderate</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>30</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>Intense</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>8</td>
<td>9</td>
</tr>
</tbody>
</table>

**Bax**

The immunoreactivity for the bax protein was also cytoplasmic (Figure 10). In contrast to p53 and bcl-2, bax protein was found to be expressed in all the 22 hyperplastic tissue samples with moderate to intense expression in 21 samples.

Figure 10. (Magnification X 45 x)
All 16 dysplastic samples also expressed bax, of which the majority (12 samples) showed mild immunoreactivity (Plate 8). Similar immunoreactivity for Bax was seen in all carcinomas, with the majority of samples showing mild expression. Expression of bax protein was absent in normal oral mucosa.

Table 4. Histopathological distribution of patients and extent of bax expression in various grades.

<table>
<thead>
<tr>
<th>Bax</th>
<th>Normal (n=12)</th>
<th>Hyperplasia (n=22)</th>
<th>Dysplasia (n=16)</th>
<th>WDSCC (n=37)</th>
<th>MDSCC (n=26)</th>
<th>PDSCC (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>0</td>
<td>1</td>
<td>12</td>
<td>36</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>Moderate</td>
<td>0</td>
<td>20</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Intense</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Proliferation marker (Cyclin D1)*

The expression of the proliferation associated protein cyclin D1 was seen restricted to the nucleus or show diffuse cytoplasmic staining depending on the stage of the cell cycle. Expression was similar (moderate to intense) in all cases of invasive cancer. Of the 22 cases of hyperplasia, cyclin D1 was mildly expressed in 19 and moderately expressed in 3 cases. In dysplastic tissue 3 of the 16 cases showed mild expression while 13 had either Class 3 or 4 (moderate to intense) immunoreactivity (Plate 10). Normal oral mucosa showed negative or mild expression.
(Figure 11. Magnification 25x).

Table 5. Histopathological distribution of patients and cyclin D1 expression in various grades.

<table>
<thead>
<tr>
<th>Cyclin D1</th>
<th>Normal (n=12)</th>
<th>Hyperplasia (n=22)</th>
<th>Dysplasia (n=16)</th>
<th>WDSCC (n=37)</th>
<th>MDSCC (n=26)</th>
<th>PDSCC (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>7</td>
<td>19</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Moderate</td>
<td>0</td>
<td>3</td>
<td>12</td>
<td>21</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Intense</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>16</td>
<td>14</td>
<td>13</td>
</tr>
</tbody>
</table>
Angiogenesis

Endothelial cell marker expression was observed as vasculature with or without diffuse immunoreactivity in surrounding cells.

Figure 12. (Magnification X 45x)

The least extent of angiogenesis was seen in normal oral mucosa and hyperplastic lesions where the mean ILVD was $15 \pm 7$. Dysplastic lesions showed more conspicuous neovascularisation (mean ILVD $41 \pm 6$). All invasive tumors showed significant angiogenesis (Plate 11). Well differentiated tumors and poorly differentiated tumors have an ILVD of $89 \pm 19$ and $95 \pm 8$ respectively, while moderately differentiated tumors it was $91 \pm 20$. 
Correlation analysis for the association of biological parameters with histological progression of disease.

Apoptosis showed a mild decline during progression. Analysis showed an inverse correlation between TUNEL reactivity and histology of the lesion ($r = -0.555$, $p < 0.01$), indicating that the extent of apoptosis decreased with increasing histological abnormality. However within the various grades in invasive lesions apoptosis failed to show any significant correlation. This is also evident from the Figure 13 and plate 6 showing various grades of TUNEL reactivity in different lesions of MDSCC. There was also good correlation between histology of the lesion and immunoreactivity of p53 ($r = 0.811$, $p < 0.001$) and presence of mutant p53 protein ($r = 0.412$, $p < 0.001$), There was also correlation between histology and both bcl-2 and cyclin D1 expression ($r = 0.724$, $p < 0.01$ and $r = 0.657$, $p < 0.01$ respectively). The expression of the bax protein however showed an inverse correlation to histology ($r = -0.679$, $p < 0.05$), suggesting that bax protein expression decreased with increasing histological abnormality. Expression of angiogenesis marker CD34 was found to correlate significantly with the histological grade of the disease ($r = 0.802$, $p < 0.001$). (Results illustrated as bar chart in Figure 13 page 79)

To analyze the predictive significance of these results, all tissue samples were grouped into either controls consisting of the benign lesions (normal oral mucosa and hyperplasia) and cases comprising dysplastic and malignant lesions. Fisher's exact test analysis revealed that the odds ratio of a tissue sample having
insignificant or low apoptotic index being a case of dysplasia or malignancy was 0.09 (p = 0.004, 95% CI: 0.11, 0.66). Such an analysis in the case of the other parameters was much more significant. The odds ratio of a lesion expressing moderate to intense levels of p53 protein being a case was 176 (p < 0.001, 95% CI: 21.5, 1436.9). For bcl-2, this odds ratio was 92.8 (p < 0.001, 95% CI: 11.7, 733.9).

Figure 13. Histograms showing mean expression of p53, extent of apoptosis, mean vascular density and expression of cyclin D1 in various histopathological grades.

**p53, Apoptosis and apoptosis regulation**

Accumulation of p53 protein detected by immunocytochemistry correlated significantly to detection of mutant p53 (r = 0.654, p < 0.001). There was an inverse correlation between TUNEL reactivity and immunoreactivity of apoptosis regulatory proteins, p53 (r = -0.639, p < 0.01) and bcl-2 (r = -0.642, p < 0.001).
Bax showed positive correlation to TUNEL and bax reactivity \((r = 0.651, p < 0.001)\).
The presence of mutant p53 protein also showed an inverse correlation to the extent of apoptosis \((r = -0.301, p = 0.01)\). Significant correlation was evident between the bax / bcl-2 ratio and TUNEL \((r = 0.652, p < 0.001)\).
The bax / bcl-2 ratio was obtained by dividing the number of bax positive cells by the number of bcl-2 positive cells. Seventy nine of the 125 samples (63%) had a bax / bcl-2 ratio of 0.5 or less. Thirty seven (47%) of these cases had Als up to 1 and the remaining 42 (53%) had Als between 1.1 and 2. However, in the 46 samples which had a bax / bcl-2 ratio of 0.6 or higher, 23 (50%) had Class 2 TUNEL reactivity (mild), 14 (30%) had Class 3 (moderate) and 8 (20%) Class 4 (intense). These results illustrated in Figure 14.
The histopathology details of the cases analyzed for bax / bcl-2 relation with TUNEL has been shown in Table 6.

Table 6. Distribution patients (n=125) based on histopathology in relation to bax/bcl-2 ratio and apoptotic index in oral lesions.

<table>
<thead>
<tr>
<th>Apoptotic Index</th>
<th>Hyperplasia</th>
<th>Dysplasia</th>
<th>WDSCC</th>
<th>MDSCC</th>
<th>PDSCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class 1</td>
<td>1</td>
<td>0</td>
<td>14</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Class 2</td>
<td>0</td>
<td>2</td>
<td>17</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>Class 3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Class 4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Apoptosis, p53 and Cyclin D1 expression.**

TUNEL reactivity also had correlation to the extent of tissue proliferation reflected by the expression of cyclin D1 (r = 0.577, p < 0.001). Cyclin D1 expression also correlated to the accumulation of p53 protein (r = 0.761, p < 0.02).
**Tumor angiogenesis**

Tumor angiogenesis was detected by CD34 expression and the extent of angiogenesis was estimated as vessel counts expressed as mean vessel density. Extent of apoptosis and expression of Bax protein showed significant inverse correlation with the expression of CD34 (\( r = -0.628, p < 0.001 \) and \( r = -0.700, p < 0.001 \)). The expression of p53 protein showed direct correlation with CD34 expression (\( r = 0.825, p < 0.001 \)), bcl-2 (\( r = 0.745, p < 0.001 \)) and cyclin D1 expression (\( r = 0.73, p < 0.001 \)). (These results are expressed as scatter plots shown in figures 15-19, pages 82-84). There was a correlation between presence of mutant p53 protein and angiogenesis (\( r = 0.508, p < 0.005 \)) (Shown as scatter plot Figure 20 page 85).

![Figure 15. Scatter plot shows direct relation between expression of p53 protein and angiogenesis. It is evident from this plot that tumor angiogenesis increases with the extent of p53 protein expression.](image-url)
Figure 16. Scatter plot shows that angiogenesis increases with the extent of bcl-2 protein expression.

Figure 17. Scatter plot shows decrease in angiogenesis with an increase in the expression of bax protein.
Figure 18. Scatter plot shows an increase in angiogenesis with the extent of tumor proliferation detected as cyclin D1 expression.

Figure 19. Scatter plot shows a decrease in angiogenesis as apoptotic index increases.
Presence of mutant p53

Note: In x axis, 1.0 (negative) and 2.0 (positive) for mutant p53 protein detected by ELISA.

Figure 20. Scatter plot shows increase in angiogenesis is associated with presence of mutant p53 protein.
DISCUSSION

Considerable interest in the role of programmed cell death during tumor development has been generated following progress in understanding the genetic basis of tissue homeostasis. A critical issue therefore is whether apoptosis is defective in tumor development and if so whether the process and its genetic regulation is of clinical relevance. Further, the survival and propagation of tumorigenic cells depend on angiogenesis. How far does angiogenesis influence proliferation or apoptosis and if so will the influence of angiogenesis can override the normal regulation of apoptosis are some of the questions that need consideration.

Implication of the decreased apoptosis in invasive cancers when compared with benign oral tissue is unclear. However it can be appreciated in the next part of the study that showed wide changes from low to high levels of apoptosis analyzed in individual cases among the invasive lesions. Contradictory results have been obtained with other tumor types. In prostate tissue, the apoptotic index has been reported to increase as prostatic epithelium proceeds from benign hyperplasia through prostatic intraepithelial neoplasia into invasive cancer (Ahira, et.al 1994). Moreover, high apoptotic index was associated with poor prognostic markers such as tumor grade, mitotic index and pathologic stage (Ahira, et.al., 1995). However in contrast to this, the apoptotic index was seen to decrease with lesion severity during colo-rectal tumor progression (Bedi, et.al., 1995). In gastric carcinomas, significant correlation was evident between the extent of apoptotic index and the
depth of tumor invasion (Koshida, et.al., 1997). In breast tumors, reduced level of apoptosis in fibroadenomas when compared with infiltrating duct carcinomas has been reported. (Kesari, et.al., 1997)

Numerous genetic factors have been shown to modulate apoptosis and interactions among these factors are complex and still incompletely defined. Mutations in p53 are frequent in oral cancer and results in the production of a functionally defective p53 protein (Munirajan, et.al., 1996 and Piffko, et.al., 1998). The mutant p53 protein attains stable conformation by forming dimers or tetramers and accumulates in the cell (Levine, 1997). Alternatively, the function of normal p53 protein may be attenuated by endogenous proteins such as mdm-2 or by exogenous proteins including the E6 of high risk human papillomaviruses, allowing the protein to be targeted for degradation (Levine, 1997). Accumulation of p53 protein in the present study correlated to the extent of apoptosis. The antibody used in the present study for the detection of p53 (DO7) can detect both mutant and the wild type p53 protein. Wild p53 protein normally has a short half-life (6-20 minutes) but mutant forms have a half-life upto 6 hours. Thus detection of p53 by immunocytochemistry is often considered to reflect the mutant form (Levine, 1997 and Piffko, et.al., 1998). However our study show that accumulated p53 detected by immunocytochemistry is of mixed type, which was confirmed by doing a mutant p53 specific ELISA. Details are discussed more elaborately in the next part of the study. Moreover a previous study from the same center with a patient population very similar to the present one revealed similar rates of frequency in the p53 gene. (Henzel, et.al., 1996).
The mechanisms of p53 induced apoptosis may be in part mediated through the differential expression of bcl-2 and bax genes. Bcl-2 functions in an anti-oxidant pathway to inhibit apoptosis (Rao, et al., 1997). The protein is localized to intracellular sites of oxygen free radical generation including nuclear membranes, mitochondria and endoplasmic reticulam. The 21kD bax protein shares extensive sequence homology with the bcl-2 protein and is capable of forming homodimers or heterodimers with the latter. An increased cellular level of bax has been found to trigger apoptosis by repressing bcl-2 (Koshida, et al., 1997). Expression of bax is known to be up-regulated by p53 (Miyashita, et al., 1995). The low levels of cellular bax protein expression or invasive carcinomas reported here, in the light of simultaneous elevated mutant p53 expression, suggests that the later is unable to up-regulate bax expression and thereby fails to repress bcl-2 expression (Plate 4). Our current results show a definite correlation between the bax/bcl-2 ratio, p53 and apoptotic index. There was also a significant positive correlation between bax expression with apoptosis as well as a negative correlation between bcl-2 and apoptosis. This pattern therefore indicates that the bax to bcl-2 ratio is critical in regulating apoptosis. The patterns of changes in bax/bcl-2 ratio are shown in plate 5.

The association between increased expression of the proliferation marker cyclin D1 with both p53 protein accumulation and presence of mutant p53 protein suggests that the over expressed p53 protein interferes with normal cell cycle regulation, possibly conferring a proliferative advantage to the cancer cells in vivo. Moreover, expression of p53 was found to be absent in normal and benign (hyperplastic) oral
A. p53 immunoreactivity showing moderate nuclear immunoreactivity.

B. Intense bcl-2 expression showing cytoplasmic and perinuclear immunoreactivity.

C. Bax immunoreactivity showing diffuse cytoplasmic immunoreactivity.

Expression of p53, bcl-2 and bax in moderately dysplastic lesion.

Plate 4
Plate 5

Legend for figures shown in Plate 5.

In the Figures (a) represents bcl-2 and (b) represents bax immunoreactivity.
(Figures 1-3 Magnification X 40 x)
(Figure 4 Magnification X 25 x)

Figure 1 (a&b)  Expression of bcl-2 and bax protein are not seen in same cells in a well differentiated squamous cell carcinoma.

Figure 2 (a&b)  Intense staining of bcl-2 protein in a dysplastic lesion showing mild expression of bax protein.

Figure 3 (a&b)  Mild expression of bcl-2 protein in a moderately dysplastic lesion showing moderate bax expression.

Figure 4 (a&b)  Mild expression of bcl-2 protein in a moderately differentiated squamous cell carcinoma showing intense bax expression.
tissue. Thus, mutant p53 by failing to regulate proliferation and allowing increased bcl-2 expression may result in the down regulation of apoptosis. Srinivas et al (1998) has previously observed a similar phenomenon to occur in pediatric acute lymphoblastic leukemia. This would thus result in a shift of tissue kinetics towards the preservation of genetically aberrant cells thereby facilitating tumor progression.

**Influence of angiogenesis on apoptosis and proliferation**

The balance of angiogenic and angiostatic factors regulates Angiogenesis. Switching to an angiogenic phenotype by cancer cells is by increasing the concentration of angiogenic peptides in the local microenvironment, which in turns leads to vascularisation at the tumor site (Hanahan, et.al, 1996). Angiogenesis ensures the survival of tumor cells by adequate supply of nutrients and most importantly the growth factors needed to protect against apoptosis. The cumulative effect of vasculature, changes in cellular genetic factors, which regulate cell division and destruction determines the extent of tumor growth potential. A fine balance between the positive and negative regulators of cell growth is essential in normal development and maintenance of tissue size and shape. In neoplasia, failure to regulate tissue size homeostasis results in the development of tumor (Wyllie, 1992).

Results from the present study demonstrate an inverse correlation between the extent of apoptosis and angiogenesis. Although such finding has been reported in other tumors (Vindigni, et.al., 1997), no such data have been previously been shown in human oral cancer. Earlier reports indicate that inhibition of angiogenesis
can up-regulate apoptosis and halt the tumor growth (Holmgren, et.al., 1995). As explained earlier, the angiogenic switch is often effected by the release of growth factors in the microenvironment. Growth factor deprivation is known to induce apoptosis (Baserga, 1994). Therefore it is possible that continued effect of growth factors on tumor cells to sustain angiogenesis and simultaneously down regulate apoptosis. Tumor progression is known to accompany hypoxia. However by physiological selection, such hypoxic cells develop apoptotic resistance (Graber, et.al 1996). The apoptotic regulatory proteins p53, bcl-2 and bax were evaluated in this study to analyze the influence of angiogenesis. The apoptosis stimulatory bax protein was found to be least in highly angiogenic lesions. There was an increased expression of bcl-2 and p53 protein in some of the highly vascularised tumors. This accumulation of p53 protein was detected as mutant variety and found associated with cyclin D1 over expression. Thus vascularised tumors with high p53 expression show higher proliferative potential. A direct effect of mutant p53 protein enhancing cellular proliferation has been demonstrated in an in vitro study (Shaw et.al., 1992). It has been reported that p53 protein regulates the expression of angiogenic factors, mutation in p53 is found to downregulate the expression of TSP-1 Thrombospondin, an inhibitor of angiogenesis leading to increase in angiogenesis (Grossfeld, et.al., 1997). It is therefore evident from this study that presence of mutant p53 protein showing significant association with increase in angiogenesis. This study showing an increase in angiogenesis during tumor progression with concomitant raise in the p53 protein level indicate that primary regulation of angiogenesis might be under the influence of p53 protein in oral cancer.
In addition to mutation in p53, the increased angiogenesis also influences the shift in tissue kinetics favoring proliferation rather than apoptosis. This is clearly demonstrated that there was a decrease in apoptosis during the progression along with an increase in angiogenesis. The alteration in apoptosis regulatory protein was also found to be associated with angiogenesis. A possibility of existence of direct influence by angiogenesis on the regulatory proteins of apoptosis remains to be understood. Unless such a relation is proved it is not possible to conclude that angiogenesis play any direct role in down regulating apoptosis by modulating the expression of apoptosis regulatory proteins. It can be concluded that angiogenesis promotes proliferation, increasing the total proliferative compartment and this relation is critical function during progression. It could therefore be logically interpreted that angiogenesis and increased proliferation and altered regulation in apoptosis could be critical in the prediction of tumor response.
CONCLUSION

The histological progression of the oral cancer is associated with increased cell proliferation and decreased cell death. The increase in levels p53 protein with raise in the levels of bcl-2 protein and concomitant decrease in the levels of bax protein, suggests that failure of mutant cells to undergo apoptosis and loss of cell cycle control. Similarly increased angiogenesis during progression also suggests that p53 expression detected might reflect mutant phenotype. Analysis of p53 protein by mutant selective ELISA confirmed these results indicating that the high levels of p53 protein detected were indeed of mutant phenotype.

In summary therefore, the present study provides data for an improved understanding of possible steps in oral tumor progression. Apoptosis apparently is important in this process and seemingly is regulated by p53 and the bcl-2/bax ratio. Further p53 also appears to influence angiogenesis leading to alterations in proliferative and apoptotic regulations. p53 seemingly to play a central role in coordinating apoptosis, proliferation and angiogenesis during tumor progression in oral cancer.