CHAPTER 6
DISCUSSION
The development of oral cancer is a multistep process. The sequential molecular alterations, accompanying/leading into OSCC, need to be established for the development of diagnostic and/or prognostic biomarkers. There are considerable difficulties in procuring human normal oral tissues and tissues of different stages of oral carcinogenesis. Hence, experimental animal models are being used for studying sequential molecular changes during oral cancer development.

Animal models of carcinogenesis allow the isolation of all stages, including normal tissues under controlled conditions. These models are amenable to pathological, genetic, and biochemical analysis and the cost of analysis is relatively low [110]. In addition, the chemical carcinogenesis models can be used to investigate the hazard risk that is caused by environmental agents and to determine which of the putative precancerous lesions will progress[111].

4NQO is a potent carcinogen and is widely used in studies understanding the experimental oral carcinogenesis. It is metabolically converted in to its active form 4 hydroxyaminoquinoline- 1-oxide (4HAQO) by enzyme NADH: 4NQO nitroreductase and NAD(P)H: quinone reductase (Figure 2.1)[63]. This activated molecule 4HAQO preferably binds to guanine residues and forms a DNA adduct. The adduct mimics UV induced pyrimidine dimer formation. It has been proposed that the carcinogenesis process induced by 4NQO shows similar molecular alterations as in human carcinogenesis[9, 112].

Earlier studies from our lab have shown that 4NQO produces all the stages of lingual carcinogenesis but not that of buccal mucosal carcinogenesis[113]. In this study 4NQO was given to rats by painting of buccal mucosa (0.25% 4NQOin Propane di-ol) as well as in normal drinking water (10 ppm of 4NQO). All the stages of lingual carcinogenesis were obtained which included dysplasia (80 and 120 days), papillomas (160 days) and carcinomas (200 days). In case of buccal mucosal carcinogenesis only papillomas were obtained in 200
days. The possible reason for not obtaining earlier stages of buccal mucosal carcinogenesis could be that the papilloma development is a rapid process and the earlier stages went undetected. The reason for not obtaining SCC at buccal mucosa could be that buccal mucosa does not get enough exposure to 4NQO because of its anatomical position[113].

Our results, using 4NQO as a carcinogen, demonstrated histopathological changes in rat tongue mucosa along a time course from normal epithelium to hyperplasia to papilloma and finally to squamous cell carcinoma (Figure 4.1). Given that these lesions did not occur in the control, it can be assumed that the tongue carcinogenesis was 4NQO-dependent[114]. The majority of lesions were at the dorsum of posterior tongue. One possible reason for this site specificity could be higher activity/expression of enzyme 4NQO reductase at the base of the tongue[115].

In the present study we treated the rats with 30 ppm of 4NQO for different time points i.e. 80, 120, 160 and 200 days in normal drinking water respectively. Histopathological analysis of lingual epithelium showed no change in 80 days treated tongue tissues however hyperplasia was observed in 120 days treated rat tongue tissues. Further we observed papilloma/carcinoma development after 160/200 days of 4NQO treatment respectively, in the tongue tissues. Results from some other laboratories were at variance with our results for example Niwa et al.(2001) and D.A. Ribeiro et al. (2007) showed the development of hyperplasia(7/10) /Dysplasia (3/10) in 12th weeks of 50 ppm of 4NQO treatment while development of dysplasia (3/10)/carcinoma (7/10) was observed in 20 weeks of 4NQO treatment[116, 117]. In both the studies development of papillomas was not observed and carcinomas were seen directly. In both these studies authors have used 50 ppm of 4NQO, which could be one of the reasons for the differences observed in our study.
An interesting observation made in our study was that two animals from same group of treatment showed two different stages of development e.g. 120 days treated rat showed hyperplasia as well as papilloma. The possible reason for this could be that, in the present study 4NQO was given in drinking water. Since the intake of water by animals can not be controlled, it is possible that the different animals from the same group got different exposure of 4NQO. Another interesting observation made in our study was the occurrence of two stages within same tissue section. This possibly can be explained on the principle of field cancerization. It is possible that the two different mutagenic events happened in vicinity, producing different sub clones which ultimately resulted in a verity of histopathologically diverse regions in the same tissue section[118].

Proteomics approaches have great potential as a means to elucidate the underlying molecular mechanisms of cancer[10]. Differential proteomics have been used to study the differential protein expression pattern in normals vs. tumors. Various differential proteomics techniques are available. In our study we have employed both gel based and gel free methods in order to obtain the differential expression pattern of normal and different stages of carcinogenesis.

We initially adopted gel based technique i.e. 2DE followed by MALDI-TOF-TOF. We performed 2-DE utilizing both 3-10 pH strips and 4-7 pH strips for IEF. Differential proteomics using 3-10 pH strips resulted in identification of limited number of proteins (Figure 4.2 and Appendix Figure A1). We identified keratin 6A, Transglutaminase 3, fatty acid binding protein 5, serum albumin precursor protein and galectin 7. Among these keratin 6a, fatty acid binding protein 5 and albumin precursor protein were found to be up regulated while transglutaminase 3 and galectin 7 were found to be down regulated.

Keratins (K) are epithelia predominant intermediate filament proteins which are expressed in a differentiation dependent and site specific manner[119, 120]. Keratin 6a expression is characteristic of squamous epithelia and is used as marker of hyper proliferation[121].
Mutations in the K6a gene result in the Pachyonychia congenital disease[122]. Proteomics study by Thiel et. al. 2011[15] on tongue carcinoma reported up regulation of keratin 6a in carcinoma as compared to normal mucosa[15]. Previous work from our laboratory using this model has also shown that K6a was up regulated in rat tongue SCC in comparison to normal tissues[113].

Intracellular fatty acid-binding proteins (FABPs) are members of a multigene family encoding ~15-kDa proteins, which bind a hydrophobic ligand in a non-covalent, reversible manner[123]. Fatty acid binding protein 5 (Fabp5) is also referred as epidermal fatty acid binding proteins and is found to be up regulated in psoriasis tissue[124] and OSCC[12, 125, 126]. It also promotes cell proliferation and invasion in oral squamous cell carcinoma[126].

Transglutaminases (TGase) are a family of cross-linking enzymes present in most cell types and catalyze (Ca++ dependent manner) the formation of Nε-(γ-glutamyl)lysine isopeptide bonds between amino acid side-chains. TGase3 (epidermal transglutaminase) has been suggested to play important role in epidermal keratinization and in the formation of the cornified envelope[62]. TGase3 was found to be down regulated in OSCC[127-129]. Interestingly, TGM3 down regulation was an early event in rat oral carcinogenesis. The early down regulation of TGM3 may drive the cells towards malignancy and might facilitate their malignant transformation. The similar observations were made by Choi et. al.2008[130].

Amongst differential proteins identified by MALDI-TOF-TOF, down regulation of galectin 7 was most interesting observation because of the fact that its over expression has been reported in human OSCC[131]. Further, down regulation of galectin 7 was confirmed by IHC as well as western blot (Figure 4.3 A and B).
Galectins are β-galactoside binding lectins involved in various cellular processes e.g. differentiation, apoptosis, metastasis, invasion etc.[132-134]. In humans, galectin 7 was found to be upregulated in tongue and esophageal cancer[131, 135, 136]. Galectin-7 was identified as PIG1 (p53 induced gene 1), one of the genes highly induced by p53 transfection into the colon cancer cell line DLD-1[137].

Down regulation of galectin 7 in our rat model can be explained by following facts. It is known that 4NQO is potent carcinogen and is physiologically converted into its active component, 4-hydroxyaminoquinoline 1- oxide (4HAQO)[63]. As a result, DNA damage is extensively induced by combination with the purine body of DNA within the nucleus to form 4HAQO-DNA adducts through reactive oxygen species.[9] In addition, 4HAQO is able to promote methylation in the promoter regions of genes[117, 138]. Taken together it can be assumed that galectin 7 down regulation may be a consequence of epigenetic regulation of its gene. Furthermore, epigenetic studies on this model will shed the light on molecular mechanisms which are involves in galectin 7 regulation.

Recently Kim et. al. 2013[139] showed that galectin 7 was downregulated in human gastric cancer tissues in comparison to normal tissues. Further studies demonstrated that this down regulation was due to DNA methylation of galectin 7 gene.

We also used 4-7 pH strips in the first dimension for 2DE. It did not result in identity of any differential proteins probably because of masking effect of keratins since pI of most of the keratins falls in 4-7 pH range (Appendix Figure A2).

It was not completely unexpected, as several limitations of 2DE based proteomics have been realized recently [140], which include
1. Because of size constrains, simultaneous resolution of very high and very low molecular weight proteins becomes difficult.

2. Poor resolution of acidic proteins (pI<3) and basic proteins (pI>8) proteins on IEF gel makes it difficult to resolve them.

3. Squamous epithelia are rich in keratins which tend to mask low abundant proteins.

Major objective of our study was to establish the differential expression pattern of proteomics at different stages of carcinogenesis. Due to limitations of gel based methods we followed the gel free and labeling method to identify a battery of differentially expressed proteins. For this purpose we utilized iTRAQ-LC-MS based protocol. The overview of iTRAQ strategy is given in Figure 2.2.

Our iTRAQ-LC-MS/MS analysis resulted in identification of 2,223 proteins. Out of these, 415 proteins were found to be differentially expressed in tumors, 333 proteins in papillomas and 109 proteins in hyperplasia. Among the differentially expressed proteins, 5 proteins were sequentially upregulated while 10 proteins were sequentially down regulated from hyperplasia to SCC. Similarly, up or down regulation of 62 and 51 proteins respectively was observed from papilloma to carcinoma tissues. Further, we observed sequential upregulation of 154 proteins while sequential down regulation of 170 proteins in SCC in comparison to normal tissues. (Appendices Table 1A)

Some of these differentially expressed proteins have already been identified in human OSCC while we have detected some novel proteins which have not been reported previously. We first validated some of these proteins well known in human OSCC (Vimentin, fascin, transglutaminase 3, periostin and cornulin) and then confirmed differential expression observed in iTRAQ studies with IHC and/or qRT-PCR experiments for a few novel proteins detected in rat OSCC in the present study (Trichohyalin, Thrombospondin2, tenascin N and coronin1a). Importantly, IHC studies with the human OSCC samples for two of the novel
candidate proteins (Tenascin N and Coronin 1a) confirmed the validity of our rat OSCC model. Furthermore, we could validate their presence even in rat hyperplasia and papillomas as well as human leukoplakia samples.

**Vimentin (Vim)**

Vimentin (Vim) is type III intermediate filament protein which is ubiquitously expressed in mesenchymal cells. This protein not only has important role in the EMT of epithelial cells but also has major role in the tumor microenvironment remodeling to facilitate the tumor cell metastasis[141]. Vimentin was found to be 3 fold upregulated in human OSCC samples in comparison to normal tissues[142]. In our proteomics study on experimental model we have observed the sequential increase in Vimentin expression (Figure 4.4A). We noted a 2 fold up regulation of vimentin in tumors as compared to normal tissues. IHC data [Figure 4.4B] revealed that vimentin expression was not detectable in normal epithelial tissues but hyperplastic tissues demonstrated weak staining in cytoplasm and suprabasal layers (Figure 4.4B 1 and 2). We noticed increased suprabasal and cytoplasmic expression of vimentin in papillomas and carcinomas as compared to normal tissues (Figure 4.4B 3 and 4). It has been shown that Vimentin expression begins in epithelial layers of variety of human cancers including head and neck [143], prostate [5] and breast cancers[144]. Recent study from our lab has shown aberrant vimentin expression in precancerous lesions and SCC of oral mucosa[145]. Chaw et al 2012[146] have proposed that aberrant expression of vimentin may be used as a potential marker for malignant transformation in OSCC.

**Fascin (Fscn1)**

Fascin (Fscn1) is an actin-bundling protein that is found in membrane ruffles, microspikes, and stress fibers[147]. It is found to be associated with tumor cell invasion and metastasis in various types of cancers including human OSCC[148, 149]. Proteomics study on human
OSCC by Chi et al 2009[150] revealed that fascin was one of the several proteins that was found to be 3fold upregulated in tumor vs. normal tissues[150]. Our proteomics study suggests it’s sequential up regulation during the process of carcinogenesis and upregulation to 3 fold in SCCs as compared to normal tissues (Figure 4.5A). IHC studies on rat tongue at different stages revealed that fascin expression was not detectable in the vehicle treated group while weak cytoplasmic staining was observed in the basal layer of hyperplastic tissues (Figure 4.5B 1 and 2). Furthermore, strong cytoplasmic, and suprabasal staining was seen in papilloma and carcinoma tissues, respectively (Figure 4.5B 3 and 4). Similar observations were made by Shimamura and colleagues in human oral dysplasia who proposed that fascin over expression in dysplastic tissues drives tumor formation[151].

**Periostin (Postn)**

Periostin (Postn) is a matricellular protein and also reported as osteoblast-specific factor 2[152]. It is also referred as a stroma-associated protein and plays an important role in tumor development and is up regulated in a wide variety of cancers including head and neck[153, 154]. It is suggested to be a strong marker for prediction of metastasis in oral cancer patients[155]. Our proteomics data demonstrated its sequential up regulation during rat tongue carcinogenesis and a 3.7-fold upregulation in SCCs as compared to normal tissues (Figure 4.6A). Immunohistochemical analysis of periostin showed that periostin was not detectable in epithelial layers of normal and hyperplastic tissues while papillomatous lesions and SCC tissues showed periostin expression only in the stromal region (Figure 4.6B 1, 2, 3 and 4). A study by Kyutoku et al 2011[156] demonstrated that it plays pivotal role in tumor progression and metastasis of murine breast cancer and proposed that this molecule can be potential drug target against breast cancer. Together, these findings along with our result of progressive expression of periostin in 4NQO induced rat tongue tumors demonstrate its potential candidature for early diagnostic and prognostic marker for tongue tumors.
Transglutaminase 3 (Tgm3)

Transglutaminases are a family of calcium-dependent acyl-transfer enzymes that are widely expressed in mammalian cells[157]. Transglutaminase 3 enzyme is required for the cross-linking of the structural protein Trichohyalin and the keratin intermediate filaments to form a rigid structure within the inner root sheath cells[62]. Marked suppression of TGM3 is associated with various cancers like HNSCC[158]. We observed sequential down regulation of Tgm3 in our proteomics study and noted a ~6 fold down regulation in tumors as compared to normal tissues (Figure 4.7A). Validation by immunohistochemistry indicates its strong cytoplasmic and suprabasal expression in normal tongue tissues. While, its cytoplasmic expression was sequentially down regulated during the process of tumorogenesis (Figure 4.7B). Ohkura et. al. 2005[128] demonstrated that TGM3 is down regulated in human OSCC and proposed that the lack of TGM-3 expression may also facilitate survival in OSCC cells[128].

Cornulin (Crnn)

Cornulin (Crnn) is a recently identified protein also known as chromosome 1 open reading frame 10(C1orf10)[159]. It has conserved S100 EF-hand calcium binding motif and is highly expressed in esophagus. It also has a glutamine rich repeats at its C-terminal region which are frequently crossed linked by TGM proteins in differentiated layers of epithelia, and forms barriers protecting regenerative basal layer from exposure to environmental agents[160]. It has been observed that forced expression of cornulin leads G1/S cell cycle arrest and a down regulation of cyclin D1 in OSCC[161]. It is considered as late differentiation marker of skin.[162] Because of unavailability of specific antibody for cornulin against rat we validated our results of proteomics analysis using real time quantitative PCR. Our proteomics and real time data demonstrated marked and sequential down regulation of this protein (Figure 4.8A) and its mRNA in hyperplasia and papillomas.
and it was undetectable in tumors (Figure 4.8B). Proteomics data revealed its 14 fold down regulation in tumor as compared to normal tissues. Real time data revealed that cornulin down regulation is an early event in carcinogenesis. This indicates that cornulin might act as strong tumor suppressor\textsuperscript{36}. Our data correlates with findings of Schaaij-Visser et al 2010[163] in that cornulin expression was downregulated in mucosal epithelium at high risk of malignant transformation, when compared to normal oral mucosa.

Overall, we were able to validate differential expression of many known proteins during different stages of rat lingual carcinogenesis, whose differential expression has been shown in human system. Since expression of these proteins has already been reported in human OSCC we did not further validate these results in human samples. Our data underlines the importance of this model system for development of biomarkers. As stated earlier, we have also detected some novel proteins whose differential expression in lingual carcinogenesis has not been documented in patients. Further, we have validated three novel upregulated proteins while one novel down regulated protein in either rat and/or in human tissues. We have taken histologically normal (tissue 2 cm. away from the tumor, n=14), leukoplakia (n=10) and tongue tumors (n=32) for validation of proteins in human tissues.

**Thrombospondins 2:**

Thrombospondins (TSP) are secreted multidomain glycoproteins. They are involved in various functions including modulating cell adhesion, proliferation, migration, and angiogenesis\textsuperscript{[164]}. They regulate cell proliferation induced by rac1 redox-dependent Signaling. TSP-2 inhibits tumor growth and angiogenesis of human squamous cell carcinomas\textsuperscript{[165]}. In our rat proteomics data we noted its upregulation by ~ 7 fold in rat tongue tumor as compared to normal tissues (Figure 4.9A). Validation by real time PCR demonstrated its progressive up regulation during rat oral carcinogenesis (Figure 4.9B). The unexpected up-regulation of TSP2 may be explained by the species specific variation in
gene expression during carcinogenesis. Another possible reason may be difference in the mode of carcinogenesis process in our rat model and human tongue cancer.

**Trichohyalin:**
It is an intermediate filament-associated protein. It interacts with intermediate filament network of the inner root sheath cells of the hair follicles and the granular layer of the epidermis[166]. It may be involved in its own calcium-dependent post synthetic processing during terminal differentiation. Our rat proteomics data revealed its sequential down regulation during carcinogenesis process (Figure 4.10A). It was 14 fold down regulated in SCC in comparison to normal tissues. Real time validation showed its progressive down regulation during rat oral carcinogenesis (Figure 4.10B).

**Tenasin N:**
Tenascin is a high molecular weight extracellular matrix glycoprotein. Its expression was detected during embryogenesis, wound healing and neoplastic processes[167]. Tenasin N (Tnn) is novel member of Tenascin family and is expressed in brain, kidney and spleen and more so in the adult than in the developing mouse[168]. Our rat proteomics data demonstrated that tenasin N (Tnn) was sequentially up regulated across the stages of rat lingual carcinogenesis. It was found to be upregulated by 2.5 fold in SCC as compared to normal tissues (Figure 4.11A). To validate our proteomics results we performed immunohistochemistry on rat tissues (Figure 4.11B). Tenasin N expression was not seen in the vehicle treated rat tissues (control groups) while hyperplasia tissues showed weak cytoplasmic staining in keratinized layer of epithelium. Tenasin N expression was also confined to keratinized layer in papillomas and carcinomas. Carcinomas showed higher expression of tenasin N as compared to papillomas and hyperplastic tissues. We further validated tenasin N expression in human tongue tissues (Figure 4.11C). Immunohistochemical staining on human tissues revealed strong basal layer and cytoplasmic
expression of tenascin N in normal tissues (12/14) while up regulation was noticed in leukoplakia (9/10) in all layers. In human tongue tumors (27/32) tenascin N was expressed in keratinized tumor cells while its basal cell expression was weak. Strong cytoplasmic staining was detected in tumor cells. Intriguingly, Tenascin N was predominantly seen in keratinizing cells of the tumor tissues and basal layer showed very weak expression (Figure 4.11 C3). The significance of this finding is unclear.

**Coronin 1a (Coro1a)**

Coronin is highly-conserved family of F-actin binding proteins. These are abundantly expressed in lymphocytes and macrophages. Coronins appear to function primarily in association with the membrane cytoskeleton through interactions with filamentous actin (F-actin) and the Arp2/3 protein complex, which plays a role in generating branches in the actin filament network. Recently Sun et al. 2014 reported that coronin3 regulates metastasis and invasion of gastric cancer cells through Arp 2 protein[169]. Coronin 1a (Coro1a) is predominantly expressed in hematopoietic cells[170]. It mediates actin dynamics in a variety of processes including cancer. It’s over expression has been associated with the breast cancer development and migration[171]. Results of our iTRAQ analysis showed sequential up regulation of coronin1a at different stages of rat oral carcinogenesis (Figure 4.12A). It was 6.5 fold upregulated in rat SCC as compared to normal tissues. Therefore, to validate results of our iTRAQ analysis we further carried out Immunohistochemistry on tissue sections at different stages of rat lingual carcinogenesis (Figure 4.12B). As Coro1a is exclusively expressed by hematopoietic cells, normal or abnormal epithelial cells did not stain for coronin1a while infiltrating dendritic cells and hematopoietic cells were stained. Examination of papilloma and tumor tissues revealed more infiltrating hematopoietic cells in tumors than papilloma thus giving more staining of coronin 1a in tumor than in papilloma. Furthermore, we carried out Immunohistochemical analysis of coronin1a expression in
histologically normal, leukoplakia and tongue tumor tissues. We noticed that coronin1a expression was restricted to only in infiltrating hematopoietic cells. None of the epithelial layer from normal or leukoplakia and tumor cells from tumor tissue showed coronin 1a expression. This can be explained on the basis of fact that normal and leukoplakia tissues have less infiltrating hematopoietic cells or dendritic cells than tumor tissues thus up regulation of Coronin 1a in tumor would be because of more hematopoietic cells in tumors (Figure 4.12C). Thus our results underline the facts that iTRAQ data needs validation using immunohistochemistry and one has to be cautious while interpreting the results of iTRAQ data. For example Kim et. al. 2009[171] have carried out proteomics analysis on breast tumor samples which has not been validated by immunohistochemistry although they have been able to give proof of principle using cell line studies[171].

Some of the sequentially altered proteins include, MMP9, Annexin A4, Secreted protein acidic and rich in cysteine (SPARC), Dermokine, Afadin etc which we have not validated further.

Matrix metalloproteases (MMPs), also named as matrixins, are zinc-dependent endopeptidases that are the major proteases involved in ECM degradation[172, 173]. MMPs can degrade a wide range of extracellular molecules and a number of bioactive molecules. MMPs play a central role in cell proliferation, migration, differentiation, angiogenesis, apoptosis and host defenses. The enzyme encoded by this gene degrades type IV and V collagens. Two different soluble gelatinases have been identified: gelatinase A, 72 kDa (MMP-2), and gelatinase B, 92 kDa (MMP-9). Both contain a collagen-binding domain within their catalytic domain, distinguishing them from other MMPs. MMP-9 is associated with the aggressive nature of many cancers, including OSCC and this aggressive nature was thought to cause type IV collagen degradation, a main component of basement membranes[174]. MMP-9 has other bioactive substrates that independently modulate
carcinogenesis, such as the pro-transforming growth factor-β1 (TGF-β1) and the pro-tumor necrosis factor-α (TNF-α)[174]. In present study we observed sequential upregulation of MMP-9 during the process of rat oral carcinogenesis.

Secreted protein acidic and rich in cysteine (SPARC) is an extracellular Ca2+-binding glycoprotein that associates with cell populations undergoing migration, morphogenesis, and differentiation[175]. It is also termed osteonectin, BM-40, and 43K protein. SPARC acts as a key regulator of critical cellular functions such as proliferation, survival, and cell migration[176]. SPARC is differentially expressed in various cancers and in the surrounding stroma compared to normal tissues and its expression pattern is variable and highly dependent on the type of cancer. High levels of SPARC expression have been reported in breast[177], prostate[72], colon rectal[178] and brain cancers[179]. On the contrary, low levels of SPARC expression have been reported in other types of malignancies, as pancreas[180], bladder cancer[181] and acute leukemia[182]. In our study we found sequential up regulation of SPARC during rat oral carcinogenesis.

Annexin IV (ANX4) belongs to the annexin family of calcium-dependent phospholipid binding proteins[183]. Although their functions are still not clearly defined, several members of the annexin family have been implicated in membrane-related events along exocytotic and endocytotic pathways. ANX4 is almost exclusively expressed in epithelial cells. ANX4 has been shown to aggregate on lipid layers upon Ca2+ binding in vitro, a characteristic that may be critical for its function[184]. Our proteomics data showed sequential up regulation of Annexin IV across the stages.

Dermokine (DK) is a gene that was first observed as expressed in the differentiated layers of skin. Its two major isoforms, alpha and beta, are transcribed from different promoters of the same locus, with the alpha isoform representing the C terminus of the beta isoform[185].

Discussion
Dermokine-β is a secreted protein abundant in stratified epithelia, and high calcium concentration markedly elevates dermokine expression. Dermokine-β/γ was expressed in keratoacanthoma and a part of well-differentiated squamous cell carcinoma (SCC)[186]. Serum DK-β/γ is the most promising of the existing tumor biomarkers for the diagnosis of early-stage colorectal cancer[187]. In contrast to the finding reported for colorectal cancer, we found sequential down regulation of DK during the process of carcinogenesis. This observation can probably be explained on the basis of species specificity.

The human kallikrein 8 protein (KLK8) is expressed in many normal tissues including esophagus, skin, testis, tonsil, kidney, breast, and salivary gland, and is found in biological fluids including breast milk, amniotic fluid, seminal fluid and serum[188]. It has also been shown to be a biomarker and prognostic factor for breast cancer. KLK8 is downregulated in breast cancer tissues and cell lines[189]. It has been suggested that expression of KLK8 may be regulated by sex steroid hormones in endometria, and that elevated KLK8 mRNA and KLK8 expression is an early event in endometrial carcinogenesis[190]. In a mouse model, KLK8 suppresses tumor growth and invasion in vivo.[191] In present study we observed sequential downregulation of KLK8 across the stages.

Afadin is an actin filament-binding protein that binds to nectin, an immunoglobulin-like cell adhesion molecule, and is colocalized with nectin at cadherin-based cell-cell adherens junctions (AJs). Afadin(-/-) mice showed developmental defects at stages during and after gastrulation, including disorganization of the ectoderm, impaired migration of the mesoderm, and loss of somites and other structures derived from both the ectoderm and the mesoderm.[192] We observed sequential downregulation of Afadin in our rat oral carcinogenesis.
As stated earlier we obtained a total identification of 2,223 proteins in our proteomics analysis. We performed Gene Ontology analysis in order to segregate the identified proteins in to two groups according to their sub cellular localizations and to their biological functions. Among the identified proteins, 1835 proteins (83%) were categorized in to their sub cellular localization. Majority of proteins were localized in to cytoplasm (20 %), nucleus (16%), mitochondria (8%), membrane (16 %) etc. while sub cellular localization of 18% proteins remained uncategorized. Amongst identified proteins, 1786 proteins (80%) belong to the category whose biological function is known. Majority of these proteins could be categorized in to metabolism (21%), cell cycle (14%), protein folding and proteolysis (17%) while 20% of these proteins remain unclassified.

We further carried out GO analysis for differentially expressed proteins in tumors. Amongst 194 upregulated proteins, 157 proteins belong to different biological pathways i.e. cell signaling, immune response, metabolism etc. Sixteen percent of these proteins are known to play a role in immune response for example proteins like S100A8 and S100A9 which are normally abundantly expressed by myeloid lineage cells like monocytes, neutrophils and macrophages[193] were found to be upregulated in tumors. These proteins have earlier been shown to be upregulated in cancers like breast cancer[194].

Amongst 221 down regulated proteins, 159 proteins belong to one of the biological pathways i.e. metabolism (19%), cell signaling (15%), cell cycle (15%). Cell cycle plays a crucial role in tumorigenesis. We observed down regulation of cell cycle related proteins for example four and a half LIM domains 1 (FHL1) and cyclin-dependent kinase inhibitor 1B (p27kip1) in tumors. FHL1 expression is found to be downregulated in several types of human tumors like breast cancer[195] and oral cancer[196]. It is known to inhibit the tumor growth through TGF beta signaling pathway[195]. Similarly p27 (Kip1) is a cyclin-dependent kinase inhibitor which regulates progression of cells from G1 into S phase in a
cell cycle. Under expression of p27 has been reported from various cancers including breast, ovary, prostate and other tissues[197]. It has been shown that over expression of p27 in oral cancer cell line resulted in growth arrest and cell death by apoptosis[198].

Thus it is evident from above discussion that further validation of these proteins in human system will help in the development of a battery of early diagnostic and prognostic marker for human oral cancer.