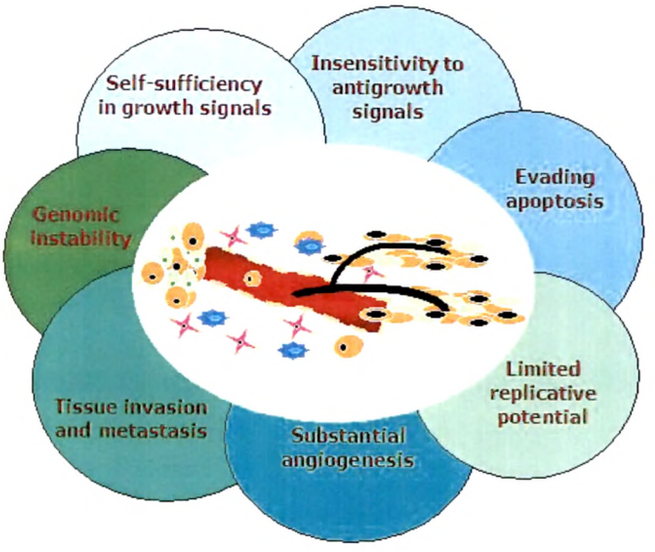
1.1 Biology of cancer

Literature defines cancer as a cellular or genetic disease. The word cancer comes from the Latin for crab; probably because of the way cancer adheres to any body parts that it seizes upon, in an obstinate manner like the crab. The word "Cancer" is used casually as a popular, generic term for malignant neoplasia (in Greek, means new formation). The cause of cancer is ambiguous which has triggered an infinite phenomenon path of research for mankind to resolve it. Ironically, the mechanistic web of cancer is apparently clear and yet presents as a complex scrabble to fill in tiny unknown virulent characteristics. This web forms the biology of cancer that needs to be understood so as to accomplish its prevention.

Ongoing progress in the field of cancer biology has resulted in increased insights into the intricate processes of malignancy. Fate of a particular cancerous cell is decided to a large extent by innate characteristics of neoplastic cell. To manifest its malignant phenotypes, neoplastic cells must acquire a set of hallmark functional capabilities during their development albeit through various mechanistic strategies including genetic instability, self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [Hanahan et al., 2003] (**Figure-1.1**). Each of these physiological changes serves not only as novel capabilities essentially acquired for hallmark of cancer during tumor development but also represents the successful breaching of anticancer defense mechanisms. Moreover, abnormal transformation initiated at genetic level affects cancer susceptibility genes like oncogenes and tumor suppressor genes by action of either accumulated mutations or carcinogens. Induced multifactoraly, it develops and manifests into a malignant tumor successfully after a normal cell undergoes a sequential series of serious multistep process. This mechanistic process involves expression of proteins like early responsive elements (transcription factors, chaperons) and cascade of downstream signal proteins (cytokines, proteases,

hormones) that serve as secondary messengers to propagate through varied mechanisms like oxidative stress, tumor microenvironment, hypoxia, ageing, inflammation, immune surveillance, malfunctioning of apoptosis and invasion and metastasis. These entire processes make tumor cells more viable, invasive and many a times resistant to conventional therapies against cancer.

Figure –1.1 Hallmarks of Cancer



1.2 Oral Cancer: Etiology and molecular pathogenesis

Progression of Oral cancer involves a multi-step carcinogenic process through various stages including hyperplasia, dysplasia, oral pre-cancers (OPC), *carcinoma in situ* (*CIS*), and invasive carcinomas finally leading to metastasis. Current evidence indicates that carcinogenesis results from a complex interaction of carcinogens and accumulated mutations in several genes. Tobacco is strongly documented playing a critical role in initiation and promotion of oral carcinogenesis via action of free radicals i.e. reactive oxygen species (ROS) and reactive nitrogen species (RNS) [Syed Sultan et al., 2004, Ray et al., 2002; Nair et al., 1995]. It contains various carcinogenic products like polyphenols, alkaloids, nitrosamines etc. that have clastogenic and carcinogenic effects [Nair et al., 2004]. The aqueous extracts of areca nut

and catechu used along with tobacco are capable of generating free radicals by auto-oxidation of these harmful products under acidic conditions (pH>6.5). Tobacco is used in different forms by different parts of the world. In the western world where chronic cigarette smoking and alcohol drinking are the main etiological risk factors while in the Asian subcontinent including India, tobacco used in varied forms, is the major culprit (**Figure-1.2.1**).

Figure–1.2.1: Radical Cause of Cancer: Tobacco in different for forms

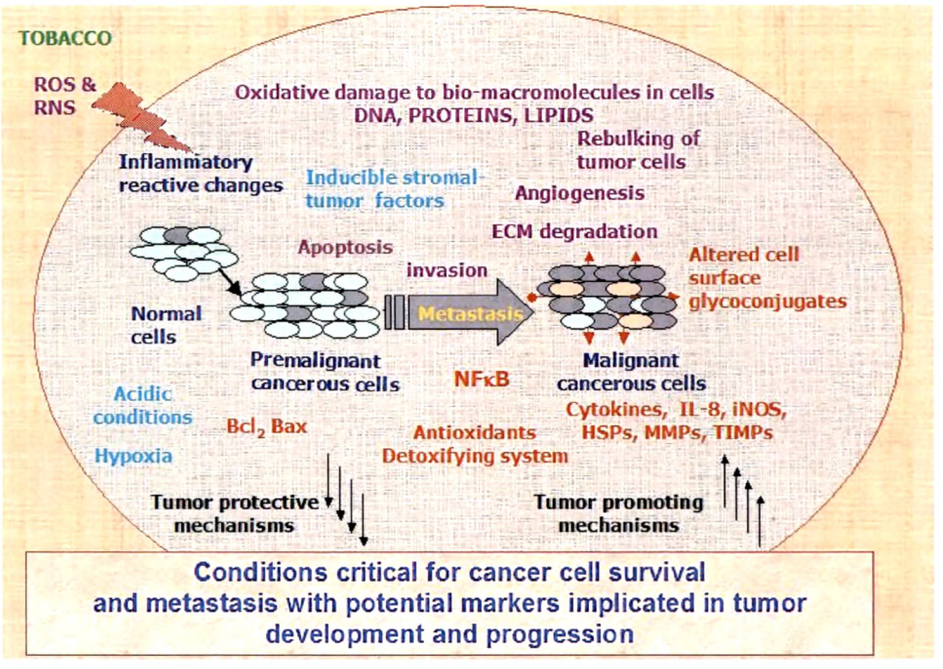
Besides ROS and RNS mediated damage, the genetic material DNA also is altered and transformed by various genomic insults like UV radiation, viruses, oxidative stress, tumor microenvironment including hypoxic and acidosis conditions. This eventually triggers the defensive mechanisms like check points of cell cycle involving activation of transcription factors like house keeping genes p53, Rb that in turn are involved in transcription of thousands of target genes for proteins involved in processes like inflammation, angiogenesis, apoptosis, invasion and metastasis. However, higher redox states with pro-oxidant shift exists in malignant cells via several mechanisms, which enable them to survive the damaging insults. Transcription factors that

are potentially known to be involved as the key molecules in cancer are nuclear factor kappa B (NF-κB), first identified in mature B cells and p53 involved as key proteins defining the hallmark of cancer. Several animal and clinical studies have revealed that in more than 80% of cancers the house keeping gene p53 is mutated unable to perform its repair function. Loss of p53 and up regulation of NFκB enables the cancerous cells to survive oxidative insults implicated in oral cancer. Accumulation of mutant form of p53 evokes and elicits an immune response producing auto p53 antibodies.

It is well documented that tumor-host interactions also influence the phenotypic expression of neoplastic changes. Microenvironments prevailing around the tumor mileu like hypoxia, acidosis, high temperature and inflammatory reactive changes modulates the expression of heat shock proteins, inflammation associated proteins like interleukin-8 (IL-8), inducible nitric oxide synthase (iNOS) and extra cellular matrix (ECM) degrading proteases notably gelatinases like matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9) that are known to be associated with angiogenic, invasive and metastatic behavior of cancer. iNOS expression is associated with nitric oxide production, that has been reported to be associated with p53 mutations through DNA damage in oral cancer [Park et al., 2003]. Moreover, the malignant cells with mutant p53 are reported to survive apoptosis through mediation of these enzymes and also known to be less susceptible to nitric oxide (NO) induced apoptosis. Expression of chaperons like 70 kDa heat shock protein (HSP-70) stabilizes not only mutant proteins like p53 but also hampers the apoptotic programme enabling the tumor cells to survive the worst environment. In oral cancerous cells, there is an imbalance of anti and/or pro apoptotic proteins that implicate in the dysfunction of apoptotic programme. The overexpression of antiapoptotic proteins like Bcl-2, survivin and down regulation of pro-apoptotic proteins like Bax is also observed.

Neooplastic changes in cell surface glycoconjugates and enzymes are expressed at or mediated through the cell membrane, leading to abnormal growth and behavior of malignant cancerous cells. Being the major component of cell membrane, various glycoprotein constituents are markedly elevated during malignant process. There is evidence that being attached to the surface of tumor cells, these sialoglycoproteins affect various important functions. Further more, expression of proteases and surface proteins enables the tumor cells to mask immune response enables cancerous cells to invade and metastasize.

Figure –1.2.2: Molecular-Pathogenesis of Cancer



Alterations in circulating levels of several glycoprotein constituents e.g. total sialic acid (TSA), lipid bound sialic acid (LSA) and seromucoid fraction [mucoid protein (MP) and hexoses] are also implicated at various stages of oral cancer. Previous studies have documented up-regulation of anti-apoptotic proteins like Bcl2, HSPs, NF-κB, MMPs, and iNOS in the pathogenesis of cancer (**Figure-1.2.2**). Although various investigators address the role of these proteins in carcinogenesis, validation of all these proteins in oral cancer

for clinical usefulness has not yet been done. Furthermore, correlation of these markers with treatment response would be substantial in improving the prognosis of oral cancer. It may constitute important avenues for therapeutic interventions for maximum benefit of cancer patients.

1.3 Aim of the study

In spite of the worldwide dedicated efforts in the research on preventive aspects of cancer, late presentation, metastasis and low overall survival rate remain strenuous problems faced by the clinicians and researchers. This evokes an urge to identify biomarkers with application to indicate early onset and occult metastasis, which can eventually aid to combat the epidemic of oral cancer in India. With nevertheless limitations the current study focuses to address a question that, according to our best knowledge, has not been explored previously to find significance of the above proteins underlying in carcinogenetic mechanism in oral cancer in the Indian context.

In this context, the present investigation evaluated efficacy of a panel of bio-molecular markers including transcription factors (p53 and NF-κB), apoptotic proteins (caspase-3, Bax and Bcl-2), HSPs (HSP-70), enzymatic antioxidants, glycoprotein constituents (TSA, LSA, MP and hexoses), inflammatory markers (IL-8 and iNOS), gelatinases (MMP-2 and MMP-9) and their tissue inhibitors (TIMP-1 and TIMP-2) aimed at exploiting their clinical usefulness. Understanding the molecular mechanism of action of these proteins in oral cancer may offer novel modes of rationally and selectively manipulating the sensitivity of tumor cells to therapy. As all these proteins have multiple functions and their expression in oral cancers may be many times associated with unexpected effects on prognosis of cancer patients. Furthermore, correlation of these markers with treatment response would be substantial in improving the prognosis of oral cancer. Therefore, the major aim of this study was to explore potential of these biomarkers all-together as cancer signatures underlying the molecular mechanism in oral cancer.

To attain the goal of the present study, these biomarkers were assessed in untreated oral cancer patients (PT) and healthy individuals as controls. Biological samples including tissue and blood specimens were collected from the subjects. Malignant and adjacent oral tissue specimens were collected from oral cancer patients at the time of biopsy or surgery. Blood samples were obtained from all the subjects. Highly specific and sensitive spectrophotometric methods were used to determine the serum levels of glycoprotein constituents and enzymatic antioxidants. Western blot method was used for expression of proteins like iNOS, Bcl-2, Bax and HSP-70 from tissue specimens. ELISA method was used determine the levels of NFκB p65, auto p53 antibodies, IL-8, MMP-2, MMP-9, TIMP-1 and TIMP-2. Oral tissue specimens from oral cancer patients were used to assess for gelatinases by gelatin zymographic method and NF-κB by EMSA method.

The major **objectives** of the study were as follows:

Comparison of circulating biomarkers between untreated oral cancer patients (PT) and controls:

- Alterations in serum levels of TSA, LSA, MP and Hexoses.
- Alterations in plasma levels of enzymatic antioxidants like GST, GR, Thiol and RBC levels of SOD and Catalase
- Serum levels of anti p53 antibodies.
- Alterations in serum levels of inflammatory cytokine - IL-8
- Alterations in levels of plasma gelatinases; MMP-2 and MMP-9 as well as their inhibitors; TIMP-1 and TIMP-2.

Study of molecular markers from malignant and adjacent normal tissues obtained from oral cancer patients.

- NF-κB activation
- Expression levels of MMP-2 and MMP-9.
- Expression levels of inflammatory markers like iNOS.
- Expression levels of Heat shock protein-70
- Expression levels of Bcl-2 and Bax

The above objectives were explored to establish clinical usefulness of these bio-molecular signatures in oral cancer.