CHAPTER 2

REVIEW OF LITERATURE
2.1 Oral cancer

Oral cancer incidence and mortality rates vary widely across the world, and the highest rates are generally registered in a few developing countries including India, Pakistan and Bangladesh, where this is the most common form of cancer [16]. According to World Health Organization report, oral cancer has one of the highest mortality ratios amongst all malignancies [17]. An estimated 300,400 new cases and 145,400 deaths from oral cavity cancer (including lip cancer) occurred in 2012 worldwide [18]. In India, the International Agency for Research on Cancer estimated indirectly that about 6, 35,000 people died from cancer in 2008, representing about 8% of all estimated global cancer deaths and about 6% of all deaths in India [19]. Most oral cancers are squamous-cell carcinomas (SCC) and it is customary to include cancers of the lip, tongue, gum, floor of the mouth, and unspecified parts of the mouth in this group. Many oral cancers arise in apparently normal mucosa, but some are preceded by clinically obvious premalignant lesions, especially leukoplakia (white patch), erythroplakia (red patch) and many others are associated with such lesions especially in South-East Asia. Most white lesions are not malignant or premalignant while erythroplastic lesions are velvety red plaques which in at least 85% of cases show frank malignancy or severe dysplasia [20]. Other potentially malignant lesions or conditions include erosive lichen planus, submucous fibrosis etc.

2.2 Risk factors:

The major risk factors for cancer are tobacco, alcohol consumption, infections, dietary habits and behavioral risk factors.

2.2.1 Tobacco: Tobacco consumption remains the most important avoidable cancer risk. Between 25 and 30% of all cancers in developed countries are tobacco-related. India is the third largest producer and consumer of tobacco. The cancer risk of tobacco use has been extensively investigated. The principle impact of tobacco smoking is seen in higher incidence
of cancers of the lung, larynx, oesophagus, pancreas and bladder. Bidi smoking is associated with cancer of oropharynx as well as larynx. Of all the tobacco consumers in India, 48% use Bidis, 14% are cigarette smokers and 38% use different forms of chewing tobacco[21]. Tobacco-related cancers account for nearly 50% of all cancers among men and 25% of all cancers among women[22]. There are predictions of incidence of 7-fold increase in tobacco-related cancer morbidity between 1995 and 2025. Further there will be an overall increase by 220% of cancer deaths simply related to tobacco use by the year 2025[22]. Smokeless tobacco products are used either alone (chewed or snuff) or in various combination with areca nut, betel leaves or lime. Different smokeless tobacco products used worldwide are known by various names: plug, gutka, khiwam, khaini, zarda, nass, toombak, gudaku and misheri [23]. High incidence of oral cancer in Indian subcontinent has been attributed to the heavy use of chewing tobacco. In India tobacco is mostly consumed with areca nut, lime or in betel quid; people using tobacco as dentifrice is also prevalent. The estimated risk for developing oral cancer in tobacco chewers is about two to four times as compared to non chewers [24].

2.2.2 Alcohol: There is a strong association between high alcohol consumption and oral cancer. Epidemiological studies carried out in India and abroad have shown that increased alcohol consumption is causally associated with cancers at various sites, mainly oral cavity, pharynx, larynx, and oesophagus [25]. Many prospective and case–control studies show a 2–3-fold increased risk for cancer of the oral cavity, pharynx, larynx and oesophagus in people who consume 50 g of alcohol a day (equal to approximately a half bottle of wine), compared with non-drinkers[26]. This effect is dose dependent. In addition, smoking has a synergistic effect. Studies demonstrate that alcohol consumption also activates carcinogens by enhancing liver metabolism, thereby working as co-carcinogen[27]. However, chronic alcohol consumption has been found to be a risk factor for the cancers of the upper respiratory and
digestive tracts, including oral cavity, hypo pharynx, larynx and esophagus as well as liver, pancreas, mouth and breast cancers [28, 29].

2.2.3 Biological factors:

Human papillomavirus (HPV) infection with high-risk types 16 and 18 has widely been reported as one of the prominent mechanisms behind the development of cervical squamous cell carcinoma. It has also been shown to be associated with oral cancer and the detection of HPV in various studies varied from 0-100% in oral premalignant and malignant tissues [30]. Thus a strong association of HPV and oral cancer is lacking as has been shown in the case of cervical carcinoma where HPV infection is necessary for disease development.

2.2.4 Diet:

Diet also appears to play an important role in oral carcinogenesis. Only a few epidemiological studies have investigated the role of vitamins and other micronutrients on oral carcinogenesis[31]. Several case control studies done have shown that higher intake of fresh fruits and vegetables have a protective influence in reducing the risk of oral cancer by 2-3 fold[32]. Studies done by Marshal et. al. 1982 has shown that deficiency of vitamin A and C doubles the risk of developing oral cancers. Thus a badly balanced diet low in micronutrients which is reflection of poor socioeconomic condition is directly associated with higher incidence of oral cancers in developing countries like India [33]. Among the most studied dietary factor in recent years is turmeric, an ingredient in the common Indian curry and a spice that has been shown to be a potent antioxidant and anti-inflammatory agent with additional promise as a chemo-preventive agent[34]. In a study in human blood cancer cell lines, turmeric suppressed and destroyed blood cancer cells. It has been shown to suppress tumour initiation, promotion, and metastasis in experimental studies[35].

2.3 Oral premalignant lesions: Oral squamous cell carcinoma is often preceded by the presence of clinically identifiable premalignant changes of the oral mucosa which are often
subtle and asymptomatic. These lesions often present as either white or red patches, known as leukoplakia and erythroplakia respectively.

2.3.A Leukoplakia:
Leukoplakia, first termed by Schwimmer in 1877 is defined as a white patch or plaque that cannot be characterized clinically or pathologically as any other disease[36]. Leukoplakia occurs most often in middle-aged and older men and arises most frequently on the buccal mucosa, alveolar mucosa, and lower lip. Most cases of leukoplakia are a hyperkeratotic response to an irritant and are asymptomatic. About 20% of leukoplakia lesions show evidence of dysplasia or carcinoma at first clinical recognition. Depending on the appearance of the lesion the leukoplakia is classified into different types[37].

2.3.A.1 Early or thin leukoplakia: it appears as a slightly elevated grayish-white plaque that may be either well defined or may gradually blend into the surrounding normal mucosa.

2.3.A.2 Homogenous or thick leukoplakia: as thin leukoplakia progresses it develops leathery appearance with surface fissures.

2.3.A.3 Nodular or granular leukoplakia: Some leukoplakias develop surface irregularities and are referred to as granular or nodular leukoplakias.

2.3.A.4 Verrucous or verruciform leukoplakia: leukoplakia having papillary surface.

2.3.A.5 Proliferative verrucous leukoplakia (PVL): It is an uncommon form of leukoplakia which is characterized by widespread, multifocal sites of involvement, often in patients without known risk factors. It begins with a white flat patch and over the time becomes papillary. This papillary growth may eventually progress to verrucous carcinoma. Such lesions have a high recurrence rate and eventually turn into an aggressive squamous cell carcinoma. Some times leukoplakia exists along with red patches or erythroplakia. If the red and white areas are inter mixed then the lesion is called a speckled leukoplakia or speckled erythroplakia.
2.3.B Erythroplakia:
An erythroplakia is a red lesion that cannot be classified as another entity. Far less common than leukoplakia, erythroplakia has a much greater probability (91%) of showing signs of dysplasia or malignancy at the time of diagnosis[38]. Such lesions have a flat, macular, velvety appearance and may be speckled with white spots representing foci of keratosis[38].

2.3.C Lichen planus:
Lichen planus is a papulosquamous eruption of the skin, scalp, nails, and mucous membranes. Although LP is more common in adults, it has become an established pediatric disorder. Its classic presentation is characterized by 4 p's: purple, polygonal, pruritic, papules[39]. It is also referred as oral inflammatory disease of unknown etiology. The World Health Organization (WHO) classifies OLP as a “potentially malignant disorder” with unspecified malignant transformation risk and suggests that OLP patients should be under close monitoring. According to reports, 1-2% of OLP patients develop oral squamous cell carcinoma (OSCC) in the long run [40].

2.3 D Sub mucous fibrosis: Oral submucous fibrosis (OSMF) is a fibrotic condition of the oral cavity and is always associated with chronic epithelial inflammation and progressive deposition of collagenous extracellular matrix (ECM) proteins in the subepithelial layer of the buccal mucosa[41]. Current evidence suggests collagen-related genes in the susceptibility and pathogenesis of OSMF. It can be assumed that the increased collagen synthesis or reduced collagen degradation as possible mechanisms in the development of the disease[42]. Malignant transformation rate of OSMF was found to be in the range of 7–13%. According to long term follow up studies a transformation rate of 7.6% over a period of 17 years was reported[42].

2.4 Conversion of premalignant lesions to carcinoma:
There may be several routes to malignant transformation of oral leukoplakia, including transformation induced by carcinogenesis due to betel quid chewing or smoking, or by HPV infection. The prevalence of malignant transformation of oral leukoplakia varies from 0.13% to 17.5%, with observation periods ranging from 1 to 30 years while the rates of five-year cumulative malignant transformation range from 1.2 to 14.5 percent (Table 2.1) [43]. Non-homogeneous leukoplakia with ulceration has a higher risk for malignant transformation and requires close follow-up and monitoring. Many investigators believe that non-homogeneous leukoplakia is a high risk factor without exception, although different terms have been used to describe those conditions [44]. Oral leukoplakia is noted to be the most common premalignant lesion of the oral mucosa and it is therefore important to clarify its clinical and histopathological characteristics. However, the mechanism of malignant transformation remains unknown.
Table 2.1: Malignant transformation potential of Leukoplakia (modified and adopted from T. Amagasa et al. 2006)

<table>
<thead>
<tr>
<th>Authors</th>
<th>Country</th>
<th>Year</th>
<th>No. of patients</th>
<th>Malignant Transformation (%)</th>
<th>Observation periods (Years)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silverman et al.</td>
<td>India</td>
<td>1976</td>
<td>4762</td>
<td>0.13</td>
<td>2</td>
<td>[45]</td>
</tr>
<tr>
<td>Gupta et al.</td>
<td>India</td>
<td>1980</td>
<td>360</td>
<td>0.3</td>
<td>1–10 (7)</td>
<td>[46]</td>
</tr>
<tr>
<td>Mehta et al.</td>
<td>India</td>
<td>1972</td>
<td>117</td>
<td>0.8</td>
<td>10</td>
<td>[47]</td>
</tr>
<tr>
<td>Gupta et al.</td>
<td>India</td>
<td>1980</td>
<td>410</td>
<td>2.2</td>
<td>1–10 (8)</td>
<td>[46]</td>
</tr>
<tr>
<td>Einhorn et al.</td>
<td>Sweden</td>
<td>1967</td>
<td>782</td>
<td>4.0</td>
<td>1–20</td>
<td>[48]</td>
</tr>
<tr>
<td>Bánócy</td>
<td>Hungary</td>
<td>1977</td>
<td>670</td>
<td>6.0</td>
<td>1–30</td>
<td>[50]</td>
</tr>
<tr>
<td>Lind</td>
<td>Norway</td>
<td>1987</td>
<td>157</td>
<td>8.9</td>
<td>6</td>
<td>[51]</td>
</tr>
<tr>
<td>Gangadharan et al.</td>
<td>England</td>
<td>1971</td>
<td>626</td>
<td>10.0</td>
<td>1–19</td>
<td>[52]</td>
</tr>
<tr>
<td>Schepman et al.</td>
<td>Holland</td>
<td>1997</td>
<td>166</td>
<td>12.0</td>
<td>6M–17 (2.7)</td>
<td>[53]</td>
</tr>
<tr>
<td>Silverman et al.</td>
<td>USA</td>
<td>1984</td>
<td>257</td>
<td>17.5</td>
<td>6M–39 (7.2)</td>
<td>[54]</td>
</tr>
</tbody>
</table>

2.5 Animal Models for oral carcinogenesis:

Animal models of cancer provide an alternative means to determine the causes of and treatment for malignancy, thus representing a resource of immense potential for cancer medicine [55].

In an attempt to develop oral carcinogenesis in animals a number of chemical carcinogens have been used. Coal tar, cigarette smoke and 20-methyl cholanghrene (20MC) were some of the chemicals, which were attempted earlier [56]. However, these chemicals either failed to produce any tumors or the tumor incidence was very low. Induction of SCC in hamster cheek pouch was first demonstrated with the help of polycyclic aromatic hydrocarbons like 9,10-dimethyl-1,2-benzanthracene (DMBA), 20MC and 3,4-benzpyrene (3,4BP) [57].
DMBA is one of the widely used carcinogens in experimental oral carcinogenesis. However, DMBA or its solvent vehicle (acetone) is a significant local irritant that causes inflammatory response, necrosis and sloughing, and hence it is difficult to study early squamous lesions[58]. Also tumors caused by DMBA in hamster cheek pouch exhibit many differences in histological features of differentiated SCC and do not closely resemble human lesions[59, 60]. Further hamster buccal pouch is immune privileged which sabotages the study of true carcinogenesis considering the important role of immune system in cancer development[59]. On the other hand 4- Nitroquinoline-1-oxide (4NQO) induced oral cancer exhibits significant similarities with human oral carcinogenesis both at histological as well as molecular level. Hence this model remains the preferred model for oral cancer studies[9].

2.5.14NQO carcinogenesis model of Rat/ mouse tongue:

One of the best studied oral carcinogenesis models is 4NQO induced rat/mouse tongue cancer model. 4NQO is a synthetic water soluble carcinogen which induces all the stages of carcinogenesis like hyperplasia, dysplasia, severe dysplasia, in-situ carcinoma and SCC[9]. It has been shown to induce SCC of the palate, tongue, esophagus, and stomach[61]. The SCC tumors produced in this fashion also displays some of the molecular changes seen in human SCC including increased expression of ras, p53, E-cadherin, Bcl-3 and Bax etc[61]. 4NQO induces a potent intracellular oxidative stress by generating reactive oxygen species [62] such as superoxide radical or hydrogen peroxide[9]. The carcinogenic action of 4NQO is initiated by the enzymatic reduction of its nitro group by NADH: 4NQO nitroreductase and NAD(P)H: quinone reductase which produces 4-hydroxyaminoquinoline 1-oxide (4HAQO)[63] (Figure 2.1). 4HAQO can be further metabolized and acetylated by seryl-tRNA-synthetase to form seryl-AMPenzyme complex [64]. This complex can also introduce quinoline groups into DNA and forms DNA adducts at various positions. However, in vivo 4HAQO reacts preferentially with guanine residues[9].
Figure 2.1: Structure of 4NQO and its metabolites (Koontongkaew et. al., 2000).[65]

2.6 Biomarkers:

The use of the term ‘surrogate marker’ in medicine dates from the late 1980s, but latter it had been modified by the term ‘biomarker’[66]. A biomarker generally refers to a measured characteristic which may be used as an indicator of some biological state or condition. A cancer biomarker is a substance that is found in an altered amount in the body and indicates that a certain type of cancer is present[67]. Ideally, a cancer biomarker should be detectable in the blood or other body fluids that can be accessed in a noninvasive manner. Clinical blood tests based on serum markers (proteins), such as CA19-9 for colorectal and pancreatic cancer, CA15-3 for breast cancer and CA125 for ovarian cancer, exhibit rather low positive predictive values. As a result, none of these biomarkers met the original goal of discovering cancer at an early stage[67]. Because of the failure to identify new single biomarker for the detection of early cancer, it has become more obvious that the simple cause and effect scenario no longer holds promise and that most physiological systems and diseases are multifactorial. Moreover, because of the genetic heterogeneity among populations, one biomarker might indicate disease in one group but be statistically non-significant in another[67]. Thus,
the human genome and proteome projects could offer distinct advantages in detection of prognostic and diagnostic proteins with higher accuracy.

2.7 Proteomics and biomarker discovery:

Proteomics term was originally introduced by Wilkins et al. in 1996 and the term “proteome” refers to the entire PROTEin complement expressed by a genOME[68]. Proteomics can be defined as the identification, characterization and quantification of all proteins involved in a particular pathway, organelle, cell, tissue, organ or organism that can be studied in concert to provide accurate and comprehensive data about that system[69]. Proteomic technologies have the potential to revolutionize the field of protein biomarker discovery and development[70]. Proteomic approaches traditionally have been divided into either gel-based or gel-free methods.

2.7.1 Gel-Based Proteomics:

2.7.1.A Two-Dimensional Gel Electrophoresis (2-DE):

It was first introduced in 1975 [71] and involves first, an isoelectric focusing of proteins [72] based on their net charge at different pH values. This is done by applying appropriate voltage for definite time point until the proteins reached to their isoelectric point. This is followed by separation in the second dimension based on the molecular weight. This technique has an excellent resolving power, and it is possible to visualize over 10,000 spots corresponding to over 1,000 proteins, multiple spots containing different molecular forms of the same protein, on a single 2-DE gel[73].

2.7.1.B Non-equilibrium pH gel electrophoresis: There is slight modification in regular 2DE named as Non-equilibrium pH gel electrophoresis (NEPHGE) technique. This technique is developed to resolve proteins with extremely basic isoelectric points (pH 7.5-11.0)[74]. Because these proteins are difficult to resolve using standard IEF due to the presence of urea
in IEF gels which has a buffering effect and prevents the pH gradient from reaching the very basic values (with a pH above 7.3-7.6). In addition, cathodic drift causes many very basic proteins to run off the end of the gel. During NEPHGE, proteins are not focused to their isoelectric point, but instead move at different rates across the gel owing to charge. For this reason, the accumulated volt hours actually determine the pattern spread across the gel. It is therefore crucial that volt hours be consistent to assure reproducible patterns[75].

2.7.1.C DIGE system (gel based labeling method):

Another modification in the classical 2 DE is use of labeling of proteins with CyDyes. This technique enables protein detection at subpicomolar levels and relies on pre-electrophoretic labeling of samples with one of three spectrally resolvable fluorescent CyDyes (Cy2, Cy3, and Cy5)[76]. These dyes have an NHS-ester reactive group that covalently attaches to the ε-amino group of protein lysines via an amide linkage. The ratio of dye to protein is specifically designed to ensure that the dyes are limiting in the reaction and approximately cover 1-2% of the available proteins where only a single lysine per protein is labelled. Intergel comparability is achieved by the use of an internal standard (mixture of all samples in the experiment) labelled with Cy2 and co-resolved on the gels that each contains individual samples labelled with Cy3 or Cy5. Since every sample is multiplexed with an equal aliquot of the same Cy2 standard mixture, each resolved feature can be directly related to the Cy2-labelled internal standard, and ratios can be normalized to all other ratios from other samples and across different gels. This can be done with extremely low technical variability and high statistical power [76, 77].

Nevertheless, several limitations of 2-DE has been realized and include issues related to reproducibility, poor representation of low abundant proteins, highly acidic/basic proteins, or proteins with extreme size or hydrophobicity, and difficulties in automation of the gel-based
techniques. Moreover, the co-migration of multiple proteins in a single spot renders comparative quantification rather inaccurate [76].

2.7.2 Gel free methods:

The gel free proteomics technology is more suitable for the analysis of proteins with low abundance in complex samples. It profits from the Liquid Chromatography [78] system to efficiently separate proteins and peptides in complex samples. Multi-dimensional chromatographic separation significantly improves the separation and identification of peptides. The advanced Mass Spectrometry (MS) systems assure the high quality of protein identification. These systems also provide more sensitive and more accurate protein quantitation. The Gel free proteomics studies significantly rely on the applied machines and experts, especially for the advanced quantification of proteins. Various LC systems are available for the separation of protein or peptide mixture complex e.g. Ion-Exchange Chromatography (IEC), Reversed-Phase Chromatography (RP), Two-Dimensional Liquid Chromatography (2D-LC) etc.

2.7.2.A Ion-Exchange Chromatography (IEC): This type of chromatography involves peptide separation according to electric charge. In cation-exchange chromatography (CX), negative functional groups attract positively charged peptides at acidic pH, while in anion-exchange chromatography (AX), positive functional groups have affinity for negatively charged peptides at basic pH. Strong cation-exchange chromatography (SCX) encompasses a strong exchanger group that can be ionized over a broad pH range. For peptide separation using SCX columns, the peptide mixture is loaded under acidic conditions so that the positively charged peptides bind to the column. By increasing the salt concentration, peptides are displaced according to their charge, while by applying a pH gradient; peptides are resolved according to their isoelectric point (pI)[76].
2.7.2. B Reversed-Phase Chromatography (RP): The separation is based on the analyte partition coefficient between the polar mobile phase and the hydrophobic (nonpolar) stationary phase. The trapped peptides are then eluted using an organic phase gradient, usually acetonitrile[79]. The ion-pair chromatography relies upon the addition of ionic compounds to the mobile phase to promote the formation of ion pairs with charged analytes. These reagents are comprised of an alkyl chain with an ionizable terminus. The introduction of ion-pair reagents increased the retention of charged analytes and improved peak shapes. Trifluoroacetic acid (TFA) and formic acid (FA) have been extensively used as ion-pairing reagents[79, 80].

2.7.2.C Two-Dimensional Liquid Chromatography (2D-LC): In this method several combinations such as AX coupled to RP (AX/RP), size exclusion chromatography coupled to RP (SEC/RP), and affinity chromatography coupled to RP (AFC/RP) are applied. In most shotgun proteomic analysis, the second dimension is performed by RP because the mobile phase i.e. acetonitrile is compatible with MS. It has been shown that SCX is an excellent match to RP for multidimensional proteomic separations[81].

2.8 Label-Based Proteomic Approaches: Various methods of MS-based quantitative proteomics have been adapted for identification and analysis of post-translational modifications and MS offers a sensitive and selective detection system. The labeling methods for relative quantification studies can be classified into two main groups: chemical isotope tags and metabolic labeling.

2.8.1 Isotope-labeled mass spectrometry: Isotope-labeling methods have been developed that introduce stable isotope tags to proteins via chemical reactions using isotope-coded affinity tags (ICAT and iTRAQ), enzymatic labeling (e.g., using $^{18}$O water for trypsin digestion), or via metabolic labeling (SILAC).
2.8.1.A ICAT technology: In this process the extracted proteins from treatment and control samples are labeled with either light or heavy ICAT reagents by reacting with cysteiny1 thiols on the proteins. Peptides containing the labeled and unlabeled ICAT tags are recovered by avidin affinity chromatography and are then analyzed by LC-MS/MS. Differential protein expression is determined by the isotope peak ratio of the peptide. However, disadvantages of ICAT analyses are obvious: it is only applicable to proteins containing cysteine; it can only identify 300-400 proteins, far fewer than 2-DE method; the peptides contain large labels, which makes database searching more difficult, especially for short peptides[82].

2.8.1.B SILAC technology: The method has the potential for high throughput and multiplexed sample analysis. It was first developed by Ong et al. [83] based on metabolic incorporation of ‘light’ or ‘heavy’ form of amino acids into the proteins in living cultured cells. Usually, heavily labeled (13C or 15N) arginine or lysine or both are used in culture medium to ensure complete labeling of every trypsinized peptide fragment. In experiments, one cell population is fed with regular amino acids and the other fed with 13C or 15N labeled amino acids. After several rounds of cell division, heavy amino acids will be incorporated into newly synthesized proteins. In the mass spectrometry spectrum, the light and heavy peptides will show up as two distinct peaks separated by the incremental mass of the labeled amino acids. By comparing the signal intensity, relative quantification can be achieved. Because of its simplicity in principle, SILAC is widely used for biomarker discovery, cell signaling dynamics, identification of posttranslational modification sites, protein-protein interactions, and subcellular proteomics[82].

2.8.1.C iTRAQ Technology for biomarker discovery:

The mass spectrometry (MS)-based quantitative proteomics is a powerful tool to discover disease biomarkers that can provide diagnostic, prognostic and therapeutic targets, and it also
can address important problems in clinical and translational medical research[84]. The isobaric tags for relative and absolute quantification (iTRAQ) technique are widely employed in proteomic workflows requiring relative quantification **figure 2.2**.

Like ICAT, iTRAQ technology also exploits an NHS ester derivative to modify primary amino groups by linking a mass balance group (carbonyl group) and a reporter group (based on N-methylpiperazine) to proteolytic peptides via the formation of an amide bond.[85] Due to the isobaric mass design of the iTRAQ reagents, differentially labeled peptides appear as single peaks in MS scans, thus reducing the probability of peak overlapping. When iTRAQ-tagged peptides are subjected to MS/MS analysis, the mass balancing carbonyl moiety is released as a neutral fragment, thereby liberating isotope-encoded reporter ions that provide relative quantitative information on proteins. Because four different iTRAQ reagents are available, comparative analysis of a set of two to four samples is feasible within a single MS run[85].
Figure 2.2 The iTRAQ reagent strategy for quantitative proteomics: Protein lysate is made from different tissues and then digested with MALDI grade trypsin to obtain the peptides. Each set of peptides is labeled with respective reporter ions. The labeled peptides are pooled, separated using cation exchange followed by reverse-phase liquid chromatography and analyzed by MS/MS. The intact mass of any peptide sequence separately labeled with the different iTRAQ tag does not differ. However, each distinct iTRAQ tag provides a unique reporter ion at m/z values 114, 115, 116 or 117 when the peptide is fragmented during MS/MS analysis. The relative intensities of these distinct reporter ions provide a measure of the relative abundance of the peptide and the protein from which it was derived across the starting protein mixtures. The amino acid fragment ions (called b and y ions) from the peptide are used in the database search to determine its sequence, leading to identification of the protein from which it is derived. Thus, relative quantification and protein identification are achieved concurrently during the MS/MS analysis procedure. Identified proteins were further validated by immunohistochemistry/RT-PCR.
2.9 Proteomics and Cancer:

With the advanced development of proteomics tools, this technology platform is being utilized to discover highly sensitive and specific protein markers for cancer diagnosis and prognosis, to elucidate the molecular determinants and key signal pathways underlying the disease mechanism, to identify novel therapeutic targets and assess drug efficacy and toxicity, and to monitor treatment response and the relapse of the cancer[86]. Proteomics has been successfully employed in studies of several cancers including oral cancer.

2.9.1 Esophageal carcinoma: Proteomics has been applied on esophageal squamous cell carcinoma (ESCC) and molecules such as pRB protein, tropomyosin isoform 4 (TPM4), prohibitin and periplakin [87-91] have been reported as potential biomarkers for the diagnosis of ESCC.

2.9.2 Breast Cancer: Proteomics has an impact on both the diagnosis and treatment of breast cancer. Measurements of the classic breast cancer biomarkers HER2, estrogen receptor (ER), and progesterone receptor (PR) are routinely done in clinical laboratories to classify tumor samples to determine treatment. A multiplexed immune selective reaction monitoring (SRM) MS assay was developed for the quantification of ER and HER2 levels in cell lines and tumor samples where in they found good correlation with ER/HER2 status measured by traditional clinical assays[92].

2.9.3 Lung Cancer: Lung cancer is generally divided into small-cell lung cancer (SCLC), representing approximately 15% of cases, and non-small-cell lung cancer (NSCLC), representing 85% of cases and can be further sub divided in to several histological types, like adenocarcinoma, large-cell carcinoma, and squamous-cell carcinoma. Proteomics can represent an important tool for the identification of biomarkers and therapeutic targets for lung cancer. A number of potential biomarkers have been identified, such as mutations in
KRAS and TP53 and alterations in expression of carcinoembryonic antigen (CEA), cytokeratin-19 fragments (CYFRA21-1), neuron-specific enolase (NSE), and cancer antigen-125 (CA-125). Glycoproteomics studies, performed by different methods for glycoprotein fractionation followed by LC-MS/MS, revealed potential lung cancer biomarkers, such as plasma kallikrein (KLKB1), pleural effusion periostin, multimerin-2, CD166, and lysosome-associated membrane glycoprotein-2 (LAMP-2)[93].

2.9.3 Liver Cancer: Hepatocellular carcinoma (HCC) is one of the most common diseases worldwide, with extremely poor prognosis due to failure in its early diagnosis. Alpha-fetoprotein (AFP) is the only available biomarker for HCC diagnosis. However, its use in the early detection of HCC is limited. A quantitative proteomic analysis approach using stable isotope labeling with amino acids in cell culture (SILAC) combined with LTQ-FT-MS/MS identification was used to explore differentially expressed protein profiles between normal (HL-7702) and cancer (HepG2 and SK-HEP-1) cells. It was found that Transglutaminase 2 can be a novel histological/serologic candidate involved in HCC[94]. In another proteomic study of hepatocellular carcinoma, it was found that HSP90 can be a potential serum biomarker[95].

2.9.4 Oral Cancer:

There are no molecular markers available to assist with the early detection, prognosis, therapeutic response prediction, and population screening of OSCC, although some genes and their products have been intensively studied during oral carcinogenesis[96]. The integration of the data from the recent -omics data-generation technologies has opened a novel path to the solution of the above issues and sheds light on the molecular mechanisms of OSCC pathogenesis[96]. Proteomics is a promising approach to understand the details of the
molecular mechanisms of OSCC, as well as in order to search for new targets for therapeutic intervention and markers for early detection[97].

Some of the recent studies have shown the potential of protein biomarkers in the prognosis of cancer prediction of relapse or metastasis. Hu et. al. 2008[5] explored the presence of informative protein biomarkers in the human saliva proteome and to evaluate their potential for detection of oral squamous cell carcinoma (OSCC). They utilized shotgun proteomics based on C4 reversed-phase liquid chromatography for prefractionation, capillary reversed-phase liquid chromatography with quadruple time-of-flight mass spectrometry, and Mascot sequence database searching for discovery of new targets that led to a simple clinical tool for the noninvasive diagnosis of oral cancer. Differential proteomics revealed several salivary proteins at differential levels between the OSCC patients and matched control subjects. Five candidate biomarkers (M2BP, MRP14, CD59, catalase, and profilin) were successfully validated using immunoassays on an independent set of OSCC patients and matched healthy subjects. The combination of these candidate biomarkers resulted in a receiver operating characteristic value of 93%, sensitivity of 90%, and specificity of 83% in detecting OSCC[5]. Ralhan et al. 2008[12] have applied iTRAQ-multidimensional liquid chromatography and tandem mass spectrometry on oral epithelial dysplasia and unmatched controls to identify the biomarkers which will discriminate oral premalignant lesions from normal tissues. In this study they identified stratifin (SFN), YWHAZ, and hnRNPKs which were highly up regulated in oral dysplasia as compared to normal tissues [12].

Hayashi E. et al. 2009[98] have used 2-DE based proteomic technology to analyze the protein expression profile in OSCC tissues and accompanying surrounding normal tissues in four oral locations (buccal mucosa, gingival mucosa, oral floor, and tongue). They have identified ten proteins that were over expressed more strongly in cancer tissues than normal ones. Among them 14-3-3 σ was found over expressed in all four sub-sites of oral cavity. This study
concluded that ten proteins identified may have important role in OSCC carcinogenesis and progression and could be used as diagnostic biomarkers of OSCC[98].

Govekar et. al. 2009[99] performed proteomic profiling of cancer of the gingivo-buccal complex using 2-DE-MS/MS approach and identified nine differentiator proteins which could distinguish normal from tumor tissues. These proteins include lactate dehydrogenase B, a-enolase, prohibitin, cathepsin D, apolipoprotein A-I, tumor protein translationally controlled-1, an SFN family protein, 14-3-3sigma and tropomyosin.

In another study Ajay Matta et al. 2010[100] showed the prognostic utility of stratifin (SFN), YWHAZ for head and neck cancer. They found significant decrease in median disease-free survival (13 months) in HNSCC patients showing over expression of both stratifin and YWHAZ proteins, as compared to patients that did not showed the expression of the same.

Chang et. al. 2011[101] have utilized iTRAQ-based quantitative proteomic approach to identify proteins that are differentially expressed between micro dissected primary and metastatic OSCC tumors. This study resulted in identification of seventy four differentially expressed proteins including PRDX4 and P4HA2. Immunohistological validation of PRDX4 and P4H4A2 revealed that over expression of these two proteins in tumors than adjacent non-tumorous epithelia was significantly associated with positive pN status. Furthermore PRDX4 over expression was a significant prognostic factor for disease-specific survival in both univariate and multivariate analyses. Moreover over expression of PRDX4 and P4H4A2 was even higher expression in the 31 metastatic tumors of lymph nodes, compared to the corresponding primary tumors [101].

Tung CL et. al. 2012[102] in their study used comprehensive patient-based proteomic approach for the identification of potential plasma biomarkers in OSCC. They have identified numerous OSCC proteins including fibrinogen (alpha/beta/gamma) chain,
haptoglobin, leucine-rich alpha-2-glycoprotein and ribosomal protein S6 kinase alpha-3 (RSK2) which have not been reported and may be associated with the progression and development of the disease[102].

Recent study by Eric Romen et. al. 2013[103] on the OSCC samples from Norway and UK demonstrated that 2D-DIGE-MALDI TOF-TOF based proteomics identification of annexin II and V, HSP-27, and SCC-Ag as a potential biomarker and might be potential drug targets for oral cancer[103].

P Chanthammachat et. al. 2013[104] performed comparative proteomic analysis of oral squamous cell carcinoma and adjacent non-tumour tissues from Thailand using 2 DE and MALDI technique and reported that KIAA1199 and Horf6 may be novel markers for oral cancer[104].

Lai et. al. 2010[14] have studied a mouse model with oral squamous cell carcinoma (OSCC) induced by 4-nitroquinoline 1-oxide (4-NQO)/arecoline in drinking water. Furthermore proteomic profiling by 2DE of mouse plasma samples indicated that haptoglobin and apolipoprotein A1 precursor were up-regulated in the mice with OSCC. They further correlated the expression of haptoglobin in human plasma samples from patients with OSCC and found that there was a strong correlation between the increasing levels of haptoglobin and the clinical stages of OSCC ($P < 0.01$). These results suggested that haptoglobin has a great potential as a sensitive plasma biomarker for early detection of patients with OSCC[14].

A number of potential diagnostic markers for oral squamous cell carcinoma (OSCC) have thus been discovered, yet none has been validated for high sensitive and reliability. Early identification of recurrence for OSCC is also a challenge. Therefore identification of a biological marker is of extreme importance, to complement clinicopathological findings for a more accurate prediction of individual patients’ prognoses and to help clinicians in planning...
more effective therapeutic strategies. In order to sequentially dissect the molecular events during different stages of oral carcinogenesis, it was proposed to carry out proteomic analysis on samples obtained at sequential stages of rat lingual carcinogenesis. We utilized both 2-DE and iTRAQ-LC-MS systems for precise detection of differences in protein profile at various stages of lingual carcinogenesis.