Aims and Objectives
Part II: Aims and objectives

Background and justification of the problem

Failure of apoptosis in genetically damaged cells may provide selective growth advantages and subsequent tumor development. Development of apoptotic resistance might undermine tumor response to therapy (Hannun, 1997 and Collins, et.al., 1994). Thus evaluation of the extent of apoptosis in tumors may therefore provide clinically relevant information.

Although many of the molecular mechanisms underlying the development of oral cancer have been elucidated during past few years, the role of apoptosis is still unclear. Pre-treatment assessment of programmed cell death and expression of various programmed cell death regulatory proteins could be of predictive value in assessing the final outcome of treatment.

However, it is necessary to consider the factors that can influence apoptosis such as proliferation and the extent of influence by angiogenesis. Tumors that are poorly vascularised, might show higher de novo apoptosis, but if treated do not produce desired apoptotic yield. This is due to poor perfusion by free radicals or drugs to target site. Secondly, if the tumor grows in a low vascular condition, vascular damage as the result of treatment might not have any influence on the tumor destruction. It is therefore essential to determine the extent to which the tumor
depends on proliferation and or angiogenesis along with apoptosis to analyze the tumor behavior.

Extensive research evidence has emphasized that apoptosis is the primary mode of cell death occurring during various modes of treatment including chemotherapy and radiotherapy (Reed, 1999). Despite of the availability of enormous data on apoptotic mechanism and introduction of newer apoptosis inducing drugs, apoptotic resistance by tumor cells continues to be a challenging issue. Recent developments regarding newer or alternate pathways in apoptosis, mechanisms involved in abrogating apoptotic pathways and possibilities of bypassing the blockades provides scope for designing alternate approaches to induce apoptosis.

Oral cancer has wide spectrum of biological behavior and assessment of programmed cell death at various stages of the disease could provide predictive information. Oral cancer is common cancer in India, with diverse molecular properties, and hence can serve as an appropriate model to study effects of treatment. Further there are no such reports available on an integrated view on apoptosis, angiogenesis and proliferation in oral cancer. Therefore we intend to analyze the significance of these parameters in oral cancer.

Factors influencing treatment response.
The main objective in treating cancer is to restore the balance of tissue kinetics. The deregulated apoptosis associated with expansion of tumor cells aided by
angiogenesis appears to be the commonest mode for the progression of cancer. However there are wide variations in the regulatory pathways of all these processes among tumors. The p53 protein plays central role in regulation of apoptosis, proliferation and angiogenesis.

Radiation and chemotherapy mediated response is dependant on p53 induced apoptosis (Hansen et.al., 1997). Immature thymocytes lacking p53 are resistant to radiation induced apoptosis. Expression of p53 influences proliferation by arresting the cell cycle through (WAF- wild type p53 activated fragment) p21 and GADD 45 pathways, as shown in Figure 1. Under normal circumstances p53 is complexed with MDM-2; upon induction p53 gets dissociated from mdm-2 and binds to DNA triggering the gene expression, resulting in the accumulation of p21. The p21 protein forms complexes with cyclins by breaking Rb-cyclin complexes. As a result the free Rb protein sequesters E2F, a transcription factor required for the synthesis of DNA synthesis machinery and thus causes cell cycle arrest (Yamasaki 1999). In a similar way the GADD 45 protein complexes with PCNA to block cell cycle. The GADD 45 protein also activated repair enzymes (Sidransky, 1998).

The wild type p53 promotes apoptosis by down regulation of bcl-2 and up-regulating bax expression. Expression of bcl-2 is associated with treatment resistance, while suppression of bcl-2 expression allows better response to treatment by both radiation and chemotherapy (Chen, et.al., 1994). Analysis of
bax and bcl-2 ratio has been reported to be of prognostic significant in Leukemia (Pepper et.al., 1997).

Angiogenesis is also down regulated by WT p53 through TSP-1 and bFGF down regulation. All these functions attributed to p53 might not occur simultaneously. Further it must be noted that in addition to p53 there may be various other factors that may also influence the proliferation, apoptosis and angiogenesis. Spontaneous apoptosis in colon cancer appears to be p53 independent (Meritt, et.al. 1994), suggesting that there are p53 independent pathways capable of inducing apoptosis (Heedt, et al., 1998). Besides bcl-2 there are more anti-apoptotic proteins that may function independent of p53 such as crmA, bcl-X(L) etc. Similarly proliferation may be enhanced by over expression or amplification of cyclin D1, which is unlikely to be regulated by p53. Angiogenesis is dependant on specific angiogenic factors, while TSP-1, bFGF are only regulatory proteins in the complex pathways. The presence of multiple, interdependent or independent pathways accounts for variations in the biological behavior and needs to be considered while interpreting the findings.

Some of the causes for defective response to treatment that may directly or indirectly involve p53 protein are listed below.

- Mutations in p53 gene or its associated proteins could lead to failure of apoptotic machinery if the cell prefers p53-mediated pathway (Pai, et.al 1998).
- Endogenous or exogenous inhibition of p53 function will also block apoptosis. Over expression of endogenous mdm2 protein (Haupt et.al., 1997) or presence of exogenous E6 protein of Human Papilloma Virus binds to wild type p53 protein and the complex is targeted for degradation by ubiquitin, leading to failure of triggering apoptotic machinery (Sidransky, et.al 1996).
Mutation in bax gene has been identified in human gastrointestinal tumors and leukemia (Rampino, et.al., 1997 and Meijerink, et.al., 1998).

Over expression of crmA and bcl-2 blocks the dissipation of mitochondrial membrane potential and thereby blocking the most potent apoptotic machinery of the cell. crma also blocks the activation of caspases ( Zarnzarni, et.al 1996 and Green, et.al 1998).

Such analysis further provides scope for inducing apoptosis by exploiting p53 independent pathways in p53 defective tumors, as shown by a novel approach using adenovirus death factors (Lavoie et.al., 1998). Oral cancer, besides being the most common cancer also show high rate of disease relapse following treatment. Various studies on prognostic markers and attempt to develop predictive tools have so far found to be insufficient. Multiple attempts to optimize treatment modalities based on proliferative potential have not been successful (Russell, et.al 1992). Thus need for predictive assays in the management of oral cancer have been stressed in many reports (Vikram, et.al 1984 and Carter et.al., 1992). An exhaustive discussion regarding the necessity and significance of pretreatment identification of prognostically important subgroups in oral cancer for the disease management has been stressed in a special feature on 'Scientific Perspectives In Management And Strategies For Cure' (Ensley, 1987). Carter (1992) suggests that an approach including the molecular aspects, functional relations could serve as better tool for prognostication. Thus considering these aspects this study was designed to analyze for prognostic markers in oral cancer.
Plan of the study.

The study was planned into three phases based on the following aspects.

Diagnosis of malignancy is made on the basis of histopathological abnormality, following which the tumor is graded clinically based on TNM classification for prognostication. The histopathological grading which has been found to be less importance in prognosis, however it is considered that as the tumor progresses show progressive changes in histology which forms the basis of histological grading. Whether or not the histopathological grades have any significance in prognosis, it is still an integral part of diagnosis. Therefore in First phase of the study, the implication of biological factors in relation to histopathological progression of the disease was analyzed. The second phase, incorporates clinical factors in addition to biological factors that are closely associated with prognosis during the follow-up of patients. Based on the results from these studies analyzed for prognostic significance a predictive assay was designed and developed in the third phase of the study.
AIMS

In light of the facts described so far, it was proposed to analyze the role of p53 protein in the regulation of apoptosis, apoptosis regulatory proteins bax and bcl-2, proliferation and angiogenesis and its implications in assessing oral tumor progression and response to treatment.

Specific objectives

1. To determine the status of p53 expression and presence of mutant p53 protein.

2. To determine the extent of de novo apoptosis, proliferation and angiogenesis in various histopathological grades of oral cancer.

3. To determine the levels of apoptotic regulatory proteins such as bcl-2 and bax in various stages of tumor progression.

4. To study the relationship between p53, apoptosis, apoptosis regulatory proteins and cell proliferation as measured by cyclin D1, AgNOR and Ki-67 in the lesions.

5. To analyze for possible de-regulation and the implications of p53 mutation and cyclin D1 over expression.

6. To assess predictive significance of pretreatment status of p53, apoptosis, apoptotic regulatory proteins, cyclin D1 and angiogenesis with regard to treatment response, disease relapse in invasive oral cancer lesions based on a follow-up study.

7. Based on the above results obtained, an attempt will be made to design a predictive assay.