Design of a Predictive Assay
PART VI: Predictive Assay

Introduction

A predictive assay is defined as a simple, sensitive and practical method capable of predicting a near accurate prognosis. The need of such predictive assays has been stressed in many studies, so as to predict possible disease outcome following treatment (Russell et.al 1992).

The ever-increasing incidence of oral cancer in many countries combined with morbidity and mortality is a cause of serious concern. Further adding to difficulty in designing and providing appropriate treatment care is that about 25-40% cases often show disease relapse and treating such cases need a different approach (Padmanaban et.al., 1990). To date there are no markers that could be used to identify patients who are at high risk of developing recurrent disease and therefore attempts on modifying treatment often fail. A commonly used method is to estimate potential doubling time based on incorporation of bromodeoxyuridine by the tumor. However, studies on oral cancer failed to show any correlation with tumor regression (Begg et.al 1990 and Corvo et.al., 1996). These studies were done with an intention that if any relation between tumor doubling and regression was found, then attempts to modify fractionation schedules could be tried. However due to lack of correlation, such attempts were abandoned. The reason for such a failure is that the tumor tissue consists of not only proliferative and apoptotic
cells but also has dormant viable cells, which are not labeled. This could explain the failure to get expected results (Potten 1996). A similar view is also applicable while evaluating apoptosis. There may be necrotic cells within the tumor, which might underestimate tumor kinetics. A study in head and neck cancers reported that low incidence of pre-treatment apoptosis showed better survival (Hotz et.al., 1999). Presence of high levels of apoptotic cells were detected in fast growing tumors, where the over growth of tumor cells leads to such an increase in apoptotic cell population. This could be a possible reason for the high levels of apoptosis in high-grade tumors. The proliferative potential determines response to treatment in such tumors. It is also difficult to determine the fractions of proliferative cells, dormant viable cells, apoptotic cells and necrotic cells in a tumor. Studies reported so far indicate that angiogenesis was alone a significant factor determines tumor relapse following treatment (Williams et.al., 1994 and Alcalde et.al 1997). But when the T stages were taken individually and analyzed for vessel density and behaviour of the tumor it failed to show any predictive value (Gleich et.al., 1996).
HYPOTHESIS

Considering the points discussed so far, this study analyzed for the behaviour of various biological factors such as angiogenesis, apoptosis and proliferation, which are considered to play a major role in both oncogenesis as well as treatment response. Based on results obtained by statistical analysis angiogenesis, apoptosis and proliferation were considered relevant for designing a predictive assay and explored for appropriateness to suit the development of a predictive assay.

Some of the factors that need to be considered include:

1. In oral cancers, some tumors grow without the need of high vascularisation. (Gleich et al., 1998).

2. Tumors have proliferative cells that proliferate and progressively displaced away from the vascular area to hypoxic zone. The displaced tumor cells in the hypoxic zone further induces angiogenesis by the release of endothelial growth factors, (Brown, et al., 1998) and modulates gene expression promoting tumor progression. (Maxwell et al., 1997). Such tumors can be considered aggressive.

3. Tumor hypoxia induces apoptosis. In general p53 protein mediates apoptosis in such tumors, such tumors might possibly respond better to treatment. (Dameron et al., 1994)
4. Tumor cells that are located near the vasculature are generally proliferative. (Brown et.al 1998). These cells are responsive to treatment, owing to high proliferative capacity and angiogenic tendency might repopulate after treatment.

5. Angiogenesis limits tumor growth, maintains proliferative cells that are displaced away from the vasculature due to accumulation of proliferative cells. As the cells get displaced away from the vascular area the growth becomes limited due to low perfusion of oxygen and essential nutrients. Under such circumstances, cells may either die or remain dormant or induce further angiogenesis (Holash et. al., 1999). Cells sensitive to hypoxic conditions might show better response during treatment provided repopulating clones are

Angiogenesis and growth limitations.
destroyed. While dormancy or angiogenic phenotype might resist therapeutic destruction.

6. Apoptosis is an important mechanism for eliminating both excess of normal cells and cells, which have genomic damage. More importantly preservation of DNA integrity in the stem cells is an essential feature of apoptosis. (Potten et.al 1997)

7. In invasive tumors, multiple colonies of tumor cells grow around vasculature, where the tumor cells are the stem cells (Brown, 1998). Presence of apoptotic cells in such area should indicate apoptosis sensitivity, while absence of apoptotic cells indicate apoptosis resistance. The natural anti-tumor response by the host mediated immunity by means of releasing cytokines. Therefore tumor cells proximal to the blood vessels should show apoptotic response.

Figures show presence of de novo apoptotic cells in the vascular area (indicated by arrow) in an untreated tumor.

The present study reports for the first time that detection of de novo apoptotic cells in the vascular area is associated with good prognosis. The figures show example of two such cases showing
de novo apoptosis and follow-up of these patients showed good response to treatment as well as disease free at the end of 36 months. Absence of such response might indicate apoptosis resistance. Our previous observations show that cells in the vascular area express bcl-2 protein to protect themselves from apoptosis (Plate 14). This raises considerable interest since such an expression of bcl-2 and escape from apoptotic signals are common in hematopoietic and endothelial cells (Suda et al., 2000). Presence of apoptotic signals and induction of apoptosis in some cells while others are resistant to apoptosis is a feature of differential regulation of apoptosis during hematopoiesis. Thus it might be possible such a mimicry by tumor cells could allow it to survive from apoptotic cell death.

8. Previous observations show that thickness of epithelium is associated with treatment outcome (Williams, et al., 1994). Concurrent observations were seen in the present study (Plate 16). A possible explanation for such result is that higher proliferative potential with low or absence of apoptotic cells accounts for thick epithelium, while higher de novo apoptosis accounts for thin epithelium. (Plate 16).

Results obtained from the previous part of the study showed vascular status and apoptosis to be relevant in predicting possible outcome of the disease.
This was based on statistical analysis, which determines the prognostic value of the markers based on an overall estimate of the results obtained from a large population (Figure 28).

![Figure 28. Line graph showing the overall trend in expression of p53 protein, angiogenesis and apoptosis with relation to follow-up status. Expression of p53 protein and apoptotic index is high in recurrent disease along with poor vascularisation.](image)

In contrast, the predictive assay is an estimate from an individual sample and therefore must be more accurate. Distinction between different types of estimates are shown in table 11.

Previous part of the study showed angiogenesis as a prognostic factor, it is evident from the scatter plot shown below that tumors showing vessel count
above 40 do not recur after treatment, while tumors showing vessel count
below 40 show mixed results showing both disease recurrence as well as

Table 11. Definition of biological and clinical cancer markers:

<table>
<thead>
<tr>
<th>Marker</th>
<th>Utility</th>
<th>Clinical value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risk</td>
<td>To assess the risk or relative probability of appearance of a tumor in a population exposed to the factor compared to general population.</td>
<td>Prevention</td>
</tr>
<tr>
<td>Diagnostic</td>
<td>To detect a new tumor or recurred tumor in the pre-clinical stage.</td>
<td>Early diagnosis</td>
</tr>
<tr>
<td>Prognostic</td>
<td>To assess the biological aggressiveness of a tumor.</td>
<td>Therapeutic strategy</td>
</tr>
<tr>
<td>Predictive</td>
<td>To predict the effectiveness of a specific therapy.</td>
<td>To plan the therapy</td>
</tr>
</tbody>
</table>

The estimated minimum period for follow-up studies to assess loco-regional control following treatment is two years for oral cancers.

(Chiesa et al., 1999)

Figure 29. Scatter plot showing relation between angiogenesis and p53 expression with disease relapse
good response (Figure 29). The objective of the predictive assay is to
determine the individual response from the later group. Despite the
regression fit line showing a decline of apoptotic activity with increase in
vasculature, by observing the scatter plot, it can be seen that tumors with
low vascularity shows wide range of apoptotic activity (Figure 30). This
factor might be responsible for possible disparity in results.

![Figure 30. Scatter plot showing relation between mean vessel density and apoptosis.](image)

The predictive assay is therefore an extension of prognostic assay, which is
more refined tailored to fit an individual sample. The present study shows
vascularity, apoptosis and proliferation status of a tissue playing a definite
role in both oncogenesis as well as treatment response. These factors are
also functionally related and therefore functional model is based on
evaluating proliferation and apoptosis around the vascular area forms the rationale for the planning this predictive assay.

**Preliminary design and investigations.**

With this objective review of sections were done to analyze for apoptosis and proliferation around vasculature.

Observations indicate that the presence of de novo apoptosis near the vasculature shows good prognosis, while absence of apoptotic cells with or without bcl-2 expression, high proliferation in the epithelial cells of the vascular area predicts poor prognosis (Plates 14-18).

Therefore we hypothesize that presence of de novo apoptotic cells around vasculature indicates apoptotic sensitivity and therefore predictive of better treatment outcome and absence of apoptotic cells around the vasculature is suggestive of resistance to apoptosis (e.g. Bcl-2 over-expression, defect in any other component of apoptotic machinery) further higher proliferative potential might undermine cell killing effect of the treatment by quickly repopulating the cells, such a repopulation is possible only if sufficient vascularity exists.
STUDY SUBJECTS AND METHODOLOGY

Therefore based on these hypothesis a study was planned with a total of 50 cases of blind coded invasive archival oral cancer samples selected at random and used for the study to evaluate the appropriateness of the hypothesis and as well as to identify criteria based on which predictions can be made.

The samples were analysed for vessels, apoptosis and proliferation based on two sets of techniques. Morphological investigation for detection of vessel, (based on criteria described by (Widner et.al., 1991 and Tipeo et.al., 1996) and apoptosis (based on criteria described by Kerr et.al., 1992) (See plate 12). (These results were supported by routine CD34 endothelial marker immunodetection and TUNEL assay. The proliferation was measured using Cyclin D1 and Ki-67 antibodies, supplemented by AgNOR (Agrophilic nuclear organizers), assay. Apoptosis and proliferation measured by various markers for validating morphological assessment. Morphological criteria were given importance in order to simplify the predictive assay and can be applied in routine practice during histological assessment of tumors by pathologists.
A. Presence of epithelial cell lining, occasional presence of RBCs (Red arrow) as identification criteria for vasculature (as described by Weidner et.al and Tipoe et.al.,) and condensed or marginated nuclei for apoptotic cells (as described by Kerr).

B. Apoptotic cells (Yellow arrow); (Brown colored labelled) in the same section detected by TUNEL assay. 

Plate 12
Justification for choosing AgNOR assay.

Nuclear Organizer Regions (NORs) are the loops of rDNA coding for rRNA existing in the nucleolus (Alberts et al., 1983). In cell division the nucleolus is located on the secondary constriction of the acrocentric chromosomes (No. 13, 14, 15, 21, 22), in which exists the agyrophilic non histone proteins called AgNORs. In addition, as soon as cell division has finished, the nucleolus reassembles from secondary constriction regions. (Stahl, 1982). In 1986, Ploton et al., by using a very simple improved silver staining technique reported that the NOR associated proteins can be observed in routine pathological tissues. In general, compared with normal cells, the nucleoli of cancer cells are larger and greater in number. Therefore Ploton suggested that the size and number of NORs might reflect or predict cell proliferation, transformation and biological malignancy. Subsequently, flow cytometric estimation of DNA content and AgNOR revealed high counts of AgNORs in S phase (Crocker et al., 1988). Various studies on oral carcinoma report the significance of AgNOR as reliable prognostic and diagnostic marker (Schwint et al., 1994; Piffko et al., 1997a. and Piffko et al., 1997b).

AgNOR and prognostication in oral cancer.

Until recently, evaluation of AgNOR performed by counting number of dots or clusters in the nuclei. (normal oral tissue 1.56+0.1 and carcinoma ranges from 2.81 to 9.16.) Further modification in the evaluation of AgNOR was introduced by Xie et al. (1997), where percentage of nuclei with more than one AgNOR (pAgNOR)
was taken and was reported to be a strongest prognostic marker in the cancer of head and neck. The proliferation marker Ki-67 was estimated along with pAgNOR showed significant association ($p=0.0042$). (The pattern of staining for Ki-67 shown in plate 13). The Ki-67 recognized as nuclear antigen is expressed from mid G1 to M phase representing the growth fraction. Ki-67 shows weak staining in G1 phase and rapidly disappears in post-mitotic cells. (Brugal et.al., 1994).

Gradual increase in the AgNOR counts was detected in various grades of tumor.

<table>
<thead>
<tr>
<th>Grade</th>
<th>AgNOR Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td>T2</td>
<td>3.4 ± 1.2</td>
</tr>
<tr>
<td>T3</td>
<td>4.0 ± 1.4</td>
</tr>
<tr>
<td>T4</td>
<td>4.2 ± 0.8</td>
</tr>
</tbody>
</table>

However this increase was not statistically significant, due to large standard deviations. A similar report based on clinical and pathological grades was shown by Hayatsu et.al., (1992).

The present study is aimed at designing predictive assay applicable for routine pathological assessment of paraffin sections and therefore methodology must be simple, rapid, reliable and cost effective. Considering the above facts this predictive assay was designed based on simple staining procedures and morphological assessment. The criteria for assessment based on the hypothesis explained earlier, is to analyze for the presence of apoptotic cells and proliferative cells in surrounding
area of the vasculature, while other areas were rejected. These counts were then analysed with follow-up status by both multivariate and univariate analysis.
Fig. 1. Dysplastic lesion.

Fig. 2, 3 & 4. Moderately differentiated squamous cell carcinoma showing various grades of Ki-67 expression.

Figure 5 & 6. Well differentiated squamous cell carcinoma showing high and low Ki-67 expression.

Ki-67 Immunoreactivity.

Plate 13
Results and Discussion.

Apoptosis and proliferation counts in the vascular area were graded as low (for count less than 10%), moderate (11-50%) and high (above 50%) and the results are shown below.

**Table: Distribution of apoptotic and proliferative grades in the vascular area and follow-up status of patients (n=48).**

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>Moderate</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
</tr>
</tbody>
</table>

From the table, it is evident that increased levels of apoptosis in 75% of cases (18/24) and or decreased levels of proliferation around the vascular area was associated with disease free status. Similarly, lower levels of apoptosis with higher proliferation in vascular area of 87.5% cases (21/24) showed poor prognosis.

The present result shows an inverse relation between apoptosis and poor follow-up status ($r = -0.30$, $p < 0.05$) and direct relation between proliferation and poor follow-up status ($r = 0.36$, $p < 0.05$). Multiple regression analysis with follow-up as dependant variable shows that reliability for using apoptosis and proliferation as independent parameters showed significance of $p = 0.0001$. However the results
obtained were not sufficient to fulfill the criteria for predictivity. This is evident from the following tables, Table 1 showing the split-up of cases based on proliferation and apoptosis and Table 2 along with follow-up status.

Table. 1

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Low</td>
<td>3</td>
</tr>
<tr>
<td>Moderate</td>
<td>0</td>
</tr>
<tr>
<td>High</td>
<td>6</td>
</tr>
</tbody>
</table>

Table. 2

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>Moderate</td>
</tr>
<tr>
<td>18</td>
<td>12 Low &amp; Moderate proliferation <em>(6 ? High proliferation)</em></td>
</tr>
<tr>
<td>6 ? High proliferation</td>
<td>12 High proliferation</td>
</tr>
</tbody>
</table>

There was an ambiguity in interpreting follow-up from 18 cases shown as red color with question marks, (such as presence of high apoptosis and high proliferation...
observed in 12 cases and 6 cases with low apoptosis and high proliferation) since these patients showed no evidence of disease. Owing to the disparity in the results obtained the sections were re-examined. The cells arising from a single stem cell show apoptosis together (shown in circle), as detected by TUNEL assay. This is shown in figure below.
Thus apoptotic counts are over-estimated when compared with proliferation. Therefore the criteria was revised to examine only the stem cells near the vasculature and the result showed presence of apoptotic cells adjacent to vasculature alone was significant criteria in determining response to treatment. All 24 cases showing apoptotic cells were disease free for 36 months following treatment while remaining cases, which did not show any apoptotic cells in the layer adjacent to vasculature had poor prognosis. The results were concurrent after re-examination of the 100 cases, which were included in the previous part of the study.

Reports on AgNOR and local recurrence or poor prognosis has been reported in other cancers, Thyroid (Yamamoto et.al., 1993), Gastric carcinoma (Nagami et.al., 1994), colorectal (Hirooka et.al., 1994), larynx and hypopharynx (Yano et.al., 1995). Thus AgNOR seems to be a potential marker for prediction of recurrence. Until the present, research on tumour cell proliferation, the observations of quantity and distribution of DNA, dynamic observations of cell division and proliferation markers by Ki-67, BrdU etc., have been performed, but still has not become a routine practice. Therefore AgNOR for simplicity and being reliable has become a potential tool in the evaluation of cell proliferation. (Yue et.al., 1999). The result from this study confirms that presence of de novo apoptotic cells near the vasculature is a good prognostic indicator, while absence of apoptotic cells in the vascular area with or without high proliferation predicts tumor relapse. This study based on functional relation between vasculature and tumor kinetics show potential clinical application.
Modulation of apoptotic response is possible by altering fractionation schedules (Haimovitz et al., 1996) therefore assessment of apoptosis is valid under this aspect in designing appropriate treatment.

Representative photographs showing various patterns including bcl-2 expression in vascular area, apoptotic and proliferative cells around vasculature, apoptosis and epithelial thickness shown in plates 14-19.

Hypothetical diagram (Diagram 1) showing possible events after treatment in relation to prognosis is shown in page 145.
Vascular area stained with CD 34 endothelial marker shown (arrow) in Figure 1A and Bcl-2 positivity in the same area is shown in Figure 1B. This patient radical radiotherapy showed poor response to treatment.
Massive de novo apoptosis in the vascular area detected by TUNEL assay shown in Figure 3A and corresponding area (arrow head in 10x magnified figure 3B) stained with CD 34 endothelial marker shown in Figure 4B. This patient showed good response to treatment.

Expression of cyclin D1 shown in Figure 4A and the corresponding area showing apoptosis detected by TUNEL assay is shown in Figure 4B. Cells adjacent to vessel show nuclear staining for cyclin D1 and the cell layers above show cytoplasmic staining for cyclin D1 (large arrow head) as well as apoptosis (arrow). Cytoplasmic detection of cyclin D1 is associated with apoptosis. This patient showed good response to treatment.
Agyrophilic nucleolar organizers (AgNORs) showing proliferative cells in basal layers (Figure 5.A) and the corresponding area stained with hematoxylin showing absence of apoptotic cells in basal layers (Figure 5.B) (Vessel indicated by arrow). Apoptotic cells are seen toward the periphery of the epithelium. This patient showed poor response to treatment.

H&E stained section showing few of apoptotic cells near vasculature (Figure 6.A), presence of multiple NORs indicating high proliferation seen in the corresponding area (Figure 6.B). This patient showed disease relapse after treatment.
A. CD-34 immunoreactivity.
B. Cyclin D1 immunoreactivity.
C. Bcl-2 immunoreactivity.
D. Bax immunoreactivity.

Figure shows proliferation (B) and profile of apoptotic regulatory proteins (C & D) in the vascular area (A). Expression of bcl-2 in cells of proliferative area (C), expression of bax is seen in cells displaced away from proliferative area (D).

Plate 17
Panel on the left side: Figure 1. Normal epithelium, Figure 2 dysplastic lesion with apoptotic cells in basal layer (indicated by arrow) showed good response to treatment, Figure 3 dysplastic lesion showing highly proliferative cells (also note the cell density) without any apoptotic cells in basal layer showed recurrent disease.

Panel on the right side: Well differentiated tumors. Figure A Absence of apoptotic cells with high density of cell population, showed recurrent disease after treatment, while Figures B & C apoptotic cells with lesser cell population showed good response to treatment.
Figure 1.  

(a) Proliferative cells in basal layer and layers above show apoptotic cells showed good response to treatment, (b) Basal layers are highly proliferative and progressively displaced cells show apoptosis though the response to treatment was good, however during the follow-up showed disease recurrence.

Figure 2.  

Progressive thickening of epithelium is observed as de novo apoptosis decreases. **Figure A.** Apoptotic cells closer to vasculature shows thin epithelium, **Figure B** Moderate apoptosis with moderately thick epithelium and **Figures C & D** Few or absence of apoptotic cells near vasculature shows thicker epithelium.  
(Apoptic cells are shown as yellow arrow and vessels are indicated by red arrow).

Plate 19
Diagram 1. shows hypothetical events in the tumor with response to treatment and follow-up status.
Conclusion

Thus analysis of apoptotic and proliferative cells around the vascular area is good indicator of tumor response. This result can therefore be applied for planning trials with modified approach in treating high-risk patients.