DISCUSSION

Cancer is the result of accumulation of changes in the excitatory and inhibitory cellular pathways, which may occur at any level of a given pathway. It has been reported that not a single, but many mutations are needed to transform a normal cell into its malignant counterpart [Vogelstein and Kinzler, 1993]. As the cell accumulates these alterations or mutations, it becomes functionally independent from the surrounding normal epithelial cells [Sidransky, 1995]. The normal cellular functions that are tightly controlled by regulatory pathways are subverted in tumor cells, thus enhancing the cell's ability to proliferate, stimulate neo-vascularization, and grow by invading locally or metastasizing to distant sites [Weiner and Cance, 1994]. The histological progression of carcinogenesis is believed to reflect the accumulation of these changes [Field, 1992; Vokes et al, 1993]. Modulation of gene expression is an important outcome in the adaptation or modification of the intracellular pathways [Bishop, 1991]. Functional interactions between different genes, their proteins as well as binding to other cellular proteins can modulate their intrinsic activities. The complexity of this intricated mechanism definitely plays a relevant role in tumor growth and progression. Metastatic potential of cancers are influenced by the local microenvironment, angiogenesis, stromal-tumor interactions, elaboration of cytokines by the local tissue, and more significantly by its molecular phenotype. This underscores the importance of understanding the intrinsic molecular metastatic process, which can be used to predict presence and location of active or occult metastatic disease. This knowledge can be useful for solid tumors like oral cancers, which are diagnosed at a late stage when most patients already have occult or overt metastasis.

Oral cancer a heterogeneous disease is the most common solid malignancy with its highest rate affecting both the genders in India. WHO research indicates a 500 percent increase in cancer cases by 2025, of which 220 percent will be due to the use of tobacco-culprit. Most of these patients are presented with local or regional lymph node metastasis resulting into overall survival rates less than 50% with high recurrence rate. Markers, which could identify metastatic potentials of oral cancer, may be useful for

treatment monitoring and identification of early recurrence in oral cancer patients. Therefore, correlation of these biomarkers in tissues and circulation with clinical findings may suggest behavior of oral tumors with pathological findings, which may be beneficial for improvements in treatment outcome and cure rates of oral cancer patients. However, a simultaneous evaluation of relationship between these marker levels with treatment outcome in patients undergoing anticancer treatment has not been systematically studied. As cancer research is more and more oriented towards the bio-molecular approach, exploiting biomolecules underlying in these mechanisms would possibly aid in the development of new anti-cancer therapies.

Targeted combination therapy plays a far-reaching role by helping to combat the cancer disease. This approach can be used for pre-screening cancer patients as suitable candidates for anticancer drugs. By pre-screening cancer patients for target proteins, we can find out in which patients these targeted therapies may be effective. Based on our analysis such patient group/s could be spared for unnecessary treatment with possible harmful side effects. Thus these findings will have important implications for clinical practice.

5.1.1 Expression of NF-κB p65 in oral cancer

Nuclear factor κB (NF-κB), a transcription factor, plays an important role in carcinogenesis as well as in the regulation of immune and inflammatory responses. It modulates multitude of critical genes including biomolecules mediating several events associated with multiple processes including acquisition of features such as promotion of cell survival and dysregulation of proliferation, angiogenesis, invasion and metastasis. It functions as regulator of genes that control cell proliferation and cell survival. Constitutive activity of NF-κB has been found to be involved in tumorigenesis in various types of cancers like breast, colorectal, ovarian cancers, and certain forms of leukemias and lymphomas. The pivotal role of the NF-κB pathway in the inhibition of apoptosis, tumor promotion and progression, and the observation that NF-κB is constitutively activated in a large number of epithelial and hematologic malignancies, strongly suggest that NF-κB inhibitors would be

useful in cancer therapy. Its activity is reported to be essential for growth and survival of many cancer cells [Furman et al., 2000; Bargou et al, 1997; Cahir-McFarland et al, 2002]. Furthermore, many cancer cells show aberrant or constitutive NF-κB activation which mediates resistance to chemo-therapy and radio-therapy [Wang et al, 1999]. Therefore, understanding NF-κB activation and its signaling pathway would enable us to monitor the efficacy of a given cancer therapy strategy. In the present study, NF-κB was estimated in nuclear extracts of oral paired tissues by western hybridization and ELISA method. The activation of NF-κB p65 was observed in 50% of the malignant oral tissues and in 27.78% of the adjacent normal tissues of oral cancer patients. NF-κB is a ubiquitous transcription factor found with baseline/basal activity to promote growth and survival of all cells, therefore it is found to be present even in adjacent normal tissues. The mean levels of tissue NF-κB p65 activation was found to be higher in malignant as compared to adjacent normal tissues. This expression of NF-κB in both tissues indicates that activation of NF-κB is an early event in oral carcinogenesis. This result was in accordance with the overexpression of nuclear Rel A in tumor tissues previously observed in larger study cohorts of gastric [Sasaki et al, 2001; Yamanaka et al, 2004; Cao et al, 2005; Lee et al, 2005], prostate [Fradet et al, 2004; Ismail et al, 2004; Ross et al, 2004; Shukla et al, 2004; Sweeney et al, 2004], endometrial [Pallares et al, 2004], hepatocellular [Tai et al, 2000] and oral [Nakayama et al, 2001] as well as cervical [Nair et al, 2003] carcinomas.

NF-κB p65 activation was observed in 87.5% of patients with habit of tobacco (WHT), 37.5% patients having tumor size 3 or 4, 37.5% and 62.5% of patients with well and moderately differentiated tumors respectively, 87.5 % of the patients with nuclear grade II, 37.5% of the patients with lymph node metastasis, and 75% of the patients with advanced disease. It is known to be aberrant in many tumors with its potential implication in early as well advanced stages of some cancers [Sweeney et al., 2004].

Important NF-κB target genes in cancer comprise of antiapoptotic genes [Barkett and Gilmore, 1999], genes involved in angiogenesis [Aggarwal,

2004], genes involved in the determination of invasiveness and the potential to metastasize [Fujioka et al, 2003]. Multivariate analysis revealed that activation of NF-κB p65 in adjacent normal as well as malignant tissues was not significantly associated with the clinico-pathological parameters. Bivariate Pearsons correlation analysis revealed a significant positive correlation between expression of NF-κB p65 in malignant and adjacent normal tissues of oral cancer patients. This probably indicates role of NF-κB as key modulator in driving cancer and also suggest the invasion of normal tissues by the tumor which might be due to tumor invasion or compression of the tumor surrounding normal tissue or tumor induced reduced blood supply as well as accompanied by inflammation in oral cancer [Haitianlu et al., 2006]. Nevertheless it indicates an advanced stage of the disease. A significant positive correlation was also observed between NF-κB p65 activation and Bcl-2/Bax ratio in adjacent normal and malignant tissues showing its protective role from/against apoptosis and also supporting the enhanced cell survival. This association confirms role of NF-κB in tumor promoter and tumor suppressor antiapoptotic as well as pro-apoptotic mechanisms, which has been reported earlier by other groups [Perkins 2004; LaCasse et al, 1998; Barkett et al, 1999;]. NF-κB contributes to cell death by transcriptionally upregulating its pro-apoptotic target genes such as Fas/CD95, FasL, death receptor 4 (DR4/TRAIL-R1) and DR5 (TRAIL-R2) [Ravi et al., 2001; Kimura et al., 2003; Wiener et al., 2004], while repressing anti-apoptotic target genes. Another possible pro-apoptotic mechanism involves p100-mediated transcription-independent cell death. The death domain in the C-terminus of p100 mediates the recruitment of p100 to death receptors, such as TNFR1 and Fas, which results in caspase-8 activation followed by apoptosis [Wang et al., 1996].

Activation of NF-κB p65 in malignant tissues was also found to correlate positively with expression of Bax. In malignancies, NF-κB has been observed to be associated with increased expression of matrix metallo-proteinases, which can promote invasiveness and metastasis formation of malignant cells [Kalkhoven et al, 1996] and induce neovascularization [Higgins et al, 1993].

In the present study, its activation was found to be positively correlated with latent and active forms of MMP-2 9 and negatively correlated with latent and active MMP-9 in adjacent normal and malignant tissues. This shows the association of NF-κB p65 activation with MMP-2 and MMP-9 involved in tumor invasion and metastasis in accordance with other reports [Novak et al., 1991; Bond et al., 1998]. NF-κB p65 activation was found to be positively correlated with serum IL-8 [Prabhudas et al.,2002] and serum glycoprotein constituents, while negatively correlated with serum p53 antibodies in oral cancer patients. Thus our results suggest that NF-κB p65 activation modulates the other proteins which are involved in the progression of oral cancer.

5.1.2 Expression of iNOS in oral cancer patients

Inflammation, angiogenesis as well as other aspects of carcinogenesis are modulated by expression of nitric oxide produced by inducible form of nitric oxide synthase (iNOS) enzyme in tumor cells, which also affects the response of patients to treatment. Human cell line studies Earlier reports have documented iNOS as the tumor suppressor and also as tumor promoter [Xie et al, 2003; Wink et al, 1998; Ambs et al, 1998; Juang et al., 1998]. The expression of iNOS protein was estimated in malignant as well as adjacent normal tissues of oral cancer patients by western blot. Constitutive expression of iNOS is demonstrated not only in cancer cell lines [Radomski et al, 1991] but also in cells of normal mucosa and tumors [Moochhala et al, 1996; Ambs Bennett et al, 1999]. In the present study expression of iNOS was observed in 87.5% of malignant tissues and in 81.3% of adjacent normal tissues of oral cancer patients. The levels of iNOS protein was found to be higher in malignant as compared to adjacent normal tissues of oral cancer patients. These results are in accordance to those reported previously by other studies [Brennan et al, 2002; Vakkala et al, 2000; Marrogi et al, 2000; Altoma et al, 2001; Massi et al,2001]. iNOS expression in adjacent normal tissues was found to be significantly higher in male oral cancer patients and non-tobacco users as compared to female cancer patients and tobacco users. These results

reflected the variability of iNOS expression in the samples. It also indicated the invasion by tumor cells of the normal tissue showing altered expression of the proteins. This was in accordance with other findings in colorectal cancer [Hao et al.2001, Moochhala et al.1996], suggesting that iNOS expression may be a very early event in progression of cancer. Further, multivariate analysis revealed that iNOS expression in adjacent normal tissues was found to be significantly higher in oral cancer patients with no habit of tobacco (NHT) as compared to patients with tobacco users (WHT). The expression of iNOS in malignant oral tissues was significantly higher in well-differentiated tissues as compared to moderately differentiated tissues of oral cancer patients. This also indicated an active participation of iNOS in development of malignant transformation in neoplastic process of oral carcinogenesis. However, the iNOS expression in malignant tissues was higher in patients having lymphatic response and tumor infiltration as compared to those patients in which no lymphatic response and tumor infiltration was present. These results confirmed the potential induction of its expression in association with inflammation and invasion of oral tissues. However, in multivariate analysis; iNOS expression in adjacent normal as well as malignant tissues was not significantly associated with other clinico-pathological parameters like age, lymphatic response, tumor infiltration, lymph-node status, different stages of the disease. Oral cancer being a heterogeneous tumor, this uneven and higher iNOS expression in malignant tumor tissues and its counter adjacent normal tissues suggested the cytotoxic effect in tumor and surrounding normal cells. This also implicated that expression of iNOS is likely to be affected by factors such as tumor microenvironment and also serves as an indirect indicator of tumor invasion and thus contributing in different stages of cellular changes that lead to oral malignancy. Though a major proportion of oral tumors and their surrounding cells express iNOS, but its expression did not show association with other clinico-pathological parameters in oral cancer patients suggesting its role as an independent variable in oral carcinogenesis.

5.1.3 Expression of Hsp-70 in oral cancer patients

Cells respond to a variety of stressful stimuli by accumulating and/or activating a set of highly conserved proteins known as heat-shock proteins (HSPs) [Steven xanthoudakis and Donald, 2000]. These molecular chaperones also play a pivotal role in maintaining a delicate balance of cellular homeostasis between survival and death. Elevated levels are reported in various tumors especially of epithelial origin [Nanbu et al, 1998; Costa et al, 1997; Vargasroig et al, 1997; Jäättela et al, 1999; Ciocca et al, 1993]. Moreover their expression is also found to be correlated to increased cell proliferation, lymph-node metastases/ metastasis, poor response to chemotherapy and poor survival [Jäättela et al, 1999; Ciocca et al, 2005; Calderwood et al, 2006].

Hsp-70 is one of the most abundant chaperones expressed in mammalian tissues and is known to be implicated in cancer development. It is known to affect treatment response by modulating the apoptotic functions in tumor cells. Overall human tumor biopsies as well as cell culture and animal experiments have indicated dual role of Hsp-70 in cancer [Alexzander et al., 2000]. Its first role is promoting cancer development by suppression of various anti-cancer mechanisms like apoptosis and senescence as well as by facilitating expression of metastatic genes and its second function is to facilitate tumor rejection by immune system [Torronteguy Carolina et al., 2006]. Hsp-70 exerts its inhibitory effect downstream of the release of cytochrome c as well as upstream of activation of caspase-3 [Li et al, 2000]. It inhibits apoptosis by binding directly to Apaf-1, and thus inhibits the eventual recruitment of procaspase-9 to the apoptosome [Beere et al, 2000; Saleh et al, 2000]. Hsp-70 protects cells from apoptotic death by acting downstream to the activation of caspase-3-like proteases [Jäättela et al, 1998], perhaps by chaperoning toxic protein products generated upon caspase activation. It probably influences through a dynamic process of physical interaction and conformational alteration affecting assembly, transport and folding of other proteins that may directly affect the execution or inhibition of apoptotic signalling pathways [Saibil, 2000]. Over expression

of Hsp70 can be used as a diagnostic or prognostic marker [Wong et al, 2005] or for assessing progression and lymph-node metastasis in cancer patients [Noguchi et al, 2002].

In the present study, both malignant oral tissues and their corresponding adjacent normal tissues were collected and assessed for Hsp-70 expression by western blot analysis. 90% malignant tissues showed the presence of Hsp-70 protein, while 80% of adjacent normal tissues showed the presence of Hsp-70 suggesting its abundance and ubiquitous expression by human tissues. This is in accordance with studies by Chuma et al (2003) propagating Hsp70 as the most abundantly upregulated gene and suggesting it as a sensitive marker for the differential diagnosis of early hepatocellular carcinoma. The expression of Hsp70 was higher in malignant as compared to adjacent normal tissues of oral cancer patients. Both paired and unpaired t- test analysis also indicated higher levels of Hsp70 in tumor tissues as compared to adjacent normal tissues. These results are in accordance with studies involving colon cancer patients, where the expression rate of Hsp70 is significantly higher in colonic cancer as compared to adjacent mucosal membrane [Wang et al, 2005].

Expression of Hsp-70 in adjacent normal tissues was found to be higher in patients with tobacco use, larger tumor size, tumor infiltration, lymphatic response, lymph-node metastasis, and moderately differentiated tumors, however not statistically significant by unpaired analysis. Hsp-70 expression in adjacent normal tissues was found to be significantly higher in advanced stage as compared to early stage of the disease of oral cancer patients. Further, multivariate analysis revealed that Hsp-70 expression in adjacent normal tissues is significantly higher in oral cancer patients with advanced stage as compared to the early stage of disease. This affirms the role of Hsp-70 in tumor development. In case of malignant tissues, the Hsp-70 expression was found to be higher in patients with NHT, early stage, larger tumor size, and moderately differentiated tumors. However, expression of Hsp-70 in adjacent normal as well as malignant tissues did not significantly associate

with other clinico-pathological parameters like age, sex, habit, tumor differentiation, lymphatic response, tumor infiltration, lymph-node status, different stage of the disease. Studies have indicated significant positive correlation of Hsp70 with axillary lymph-node metastasis [Lazaris et al, 1997]. Though in the present study, statistical significance was not found, however expression of Hsp70 was also found to be higher in lymph-node positive as compared to lymph-node negative tissues of oral cancer patients. Thus higher expression of Hsp70 in both malignant and adjacent normal oral tissues in the current study is suggestive of enhanced cell survival facilitated by higher induction of these stress proteins modulating and abrogating regulatory pathways in these oral cancer patients.

5.1.4 Expression of Bcl-2 and Bax in oral cancer patients

Apoptosis plays a significant role in the onset and/or development of cancer. Defects in this cellular program can lead to disturbances in tissue homeostasis, i.e. balance of cell proliferation and cell death. Among the most crucial regulators of this process are members of the Bcl-2 gene family. Gene products of the Bcl-2 family can form homo- and heterodimers with each other. Bcl-2 and Bax are the key molecules playing critical role in apoptosis. Bcl-2 also known to be an oncogene, has the ability to block a wide variety of apoptotic signals, and its over expression has been reported in a large number of human neoplasms, including breast, prostate and thyroid carcinoma and large cell carcinoma of the lung [Le et al,1999; Perez et al, 1997; Soda et al, 1999; Eerola et al, 1999]. Its abnormal expression usually in terms of overexpression in genetically modified cells such as tumor cells, contributes to the expansion of the damaged cell clone by preventing cell turnover due to programmed cell death, leading to cellular immortalization. Moreover, this increased bcl-2 expression in oral cancer possibly reflects tumor cell resistance to apoptosis and may have implications for their responsiveness to different treatments.

Bax is another member of the Bcl-2 family, but in contrast to Bcl-2 it has an apoptosis-stimulating function. Bax protein has been established as a tumor suppressor, because its inactivation leads to rapid tumor growth by decreasing the extent of spontaneous apoptosis of tumor cells. The pro-apoptotic action of Bax protein is dependent on the formation of Bax homodimers on the outer mitochondrial membrane. The antagonistic effect of Bcl-2 protein has been at least partially accounted for its ability to form Bcl-2-Bax heterodimers, thus preventing the formation of Bax homodimers. Hence, it has been proposed that the cellular Bcl-2/Bax ratio is a key factor in the regulation of apoptosis; a high Bcl-2/Bax ratio makes cells resistant to apoptotic stimuli, while a low ratio induces cell death [Sedlak et al, 1995; Hanada et al, 1995].

Student's *t*-test was carried out to compare the bcl-2 and bax expression between malignant and adjacent normal tissues as assessed by western blot analysis. In the current study, the expression of bcl-2 onco-protein was found to be present in 71.4 % of malignant and 14.9% of adjacent normal tissues of cancer patients. These levels were found to be significantly higher in malignant as compared to adjacent normal tissues of oral cancer patients. Previous studies postulated a role for bcl-2 in the progression of squamous cell carcinoma and one such study demonstrated higher bcl-2 mRNA expression and stronger bcl-2 protein immunostaining in OSCC in comparison with the adjacent normal oral epithelium [Chen et al, 2000] highlighting an inverse correlation between bcl-2 expression and tumor differentiation. The expression of bcl-2 in malignant tissues was found to be significantly higher in oral cancer patients with larger tumor size as compared to lower tumor size.
The Bax protein was detected in 68.8% of both malignant and adjacent normal tissues as assessed in paired oral tissues. The levels were found to be higher in malignant as compared to adjacent normal tissues. The mean levels of band intensities for Bax proteins were found to be higher in malignant as compared to adjacent normal tissues of oral cancer patients. The ratio of bcl-2/bax was also calculated as it serves as a better index. A higher expression

and phosphorylation of Bcl-2 and high ratio of Bcl-2 to Bax (which promotes apoptosis) are known to be possible resistant determinants of various anticancer agents [Sinoura et al, 1999; Violette et al, 2002; Huang et al, 2000]. The ratio of bcl-2/bax was higher in malignant tissues as compared to its normal counterpart. Multivariate analysis revealed that expression of these apoptotic proteins (bcl-2 and bax) in adjacent normal as well as malignant tissues did not associate significantly with clinico-pathological parameters in oral cancer patients. Thus, a higher expression of Bcl-2 and higher ratio of Bcl-2 to Bax showed a distinct role in oral pathogenesis and this may be employed as molecular signature of oral cancer. The results of the current study highlight the possibility that bcl-2 may be effectively employed for better characterization of oral cancer and to possibly predict its biological behaviour.

5.1.5 Tissue Gelatinases in oral cancer

Transgression of basement membranes is facilitated by expression of MMPs, and the increased expression of a number of different MMPs has been associated with malignancy and metastasis [Egeblad et al, 2001; Kleiner et al, 1999]. Metastasis cascade requires survival of tumor cells with altered communication skills with tumor mileu involving varied cell- stromal interactions. Among MMPs, MMP-2 (Gelatinase-A) and MMP-9 (Gelatinase-B) have been regarded as major critical molecules assisting tumor cells during metastasis [Sternlicht et al, 20001; Fingleton et al, 2006]. These gelatinases are known to have significant clinical usefulness in tumor progression [Kusugawa et al, 1993; Thomas et al, 1999] and are also known to correlate well with metastatic potential of tumor cells [Nakagawa et al, 1996; Lengyel et al, 1995]. These gelatinases are expressed in latent and active forms. Latent form of both gelatinases are secreted as zymogen (inactive) and expressed constitutively. While the latent forms of gelatinases undergo proteolytic processing into an active form and its expression is induced to functionally exert their biological activities. Both the tissue gelatinases, MMP-2 and MMP-9 were assessed by gelatin zymography (substrate-zymography), a

sensitive and specific functional assay to discriminate both latent and active forms of gelatinases by gel analysis. It is one of the novel methods to identify MMPs based on their ability to degrade their preferred substrates impregnated in-gel and recognized by their respective molecular weights.

5.1.5 A Tissue Gelatinase-A (MMP-2) in oral cancer

Expression of both forms i.e. latent as well as active MMP-2 were observed in adjacent normal, malignant and positive lymph-node and negative lymph-node oral tissues. Interestingly the expression of MMP-2 activities is more prominent in malignant tissues as compared to adjacent normal tissues. This is due to expression of MMP-2 in both tumor and stromal cells but secretion seems to be higher in the former. Paired and unpaired student's *t*-test was performed to compare the levels of different forms of MMP-2 in oral tissues. Both the forms were higher in malignant tissues as compared to adjacent normal tissues. Active form and activation ratio of MMP-2 were significantly elevated in malignant tissues as compared to adjacent normal tissues. However, no significant difference in latent form of MMP-2 between normal and malignant tissues was found. Similar findings are documented by Tokumaru et al (2000), where they showed that active MMP-2 was significantly elevated in malignant head and neck SCCs tissues as compared to normal tissues. Other study groups also reported significant elevations in activation ratio of MMP-2 in malignant tissues as compared to normal counter part which is in accordance with the present study [Komaya et al, 2000; Tokumaru et al, 2000]. Total MMP-2 was significantly higher in malignant tissues as compared to adjacent normal tissues as observed by other investigators [Hong et al, 2000]. The mean values for active form of MMP-2 were found to correlate significantly with lymphatic response and tumor size. Active MMP-2 expression in malignant tissues was significantly higher in oral cancer patients with lymphatic response as compared to those with no lymphatic response. While its expression in adjacent normal tissues was also found to be significantly higher in oral cancer patients with larger tumor size as compared to those with smaller tumor size. Multivariate analysis revealed

that no significant correlation was observed between clinico-pathological parameters and MMP-2 expression and activation in adjacent normal as well as malignant tissues which is in accordance with other study in oral cancer patients [Yorioka et al, 2002].

5.1.5 B Tissue Gelatinase-B (MMP-9) in oral cancer

Both, latent MMP-9 as well as active MMP-9 were expressed in adjacent normal, malignant and lymph-node positive tissues. Interestingly, the expression of MMP-9 was more prominent in malignant tissues as compared to their normal counter parts. Student's *t*-test showed that the levels of both the forms of MMP-9 were higher in malignant tissues as compared to adjacent normal tissues.

Mean levels of latent MMP-9 were higher in adjacent normal as compared to malignant tissues. Total MMP-9 was higher in malignant tissues as compared to adjacent normal tissues which is also demonstrated by other research groups [Hong et al, 2000]. Active form and activation ratio of MMP-9 were significantly higher in malignant tissues as compared to adjacent normal tissues. The expressions of all forms of MMP-9 in adjacent as well as malignant tissues were comparable with the clinical details except for size of the tumor. Expression of active MMP-9 in adjacent normal tissues was found to correlate significantly with tumor size. Moreover, expression of active MMP-9, total forms and activation ratio for MMP-9 in malignant tissues were significantly higher in oral cancer patients with larger tumor size as compared to those with the smaller size. Multivariate analysis revealed that expression of active MMP-9, total MMP-9 and activation ratio of MMP-9 in malignant tissues were significantly correlated with tumor size, different stage of the disease and early and advanced stages of the disease in oral cancer patients. However, expression of latent, active, total and activation ratio of MMP-9 in adjacent normal as well as malignant tissues did not reveal significant association with other clinico-pathological parameters.

Comparison of Tissue MMP-2 and MMP-9 in oral cancer

Gelatinase–A (MMP-2) expression is constitutive and is also involved in activation of MMP-9 which is inducible. Moreover, since these gelatinases biologically exist in different forms like latent (zymogen), active and in complex with their tissue inhibitors of MMP (TIMP's), therefore both the forms of MMP-2 and MMP-9 were correlated in the oral cancer patients. The bivariate correlation, Spearmans rho and Pearsons coefficient revealed positive correlation between latent and active forms of both MMP-2 and MMP-9 in tissues. Latent MMP-2 correlated positively with active MMP-9, and significantly with latent MMP-9 and active MMP-2. While active MMP-2 correlated positively with active MMP-9, and significantly with latent MMP-9 and active MMP-2. In adjacent normal oral tissues, latent MMP-2, active MMP-2 and total MMP-2 were significantly higher as compared to latent MMP-9, active MMP-9 and total MMP-9. Latent MMP-9 was significantly correlated with active MMP-9, latent MMP-2 and active MMP-2. Active MMP-9 positively correlated with latent and active MMP-2, latent MMP-9. While in malignant oral tissues, the expression of all the forms i.e. latent, active, total and activation ratio of MMP-2 was significantly higher as compared to all other forms of MMP-9.

The percentage activity of both latent MMP-2 and latent MMP-9 was higher in adjacent normal tissues as compared to malignant tissues, while percentage activity of both active MMP-2 and active MMP-9 was significantly higher in malignant tissues as compared to adjacent normal tissues. Percentage activity in the respective tissues when compared, it was observed that latent MMP-2 activity in adjacent normal tissues was higher than latent MMP-9, while percentage activity of active MMP-9 was higher than active MMP-2. Interestingly, the percentage activity of latent MMP-2 was significantly lower and percentage activity of active MMP-2 was significantly higher in malignant oral tissues as compared to latent MMP-9 and active MMP-9 respectively. This was in contrast to other study groups presenting malignant oral SCCs as compared to adjacent normal tissues [Hong et al, 2000].

5.2.1. Serum p53 autoantibody analysis

p53 protein is involved in the maintenance of cellular integrity after DNA damage. Disruption of the p53 pathway leads to intense genomic instability and trigger carcinogenesis marking an early event in oral cancer. Its disruption is due to alterations attributed to either mutations, loss of heterozygocity or interaction with viral proteins. Mutations in the p53 tumor-suppressor gene are found at a high frequency in a wide variety of primary human cancers. In cells and tissues with wild-type p53, the protein is difficult to detect, however, in cancer tissues, the mutant proteins are degraded less rapidly than wild-type p53, which has a half-life of 20 mins making mutant forms of p53 easily detectable in tumors. p53 alterations have been reported in about 50-90% of head and neck squamous cell carcinoma [Ahomadegde et al, 1995; Boyle et al, 1993; Sakai and Tsuchida, 1992]. In the present study, serological analysis was used to detect anti-p53 autoantibodies in serum of cancer patients with an immune response against abnormally high levels of p53 protein inside the tumor cells. The serum anti-p53 antibodies are indirect results of a missense point mutation in p53 gene. In head and neck squamous cell carcinoma, significant association has been observed between anti p53 antibodies and poor clinical outcome, i.e. increased risk of relapse and death [Bourhis et al, 1996]. Ralhan et al (1998) suggested potential usefulness of p53 antibodies in tobacco and betel quid abused populations for identifying high risk individuals. Anti-p53 antibodies could serve as a surrogate marker for early p53 alterations [Ralhan et al, 1998] which can be a potential aid in early detection of oral cancer.

In the current study, presence of serum p53 autoantibodies was seen in about 19% oral cancer patients, while controls showed complete absence of serum p53 autoantibodies. This was in accordance with the data from our laboratory [Sainger et al, 2006] and other studies [Gottschlich et al, 2003]. Oral cancer patients showed significantly higher mean values than the controls for p53 autoantibodies. The etiology of most p53 mutations is clearly related to tobacco carcinogens. Association of tobacco habits and p53

antibodies was also evaluated. 21.7 % of oral cancer patients were sero-positive for p53 autoantibodies and these patients were found to have habit of tobacco (WHT). This observation revealed that all patients who showed serum p53 positivity were tobacco users while patients with no habits of tobacco showed complete absence of p53 autoantibodies. In subjects with WHT, the mean levels were significantly higher in oral cancer patients as compared to healthy controls. Moreover mean values of serum p53 levels were also significantly higher in WHT patients with habit of tobacco as compared those with NHT. This suggested the importance of tobacco associated p53 alterations in oral cancer patients.

Correlation of serum p53 antibodies with various clinico-pathological features was determined. Serum p53 antibodies were present in 26.7% of patients with smaller (T1+T2) tumor size and 47.6% of patients with larger tumor size (T3+T4). There was a significant increase in levels of serum p53 autoantibodies in early stage patients to the advanced stage patients showing its importance in disease progression as observed in other studies [Sangrajrang et al, 2003; Maass et al, 1997]. Serum anti-p53 antibodies were present in 21% of patients with lymph-node metastasis. 27.5% and 19.1% of oral cancer patients with well differentiation and moderate differentiation was observed respectively. 18.6% and 27.3% of tumors of nuclear grade I and II showed the presence of p53 autoantibodies respectively. Among the oral cancer patients, univariate analysis revealed that the presence of serum p53 antibodies was significantly associated only with lymph node metastasis positivity but not with other clinico-pathological factors. This was also in agreement with the previous reports from our laboratory [Sainger et al, 2006].

Many of the studies also focused on the role of p53 in diagnosis, prognosis and treatment monitoring of cancer patients [Angelopouloiu et al, 1997; Rawal et al, 2001; Sainger et al, 2006]. Seropositivity of p53 autoantibodies was observed in 23.7% of follow-up patients. Our analysis of comparison of

serum p53 autoantibody levels between untreated oral cancer patients (PT) and their complete responders (CR) revealed a strong correlation between antibody levels and treatment response. The p53 autoantibody levels though not statistically significant but were higher in untreated oral cancer patients as compared to their complete responders. Nevertheless the mean values of serum p53 autoantibodies were lower in untreated oral cancer patients as compared to their non-responder (NR) patients. The results suggest that serum p53 autoantibody levels act as a good indicator of disease status which may have clinical usefulness in treatment monitoring of oral cancer patients.

5.2.2 Serum IL-8 analysis

Interleukin-8 (IL-8) was originally discovered as a chemotactic factor for leukocytes and was also identified to be an angiogenesis-regulating molecule that induced angiogenesis [Neufeld 2006]. It is known to contribute to cancer progression through its potential functions as a mitogenic, angiogenic, and motogenic factor [Xie, 2001] with inflammatory activity [Baggiolini, 1989]. It is also known to be a potent angiogenic factor in several cancers and is associated with metastasis [Singh et al, 1994; Luca et al, 1997]. Besides its crucial role in inflammatory and allergic responses, it plays a vital role in tumor-associated angiogenesis and tumor progression [Sunil Manna et al, 2005]. It has recently been reported to have angiogenic activity and to play an important role in tumor-associated angiogenesis in several solid cancers such as melanoma, colorectal carcinoma, glioblastoma and non-small cell lung cancer [Sadick et al, 2005; Luca, 1997; Ueda, 1994; Desbaillets, 1997; Smith, 1994]. The progression of neoplasms from the benign to malignant state is often associated with a switch to an angiogenic phenotype, representing an increase in pro-angiogenic molecules produced by the tumor cells and organ-specific environments [Suyun Huang et al, 2002]. Since interleukins may stimulate cancer cell growth and contribute to locoregional relapse as well as metastasis, constitutive synthesis and release of these cytokines leads to augmentation of their serum concentration that might be utilized as a marker for prognostication and monitoring of the course of cancer treatment. IL-8 is

secreted by a variety of tumor cells, and it has been shown to be a positive regulator of tumor cell proliferation and angiogenesis, and correlates with the metastatic potential of tumor cells [Christensen et al, 2005].

In the present study, serum IL-8 was estimated by ELISA and ROC curve analysis was carried out. It revealed that mean levels of serum IL-8 were significantly elevated in oral cancer patients than the healthy controls and also had good discriminatory efficacy to discriminate between the two groups. The elevations in serum of IL-8 levels in patients may be caused by an excessive production in tumor cells and subsequent release into the circulation. This is in accordance with other reports where IL-8 was found to be over expressed in the hepatocellular carcinoma (HCC) tumor cells compared with non tumorous livers [Akiba et al, 2001]. This elucidates the role of serum IL-8 as a biological tumor marker in oral cancer patients.

The mean serum IL-8 levels were significantly higher in oral cancer patients having habit of tobacco (WHT) as compared to healthy controls. The data analysis revealed that the mean values for serum IL-8 were significantly higher in cancer patients with larger tumor size, showing lymphatic response, tumor infiltration and those being in stage III compared to stage II patients. Moreover, the mean values were also higher in advanced stage of patients as compared to those in early stage of the disease suggesting its role in disease progression. Moreover, significant elevation in IL-8 levels were observed in oral cancer patients having tumor infiltration and lymphatic response as compared to their normal counterparts. This is compatible with an IL-8 mediated stromal cell modulation of tumor growth. These tumor-induced responses in the local tumor microenvironment have been associated with increased growth and metastasis and decreased survival in cancer patients. Association of these clinico-pathological parameters was determined using univariate analysis. Serum IL-8 was significantly associated in patients with larger tumor size but not with other clinico-pathological parameters. Follow up studies were carried out to evaluate its role in treatment monitoring in oral cancer patients. The mean values for serum IL-8 were lower in untreated oral cancer patients as compared to their complete responders. While the mean

values of serum IL-8 levels were higher in untreated oral cancer patients as compared to the NR. In the present study, significant correlation between serum levels of IL-8 and oral cancer patients of different stages was found. Serum IL-8 levels were significantly higher in untreated oral cancer patients as compared to controls. Serum IL-8 levels were also higher in untreated oral cancer patients as compared to NR and lower as compared to CR. Thus, the results indicated that stromal cells in tumor microenvironment mileu might be involved in the production of such angiogenic factors by cancer cells, thereby, anti-inflammatory agents might have the potential to impede the pathway of IL-8 induction. Increased serum IL-8 were correlated with accelerated clinical course, a higher tumor load and advanced tumor stage. Significant correlation of serum IL-8 levels with tumor size suggest that IL-8 is involved in the progression of oral cancer. These findings indicate that serum IL-8 may be a useful biological marker of/for tumor invasiveness and an independent prognostic factor for oral cancer patients. It may be useful in selecting patients with more aggressive tumors for neoadjuvant treatment. Therefore, targeting IL-8 can be a potential approach to control angiogenesis and invasion in oral carcinoma.

5.2.3 Serum Glycoproteins constituents

A number of glycoconjugates are altered in primary cancerous and metastatic disease. Increase density of SA at the cell surface of malignant or transformed cells has been reported from studies of animal models systems and human systems. These surface concentrations have been reported to alter malignant potential and changes in immunogenecity [Stoyloff and Ivanov, 2005]. Overall increase in cell surface SA content inturn reduces the attachment of metastatic tumor cells to collagen type IV and fibronectin. Moreover, SA is also important for biological interactions including cell adhesion to selectin and lectins. Because of their probable importance for the main qualities of transformed cells (disturbed cell-cell recognition and cell-adhesion, invasiveness and metastatic potential), it has been suggested that these cell surface changes are triggered by specific oncogenes activation and

may manifest at an early stage of tumorigenesis. Various investigators have reported increase of SA in sera of cancer patients. In a malignant cell, the membrane glycoproteins and glycolipids have altered carbohydrate metabolism, which could be responsible for all the abnormal behaviour i.e abnormal cell recognition cell adherence, antigenecity and invasiveness. Estimation of glycoprotein (TSA, LSA) and glycolipid (seromucoid fraction quantitated in terms of mucoid protein [MP] and hexose contents) constituents in sera of cancer patients are of considerable interest because of their potential application as diagnostic and prognostic indicators for cancer patients [Bhuvarahamurthy t al., 1995; Suer et al, 1996, Riley et al., 1990]. Therefore, the current study aimed to assess the expression of glyconconjugates like total sialic acid (TSA), lipid bound sialic acid (LSA), mucoid proteins and hexoses (galactose and mannose) in patients with OSCC and to determine association of alterations in serum levels of sialic acid forms, and seromucoid fractions with diagnosis and treatment monitoring of oral cancer patients. It also aims to investigate whether expression of these biomarkers correlated with tumor stage, disease status and outcome. Future studies evaluating the role of biomarkers in OSCC are warranted.

5.2.3 A Total sialic acid (TSA)

Total sialic acid (TSA) was significantly elevated in untreated oral cancer patients as compared to controls and was significantly able to discriminate between the two groups in accordance with earlier reports [Patel et al., 1993]. This implies use of TSA as diagnostic marker in oral cancer which is also reported from other studies [Xing et al., 1994]. The mean serum levels of TSA were higher in oral cancer patients with WHT as compared to those NHT. In both WHT and NHT, the mean serum TSA levels were found to be higher in oral cancer patients as compared to controls. Serum TSA levels were higher in patients with advanced stage as compared to patients with early stage of the disease in accordance with other study [Romppanen et al., 1997]. Moreover, mean serum TSA levels were also higher in patients with tobacco habit, lymph-node metastasis, and moderately differentiated and large sized tumors. The mean values of TSA were higher and lower in untreated oral

cancer patients as compared to NR and CR respectively. Other study also reported similar findings [Rawal et al., 1998]

5.2.3 B Lipid bound sialic acid (LSA)

The mean serum Lipid bound sialic acid (LSA) levels were significantly elevated in untreated oral cancer patients as compared to the controls. In both tobacco users and nonusers, the mean serum LSA levels were found to be significantly higher in oral cancer patients as compared to controls. The mean LSA levels were significantly higher in patients with tumor infiltration as compared to those showing no tumor infiltration suggesting its role in tumor invasiveness. The mean serum LSA levels were higher in patients with advanced stage as compared to patients with early stage of the disease reported similarly from Romppanen et al., (1997). The mean values of serum LSA levels were significantly higher in untreated oral cancer patients as compared to complete responder (CR) patients. This was in accordance with reports from other studies [Bhatavdekar et al., 1988; Rawal et al., 1998; Tomaszewska et al., 1997]. This strongly suggest the use of LSA as a tumor marker for oral malignancy [Lopez-saez et al., 1995; Schutter et al., 1992].

5.2.3 C Mucoid protein (MP)

Mucoid protein (MP) levels were significantly higher in untreated oral cancer patients as compared to healthy controls. The mean serum MP levels were higher in oral cancer patients with WHT as compared to those NHT. In both tobacco users and nonusers, the MP levels were found to be higher in oral cancer patients as compared to controls. Univariate analysis revealed that serum MP levels were significantly associated with tumor size, nuclear grade and stage of disease showing its correlation with tumor size [Fischer et al., 1990; Riley et al., 1990; Feijoo et al., 1997; Romppanen et al., 1997; Tomaszewska et al., 1997]. The mean serum MP levels were higher in patients with advanced stage as compared to patients with early stage of the disease as well as in patients with tobacco habits, lymph-node metastasis, and moderately differentiated and large sized tumors. The mean levels of serum MP was significantly higher in untreated oral cancer patients as compared to CR while the mean levels of serum MP were lower in untreated

oral cancer patients as compared to NR. Other study also reported similar findings [Rawal et al., 1998]

5.2.3 D Hexose

Hexose levels were significantly elevated in untreated oral cancer patients as compared to controls. The hexose levels were higher in oral cancer patients with WHT as compared to those with NHT. In both tobacco users and nonusers, the hexose levels were found to be higher in oral cancer patients as compared to controls. Moreover, the mean serum hexose levels were significantly higher in cancer patients with larger tumor size, and moderately differentiated tumors as compared to those with smaller sized and well-differentiated tumors suggesting its role in tumor progression and differentiation. The results indicated clear association of tumor burden and elevations in marker levels.

Univariate analysis revealed importance of serum hexoses in tumor burden of oral cancer patients, which was significantly associated with larger tumor size, nuclear grade and stage of the disease. The mean serum hexose levels were higher in patients with advanced stage as compared to patients with early stage of the disease. Moreover, serum levels of hexose values were also higher in patients with tobacco habits, lymph-node metastasis, and moderately differentiated and large sized tumors. The mean of serum hexose levels were significantly higher in untreated oral cancer patients as compared to CR, while the mean levels of serum hexoses were lower in untreated oral cancer patients as compared to their NR. Other study also reported similar findings [Rawal et al., 1998]

5.2.4 Plasma Gelatinase-A (MMP-2) and its inhibitor TIMP-2

Several reports are available on the analysis of MMPs and TIMPs in blood serum suggesting that both MMPs and TIMPs are released from platelets and leukocytes into serum during blood collections [Hiller et al, 2000], and plasma should be used to determine circulating MMPs and TIMPs [Jung et al, 1996; Sobin et al, 1997]. There is a dearth of reports on MMP-2, MMP-9, TIMP-1

and TIMP-2 in blood plasma as non-invasive tumour markers for oral cancer. This study is performed to investigate the impact of MMP-2, MMP-9, TIMP-1, TIMP-2, and their indices in blood plasma for non-invasive diagnosis of oral cancer.

In the current study, unpaired student's *t*-test analysis of our results revealed no significant changes in circulating levels of MMP-2, TIMP-2, MMP-2/TIMP-2 and TIMP-2/MMP-2 in plasma of oral cancer patients and healthy controls. The present study findings were in accordance to other reports studied in lung cancer patients and controls [Ylisirno et al., 2000; Komorowski et al., 2002] but contrast to some other reports [Lein et al., 2000]. MMP's and TIMP's play important role in normal physiological processes which could be relative reason of overlapping concentrations of MMPs and TIMPs between controls and cancer patients. The mean MMP-2/TIMP-2 ratio was significantly higher in oral cancer patients having WHT as compared to those with NHT, while no significant differences were found for MMP-2, TIMP-2 and TIMP-2/MMP-2 ratio. In subjects with NHT, MMP-2 and MMP-2/TIMP-2 were significantly lower in oral cancer patients as compared to controls. While in subjects with WHT, no significant variations were found. MMP-2 levels were lower, while TIMP-2 levels were higher in moderately differentiated tumors and in patients with lymph-node metastasis. Both MMP-2 and TIMP-2 levels were lower in patients with advanced disease as compared to early disease. The levels were also higher in NG-II tumors and in patients with lymphatic response, while lower in patients with tumor infilteration. The alterations in plasma levels of MMP-2 and TIMP-2 may be due to complex role of TIMP-2 in activation as well as inhibition of MMP-2. Besides its anti-MMP activity [Jiang et al., 2002], TIMP-2 is also known to regulate its activation in combination MT-MMP1, as well involved in growth promoting and antiapoptotic functions favouring thus tumor progression [Butler 1998; Shofuda et al., 1998]. However, the plasma levels of both MMP-2 and TIMP-2 and the indices did not correlate with any of the clinico-pathological parameters of the oral cancer patients. The mean values of plasma MMP-2, TIMP-2 and indices were

compared between untreated oral cancer patients (PT) and their CR and NR by paired student's *t*-test. The analysis revealed that the mean values of MMP-2 and TIMP-2 were found to be significantly lower in untreated oral cancer patients as compared to their CR. While the mean values of MMP-2 and TIMP-2 were lower in untreated oral cancer patients as compared to NR and remained persistently higher. However because of the small sample size (n=9), no significant difference was observed. Study with large sample size needs to be addressed to affirm its role.

5.2.5 Plasma Gelatinase-B (MMP-9) and its tissue inhibitor TIMP-1

Student's *t*-test analysis revealed no significant differences in the mean values of MMP-9, TIMP-1 and ratio of MMP-9/TIMP-1 and TIMP-1/MMP-9 between plasma of oral cancer patients than the healthy controls in accordance with study by Holten-Anderesen (2004) for TIMP-1. The analysis suggested that the plasma MMP-9/TIMP-1 ratio was able to discriminate between controls and oral cancer patients. Further no significant alterations were found for these parameters when compared with tobacco habits. Multivariate analysis revealed that the plasma levels of MMP-9 and TIMP-1 were lower in old age patients, but were higher in male patients compared to female patients. MMP-9 levels were lower, while TIMP-1 levels were higher in patients with tobacco habit. Both MMP-9 and TIMP-1 were higher in moderately differentiated and NG-II and large sized tumors. MMP-9 levels were lower, while TIMP-1 levels were higher in patients with lymph-node metastasis as compared to those without lymph-node metastasis. The plasma levels were also higher in advanced disease stage patients as compared to early stage patients. MMP-9 levels were lower in patients showing lymphatic response and tumor infilteration. TIMP-1 levels were higher and lower in patients with lymphatic response and tumor infilteration respectively. The plasma levels of MMP-9, TIMP-1 and the indices did not correlate with none of the clinico-pathological parameters of the oral cancer patients. The mean values of plasma MMP-9 and TIMP-1 were significantly lower in untreated oral cancer patients as compared to their CR. While the mean values of plasma MMP-9 and TIMP-1

were lower in untreated oral cancer patients as compared to their NR. However because of the small sample size (n=9), no significant difference was observed. Study with large sample size needs to be addressed to affirm its role.

Plasma levels of MMP-2 were positively correlated and were significantly lower in oral cancer patients as compared to MMP-9. Plasma levels of TIMP-2 were significantly lower as compared to plasma TIMP-1 in oral cancer patients with positive correlation. Plasma levels of MMP-2 and MMP-9 were significantly higher than both the natural tissue inhibitors TIMP-1 and TIMP-2 in oral cancer patients. Comparison of indices revealed that MMP-2/TIMP-2 was significantly higher and lower than TIMP-2/MMP-2 and MMP-9/TIMP-1 respectively. MMP-9/TIMP-1 index was significantly higher than TIMP-1/MMP-9. TIMP-1/MMP-2 ratio was significantly higher as compared to TIMP-2/MMP-2 in oral cancer patients.

Spearmans rho bivariate coefficient of correlation was used to study association between the levels of gelatinases, both total MMP-2 and total MMP-9 in tissue and plasma samples of the oral cancer patients. Plasma MMP-2 levels showed significant positive correlation with plasma levels of TIMP-2 and MMP-2/TIMP-2 index and significant negative association with TIMP-2/MMP-2 index. Whereas, plasma MMP-9 levels revealed positive correlation with both TIMP-1 and MMP-9/TIMP-1 index and negative correlation with TIMP-1/MMP-9 index. Tissue levels of gelatinase-A (MMP-2) revealed positive correlation with plasma levels of plasma MMP-2, MMP-9, TIMP-1 and negative correlation with TIMP-2. While, tissue levels of gelatinase-B (MMP-9) exhibited positive correlation with plasma levels of MMP-9, TIMP-1 and TIMP-2 negative correlation with plasma MMP-2 in oral cancer patients. This affirms the role of association of TIMP-1 and TIMP-2 in regulation of MMP-9 and further evaluation of tissue and plasma levels pf these parameters.

5.2.6 Circulating levels of antioxidant enzymes and oxidative stress related biomarkers

Free radicals are highly reactive species that have been implicated in the pathogenesis of many diseases. If the body's defense system malfunctions, or is impaired then the consequences of ROS and RNS cause damage to vital biomolecules including lipids, proteins, DNA leading to mutagenesis and carcinogenesis [Matés et al, 1999]. Oral cancer is manifested due to mutagenic and genotoxic effects of ROS and RNS generated by tobacco usage [Jeng et al, 2001]. The cumulative production of ROS/RNS through either endogenous or exogenous insults is termed as oxidative stress and is common for many types of cancer cells that are linked with altered redox regulation of cellular signalling pathways. Oxidative stress induces a cellular redox imbalance which has been found to be present in various cancer cells compared with normal cells; the redox imbalance thus may lead to oncogenic stimulation. High levels of oxidative stress have also been linked to cancer development. This damage is prevented by phase-1 and phase-2 enzymes involving antioxidant and detoxifying enzymes. Depletion or variations in these enzymes in various malignancies have been reported [Cook et al, Saydam et al, 1997; Rawal et al, 1999] which have potential implication to serve as biomarkers useful in diagnosis, prognosis and treatment monitoring of cancer patients. The enzymes involved in the phase-1 as first line of defense against free radical damage in cancer like SOD, Catalase and Gpx, and those involved in phase-2 as second line of defense as GST isoforms, plays a central role in the pathogenesis as well prevention of cancer. Therefore, these biomarkers were assessed in oral cancer patients as well as healthy controls as they could aid in early identification of tobacco-associated oral carcinogenesis.

Erythrocyte SOD and Catalase are enzymes involved in phase-1 as first line of defense to eliminate and reduce harmful effects of free radicals and other toxic by oxidizing them and converting into reactive metabolites that have implications in causing altered membrane permeability, DNA adducts and mutations that may gradually lead to neoplastic transformation. In the current study, significantly lower mean levels of erythrocyte SOD and catalase were observed in oral cancer patients as compared to healthy controls. This

indicates a reduced capacity of these enzymes to quench the free radicals generated due to tobacco induced damage and thus ultimately leading to clinical and pathological appearance of oral cancer. This was in contradiction to reports from other groups [Abou-Seif et al, 1996] who observed higher activities of the same.

Further, in this study, mean plasma levels of antioxidant like thiol was significantly lowered in oral cancer patients as compared to controls. This suggests higher oxidative stress in these patients attributing to tobacco consumption, which was also observed to be higher in former subjects. This is in accordance with the other studies by Rovere et al, (2000) reporting lower thiol levels in cancer patients as compared to controls. Decrease in thiol levels in cancer patients may be due to the consequence of oxidative processes elicited by free radicals. It has also been documented that cancer patients show an accelerated shift to more oxidized conditions and also possess reduced antioxidant buffering activity as tumor progresses [Bounous and Molson, 2003; Hack et al, 1990].

In response to reduced efficiency of the first line of defense enzymes and significantly reduced thiol levels or a higher oxidative stress evokes the counter action of enzymes implicated in phase -2 as the second line of defense involving GSH depletion and replenishing enzymes GST and GR respectively to fight against free radical scavenging system in cancer. The mean levels of GST (plasma and erythrocyte) and erythrocyte GR were found to be significantly elevated in oral cancer patients as compared to healthy controls. The increase in these enzymes reflects the higher oxidative stress which was as a result of excess generation of reactive species unable to be detoxified by enzymes involved in the first line of defense against tobacco induced damage implicated in carcinogenesis. Since both GST and GR were associated with metabolism of glutathione pool in the body, alterations in their levels in both plasma and erythrocyte levels indicate altered antioxidant capacity. Higher levels of plasma and erythrocyte GST and erythrocyte GR in oral cancer patients could be due to tobacco associated detoxification of carcinogenic products of activated metabolites of tobacco in conjugation with

glutathione [Ambrosone, 2000]. Oral cancer patients also showed lower mean levels of plasma GR, which was due to significant low plasma levels of thiol as compared to controls. This might attribute to higher oxidative stress existing in cancer patients. Significant changes in antioxidant and enzymatic activities are suggestive of role of tobacco as the major mediator of ROS induced damage. Human cells generally function in a reduced state but oxidative stress results into imbalance towards more oxidized state resulting into lower levels of antioxidants [Halliwell, 2000]. Lower levels of thiol indicate more of oxidized state in oral cancer patients as compared to controls. Further, ROC curve analysis confirmed that these antioxidant enzymes and plasma thiol levels could discriminate between the two groups of subjects. Mean levels of erythrocyte GST, GR, SOD were higher, while plasma levels of GST, GR, and Thiol were lower in advanced stage of patients as compared to early stage patients. However, catalase showed no change. These observations may be due to induction of GST activities in response to carcinogens reflected in terms of detoxification of environmental metabolites in oral cancer.

Further in this study, the oral cancer was followed up and assessed for the biomarkers after receiving anticancer treatment in CR's and NR's to evaluate their role in treatment monitoring in these patients. In case of complete-responders (CR), paired student's *t*- test revealed that the mean levels of GST (plasma and erythrocyte), plasma GR were lower than untreated oral cancer patients (PT). However, circulating levels of Thiol, erythrocyte GR, SOD and catalase were higher in CR than PT. Thiol levels were significantly higher in CR as compared to PT, while other biomarkers like levels of GR (plasma and erythrocyte), erythrocyte SOD and catalase were comparable between CR and PT. These alterations in the thiol and enzymatic antioxidants may be due to two reasons. First the production of these enzymes was reduced as the oxidative stress in the patients is declined owing to surgical removal of tumor (post-surgery) as indicated by significantly higher thiol levels in CR showing good response to therapy. Secondly increase in enzymes like erythrocyte levels of SOD and catalase is due to their efficiency to quench or fight the free

radicals generated due to post radiotherapy treatment and infilteration of stromal cells like inflammatory cells from tumor deceased during surgery which is in accordance with the reports indicating the production of ROS after surgical trauma [Bulkley, 1983; Billing et al, 1997]. Decline in plasma levels of GST, GR and comparable increase in SOD and catalase showed that primary effect of ROS is handled effectively by the first line of defense enzymes not further requiring synthesis of GST, GR enzymes; which is also in accordance with studies from Bogaards et al, (1994). Besides this GSH replenishment was observed/done after successful treatment in these patients, and due to radiation induced activation of enzymes to maintain GSH pool. In NR patients, altered antioxidant enzymes and lower thiol indicates of higher oxidative stress which might be due to presence of residual tumour harboring such alterations. The mean levels of erythrocyte GR and catalase were higher, while mean levels of plasma GST, GR, Thiol, erythrocyte GST and SOD were remained lower in NR as compared to PT. This clearly showed the significance of ROS and higher oxidative stress in NR showing failure of the cancer treatment.

Summarizing the results, the present study revealed that oral cancer patients had squamous cell carcinoma. Majority of the patients were diagnosed at an advanced stage showing late presentation of the disease. Expression of NF-κB in terms of activity and protein levels was found to be higher in malignant oral tissues as compared to adjacent normal oral tissues, which was confirmed by using Western blot. The higher expression suggested its critical role in oral pathogenesis and positive association of the same with other biomarkers indicate its potential transcriptional modulation. The higher expression of Bcl-2/Bax ratio malignant as compared to adjacent normal tissues indicates a higher proliferative capacity and lower apoptosis in malignant tissues. The altered expression of iNOS and Hsp-70 in malignant tissues as compared to adjacent normal tissues of oral cancer patients reflected the early changes with respect to ROS, inflammation and tumour -stromal interactions. Higher serum IL-8 levels in untreated oral cancer patients as compared to post

treatment follow up patients indicate a potential role for the cytokine in prognostication of oral cancer. Invasion and Metastasis is associated with activation of matrix degrading proteases with malignant potential of the tissues. Active, total and activation ratio of MMP-2 and MMP-9 levels were higher in malignant tissues as compared to adjacent normal tissues. A significant activation of both MMP-2 and MMP-9 was observed in lymph node tissues positive as compared to negative for metastasis. Significant alterations were found in expression of tissue levels of Bcl-2, active MMP-2, total MMP-2, activation ratio of MMP- 2, active MMP-9 and activation ratio of MMP-9 and circulating levels of serum p53 autoantibodies, IL-8, TSA, LSA, MP, Hexoses, plasma GST, erythrocyte GST and GR in oral cancer patients. ROC curve analysis suggested that tissue levels of Bcl-2, MMP-2 and MMP-9, serum levels of p53 antibodies, IL-8, TSA, LSA, MP, Hexoses and plasma levels of TIMP-2/MMP-2, MMP-9/TIMP-1, GST, GR, Thiol, erythrocyte GR, SOD, and Catalase had good efficacy to discriminate between controls and oral cancer patients indicating their usefulness as potential diagnostic biomarkers in oral cancer patients.

These biomarkers were also compared with clinicopathological parameters in the present study as they serve as important prognostic indicators in oral cancer patients. No significant association was observed for NF-κB p65 activation, Hsp-70, Bcl-2, Bax, different forms of MMP-2 with clinico-pathological parameters, suggesting their role as independent prognostic variables in oral carcinogenesis. In adjacent normal tissue, expression of iNOS showed significant association with age and sex of oral cancer patients, while Hsp-70 showed significant association with stage of the disease. In malignant oral tissues, the expression of iNOS was significantly associated with tumor differentiation; and active, total and activation ratio of MMP-9 expression showed significant association with tumor size and different stages of the disease. Thus the data presented in this study provides evidence for the importance of tumour-host interactions in sustaining tumour growth and metastasis and therefore bolsters the rationale for continued development of anticancer agents which target not only the tumour cells but their support

systems as well. Significant higher levels of serum p53 autoantibodies and LSA was observed in patients with lymph-node metastasis and advanced stage. Serum IL-8 was significantly higher in patients with larger tumor size, lymphatic response, tumor infilteration and advanced stage. Serum Hexoses were significantly higher in patients with NG II and larger tumor size. Multivariate analysis of circulating biomarkers with clinicopathological parameters revealed significant association of serum levels of p53 antibodies, IL-8, LSA, MP and hexoses with lymph node metastasis, larger tumor size, tumor infilteration, nuclear grade, stage of disease and larger tumor size of oral cancer patients respectively. However, the plasma levels of MMP-2, MMP-9, TIMP-1 and TIMP-2 did not correlate with any of the clinico-pathological parameters of the oral cancer patients.

The levels of the circulating biomarkers were also assessed in the follow-up samples of oral cancer patients grouped into complete-responders (CR) and non-responders (NR) according to their treatment outcome. Serum levels of p53 autoantibodies, IL-8, TSA, LSA, MP, Hexoses and plasma levels of GST, GR, Thiol and RBC GR, Catalase was higher in PT as compared to CR and plasma levels of TIMP-1, TIMP-2, were lower in PT as compared to NR that correlated with treatment outcome of the disease. The mean levels of TSA, MP, and Hexoses were significantly elevated in PT as compared to CR. Serum p53 autoantibodies, IL-8, MP, Hexoses, plasma MMP-2, TIMP-1, RBC and plasma GST activities, erythrocyte GR, SOD, Catalase and thiol declined on complete response to therapy while elevated in case of no response to therapy as compared to PT levels. Thus, follow-up analysis of patients in this study revealed that combined evaluation of these molecules could serve as decision making biomarkers aiding in treatment monitoring and management of oral cancer patients.