Global protein profiling during rat lingual carcinogenesis and validation of differentiator proteins in human tongue

By

Bihari Lal Soni

[LIFE09200704007]

Tata Memorial Centre

Mumbai

A thesis submitted to the

Board of Studies in Life Sciences

In partial fulfillment of requirements

For the Degree of

DOCTOR OF PHILOSOPHY

Of

HOMI BHABHA NATIONAL INSTITUTE

May, 2015

Homi Bhabha National Institute

Recommendations of the Viva Voce Committee

As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by Bihari Lal Soni entitled "Global protein profiling during rat lingual carcinogenesis and validation of differentiator proteins in human tongue" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

Vinay Kumar
----------Date: 19/05/2015

Chairperson: Dr. Vinay Kumar

M M Vaidya
----------Date: 19-05-2015

Guide/Convener: Dr. Milind M. Vaidya

----------Date: 19.05.2015

Member 1: Dr. G. B. Maru

----------Date 19/05/15.

Member 2: Dr. Rukmini Govekar

----------Date: 19.5.2015

Invitee: -- Dr. Anita Borges

Neeta Singh
----------Date: 19.5.15

External examiner: Dr. Neeta Singh

The final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to HBNI. I hereby certify that I have read this thesis prepared under my direction and recommend that it may be accepted as fulfilling the thesis requirement.

Date: 19th May2015

Place: ACTREC, Mumbai.

M M Vaidya

Dr. Milind M. Vaidya

Guide

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at Homi Bhabha National Institute (HBNI) and is deposited in the Library to be made available to borrowers under rules of the HBNI.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the Competent Authority of HBNI when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

Navi Mumbai, Bihari Lal Soni

Date: 19th May 2015

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. This work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Navi Mumbai, Bihari Lal Soni

May 2015

List of Publications arising from the thesis

Journal: Clinical Communications Oncology

1. Soni BL, Marimuthu A, Pawar H, Sawant SS, Borges A, Kannan R, Pandey A, Ingle AD, Harsha HC, Vaidya MM. Quantitative proteomic analysis of different stages of rat lingual carcinogenesis. Clin Commun Oncol 2014;1:2

National/International conferences attended:

1. Presented Poster in keystone F2: "Proteomics, Interactomes", entitled "Quantitative Proteomics Analysis of Different Stages of Experimental Tongue Carcinogenesis for Biomarker Discovery", May 7, 2012-May 12, 2012, at the Clarion Hotel Sign, Stockholm, Sweden.

2. Presented Poster in American Association of Cancer Research conference entitled "Sequential changes in galectin 7 expressions during rat lingual carcinogenesis" in New Horizons in cancer Research: Biology to prevention to Therapy, Gurgaon, Delhi, Dec. 13-16, 2011.

3. Presented Poster entitled "Global protein profiling of sequential changes during rat lingual carcinogenesis" in Clinical Proteomics: technology and beyond, ACTREC, Mumbai, Feb 2011.

4. Participated in 5th AOHUPO Congress. 14th ADNAT Convention & 1st PSI Conference On New Perspectives in Proteome Research:Feb 21-25, 2010, Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500007, India

5. Poster Presentation "Global protein profiling of sequential changes during rat lingual carcinogenesis"in the International Symposium on: Mass Spectrometry in Life Sciences. NCBS, Bangalore. September 27, 2009 to September 29, 2009.

Bihari Lal Soni

DEDICATED TO AMMA AND BABU JI

ACKNOWLEDGMENT

This PhD work would have been impossible without the help and support of many people and I cherish this opportunity to thank them all for their contribution. First and foremost, I would like to thank Dr. Vaidya for being a wonderful and a very patient mentor. He himself became an example to remain enthusiastic no matter how difficult the time, take unorthodox approaches to solve a problem, and multi-task with ease has been the lesson for life.

I am thankful to Dr. S. Chiplunkar (Director, ACTREC), Dr. R. Sarin (Ex-Director, ACTREC) and Dr. S. Zingde (Ex-Deputy Director, ACTREC) for providing the infrastructure and constant encouragement to carry out research in the premier cancer centre, ACTREC, Tata Memorial Centre.

I have been most fortunate to have eminent DC members on my panel: Firstly, I wish to thank Dr. Zingde, the Chairperson of the first doctoral committee, for all her efforts towards motivating me and I also thank her for expert comments on proteomics part of my study. I am also grateful to have Dr. Maru as my DC member. His expert comments and advices helped me a lot to develop the rat model and understand the carcinogenesis process. I would like to also thank Dr. Anita Borges whose expertise in analysis of histology gave me insight of understanding of histology. I am highly indebted to Prof. Vinay Kumar, the chairperson of the present DC, for his expert comments on my project. I also want to extend my thanks to Dr. Rukmini Govekar whose expertise in 2DE helped me a lot in understanding the initial proteomics study.

I would like to specially thank Professor Akhilesh Pandey, Founder and Director of Institute of Bioinformatics for agreeing to collaborate with us and also giving me opportunity to work in his institute. I am also thankful to Dr. Harsha Gowda for his valuable support and guidance in iTRAQ study during my visit to IOB.

I wish to convey my warmest thanks to all my lab members (Past and Present). I would like to thank my seniors Dr. Deepak Kanojia, Dr. Hunain Alam and Dr. Sapna Iyer for being such wonderful and inspirational colleague of mine. I would specially like to thank Deepak and Alam whose scientific discussion in lab shaped my thinking in science. A special thanks to my present colleagues Crismita, Richa and Pratik whose cheerful attitude made the lab environment joyful and alive. I am also thankful to Vinita, Vaishali, Rajesh 'Bhaiya' and Sridhar for their support and help in all respect.

It is my pleasure to thank all my batchmates (batch 007) whose constant encouragement and support paved my path to PhD so simple and joyful. I specially want to thank Harsh Pawar "Mota Bhaee" for helping me a lot in my struggling days. I want to thank Ajit, Akhil, Dilip, Dimpu, Hemant, Sumeer, Gaurav and Vinayak for being such a wonderful friend. I specially want to thank the one and only lady in our batch Ratika for tolerating us for so many years. I am

indebted to my entire friend circle whose constant support and love, made my PhD journey such a joyful. I would also like to thank my M.Sc. friends Ahan, Akhilesh. Subhanarayan Das, Ravi, Pawan, Deepak, Hari, Saiyyad, Ganesh and many more for their constant love and support.

I am extremely thankful to Vaishali Kailaje, Tanuja, Jairaj and Mansi from Imaging Facility for helping me acquire all the microscopic images. I am also thankful to Mr. Uday Dandekar for his efforts towards maintaining the Common Instrument Facility. I wish to thank Mr. Sawant and specially Shyam for their help and support. I am extremely thankful to Dr. Ingle and their staff, Shashi, Mahesh and Sada for helping me with animal handling, and I also thank Chavhan ji and Sakpal ji for providing me with tissue sections and H&E slides. I want to thank Mr. Shashi Dolas, proteomics facility for his immense support provided to me for the MALDI-TOF-TOF. I also would like to thank all the rats whose precious tissue samples helped me to achieve my PhD thesis.

I am also grateful to CRI for my fellowship and DBT for funding my project.

Last but not least, I am thankful to GOD and my family who gave me strength during struggling times of my life. I am indebted to my parents whose constant support, sacrifices and struggle for me can never be paid back. I am also thankful to my brother and sisters whose love, guidance, belief and support made me strong enough to travel the path of PhD. You all have been my source of strength and inspiration throughout my life.

Bihari Lal Soni

CONTENTS Page No.

Homi Bhabha National Institute

Ph. D. PROGRAMME

1. Name of the Student:	Bihari Lal Soni
2. Name of the Constituent Institution:	Tata Memorial Centre**,** Advanced Centre for Treatment, Research and Education in Cancer.
3. Enrolment No. :	LIFE09200704007 (3rd September 2007)
4. Title of the Thesis:	Global protein profiling during rat lingual carcinogenesis and validation of differentiator proteins in human tongue
5. Board of Studies:	Life Sciences.

SYNOPSIS

1. Introduction:

Oral squamous cell carcinoma (OSCC) remains a major cancer in the Indian subcontinent, comprising more than 30 % of all cancers. The most commonly involved sites of tumor development in the Indian population are buccal mucosa and tongue[1]. The major risk factors for oral cancer are chewing tobacco either alone or with allied products and alcohol consumption. Precancerous lesions of leukoplakia and sub mucous fibrosis are also prevalent in India due to these habits[2]. The five year survival rate of OSCC has not changed in the last few decades.

In patients, the molecular analysis of multiple steps is hampered by the unavailability of biopsies of all the stages of carcinogenesis. However, animal models of carcinogenesis allow the reproducible isolation of all stages, including normal tissues, which are then amenable to pathological, genetic and biochemical analyses[3]. To this end, 4 Nitro-quinoline 1 oxide (4NQO) induced rat model of carcinogenesis remains the preferred model for studies related to oral carcinogenesis because it mimics molecular and pathological changes observed in humans[4].

Proteomics has grown as a powerful tool for biomarker discovery[5]. iTRAQ-based LC MS-MS is a powerful tool which is utilized in large number of proteomics studies to understand the difference between protein expression profiles of normal vs. diseased samples[6].

In spite of the fact that a large number of molecules have been identified as potential early diagnostic and prognostic markers for oral cancer, none of them has reached the clinics. Possible reasons could be, 1. Most of the studies do not specify which sub sites were studied and 2. Very few studies have attempted sequential analysis. In

order to sequentially dissect the molecular events during different stages of carcinogenesis, it was proposed to carry out proteomic analysis on samples obtained at sequential stages of rat lingual carcinogenesis. Thus the work in this thesis is towards dissecting sequentially molecular alterations occurring at a single subsite i.e. tongue using a rodent model and validating the observations in cancer of human tongue. The objectives were thus as follows:

OBJECTIVES:

1. Establishment of rat lingual cancer model induced by carcinogen

2. Identification of differentially expressed proteins at different stages of lingual cancer development in a rat model using quantitative proteomics

3. Validation of results obtained from quantitative proteomic study

4. Correlation of the data with human samples.

2. Materials and Methods:

2.1 Establishment of rat lingual cancer model induced by carcinogen (4NQO)

The study was approved by the Institutional animal ethics committee. Approximately 5-6 weeks old Sprague Dawley rats (SD rats) were given 4NQO in drinking water at a concentration of 30 ppm. Rats were divided in three groups and each group was kept for 80, 120, 160 and 200 days respectively. All the animals in a group were sacrificed at each time point, the tongue examined for lesions and the respective tissues were collected and stored at -80^0C.

2.2. Histology

5-8µm thick sections of the tissues cut from paraffin blocks were stained with hematoxyline and eosin, and were examined under upright microscope (Axio imager Z1, Zeiss). The pathological status of the tissues was defined by the pathologist.

2.3. Quantitative proteomic studies of rat tissues

2.3.1.1: 2-Dimensional gel Electrophoresis

Total cell lysate of tongue tissue was prepared in Urea lysis buffer. Protein estimation was done by using RC-DC kit (Sigma, USA). Two hundred µg of lysate proteins were resolved on the first dimension using 17 cm strips of either pI range 3-10 or 4-7 (Bio-Rad).

After completion of IEF, the strips were equilibrated in Equilibration buffer I and II for 15 min. respectively. Each strip was placed on to 12% SDS polyacrylamide gel and resolved as per Laemmli protocol[7].

The gels were washed and stained with silver essentially according to Fulzele et. al. 2013[8]. The stained proteins on the gels were scanned and proteins which were differentially expressed were subjected to mass spectrometry.

2.3.1.2 Mass spectrometry analysis

Differentially expressed protein spots were cut out from the gel, destained in destaining solution and were subjected to in-gel digestion with Trypsin (20ng/gel piece). The peptides were extracted, reconstituted in 1% Trifluroacitic acid (TFA) and anlyzed on the MALDI TOF-TOF Ultraflex-II from Brucker Daltonics, Germany.

2.3.2: iTRAQ analysis

Hundred µg of protein was pooled from each group of normal (n=10), hyperplasia (n=5), papilloma (n=5) and tumor (n=5) tissues and digested with proteomics grade Trypsin. Peptides generated from normal, hyperplasia, papilloma and carcinoma tissues were labeled with reporter ions of m/z 114, 115, 116 and 117 respectively as per manufacturer's protocol. Labeled samples were then pooled and subjected to strong cationic exchange chromatography (SCX). SCX fractions were subjected to nanoflow LC system (Agilent 1200 Series) interfaced with LTQ-Orbitrap Velos mass spectrometer. Spectra obtained by mass spectrometer were analyzed by Proteome Discoverer software (Thermo Scientific).

2.4. Bioinformatics analysis of proteomics Data

All differentially expressed proteins were subjected to Gene Ontology (GO) analysis.

2.5. Validation of results obtained by quantitative proteomic study

Some of the differentially expressed proteins identified using either 2DE or iTRAQ-LC- MS/MS analysis of rat tissue samples were further validated by Immunohistochemistry (IHC) and/or RT-PCR. Human tongue samples were also used for validation of novel proteins.

2.5.1 Antibodies

The following antibodies were used

Antibody	Dilution	Clone	Company	Catalog no.
Vimentin	1:400	V9 clone, Mouse monoclonal	Sigma	V 6630
Transglutaminase 3	1:8000	Mouse monoclonal	SantaCruze	sc-101366
Periostin	1:50	Rabbit polyclonal	SantaCruze	sc 49480
Coronin 1a	1:1000	Rabbit polyclonal	Covance	PRB-5002-100
Tenascin N	1:100	Rabbit polyclonal	HPA	HPA-026764
Fascin	1:200	Mouse monoclonal	Thermo Fischer	MA1-20912
Galectin 7	1:300	Mouse monoclonal	SantaCruze	Sc 166222 (H8)

2.5.2 Immunohistochemistry

Formalin-fixed, paraffin-embedded, 5 μm thick rat tissue sections were mounted on poly-L-lysine coated glass slide. IHC was carried out with respective antibody as per standard protocol. Diaminobenzidine was employed as the chromogen and slides were counterstained with Mayor's hematoxylin.

2. 5.3 RNA isolation and qRT-PCR

To validate the proteomics data qRT-PCR was performed whenever the respective antibodies were not available. Total cellular RNA was extracted from the tissue by Tri-reagent (Sigma-Aldrich, USA) as per manufacture's protocol. RNA was estimated by measuring absorbance at 260 nm and 280 nm using nanodrop (ND-1000 Spectrophotometer, Wilmington, USA). cDNA synthesis was carried out as per the manufacturer's protocol (Fermentas, Thermo Scientific, Waltham, MA). Obtained cDNA was used as template for qRT-PCR. Master Mix SYBR Green (Applied Biosystems, Bedford, MA) was used with 5 nM of forward and reverse primers. Real-time quantitative PCR was performed with the ABI PRISM7700 Sequence Detection System. Beta actin gene was used as endogenous control. All amplifications were done in triplicate. Results were expressed as relative gene expression using the $2^{-\Delta\Delta}$Ct method[9].

2 Results

2.1 Development of rat model of carcinogenesis

The animals treated with milliQ water, acetone or 80 days 4NQO did not reveal any alterations at the dorsum of the tongue. Hyperplasia/atypical hyperplasia was observed after 120 days and papilloma/atypical papilloma was observed after 160 days at the dorsum of the tongue. Squamous cell carcinoma developed at the dorsum of the tongue in 200 days in rats treated with 4NQO.

3.2 Differential Proteomics

3.2.1: 2DE gel electrophoresis

In the initial study using 2DE gel electrophoresis, five differentially expressed proteins were identified. These included three upregulated proteins; fatty acid binding protein 5, keratin 6 A and serum albumin precursor protein and two down regulated proteins; galectin 7 and transglutaminase 3.

Due to limitations in proper resolution in 2DE gel and poor identification of proteins by MALDI-TOF-TOF, we employed iTRAQ-LC-MS/MS based quantitative proteomics technology for better proteome coverage.

3.2.2: Proteomics study using iTRAQ technology

Four plex iTRAQ LC-MS proteomics analysis at each stage during the tongue tumerogenesis induced by 4NQO in Sprague Dawley rats resulted in identification of 2,223 proteins from the rat tongue SCC of which 415 proteins were found to be differentially expressed in comparison to normal (untreated tissues). Of these 415 proteins, 194 proteins were up-regulated while 221 proteins were down-regulated in SCC of tongue tissues. Table 2 describes the details of differentially expressed proteins at each stage.

Stages	No. of Up regulated proteins	No. of Down regulated proteins	Total proteins
Hyperplasia	**35**	**74**	**109**
Papilloma	**155**	**178**	**333**
SCC	**194**	**221**	**415**
Total	**384**	**473**	**857**

Among the differentially expressed proteins, 5 proteins were sequentially upregulated while 10 proteins were sequentially down regulated from hyperplasia to SCC. Similarly, sequentially up or down regulation of 62 and 51 proteins respectively was observed from papilloma to carcinoma tissues.

3.3 Validation of differentially expressed proteins

3.3.1 Proteins from gel based study

From the five differentially expressed protein spots identified in the 2DE based study galectin7 was validated because it showed down-regulation as opposed to available literature showing its upregulation in human SCC[10]. Galectin7 was validated by IHC and found to be down-regulated in rat tongue SCC.

3.3.2 Proteins from iTRAQ based study

Several proteins like Vimentin, K14, K17, MMP9, TGM3 and Periostin, had been reported earlier in human OSCC and were also found to be differentially expressed in rat tongue SCC. The analysis, in addition, detected number of novel proteins which have not been reported previously in human OSCC. In this study some of the known candidate proteins whose differential expression in human oral carcinomas has been shown previously by us and others were validated by IHC or qRT-PCR. Vimentin, Fascin, Periostin and Transglutaminase3 were validated by IHC while Cornulin was validated by qRT-PCR. Vimentin, Fascin and Periostin were found to be sequentially up-regulated while Transglutaminase 3 and Cornulin were found to be sequentially down regulated.

3.3.3 Validation of Novel molecules

Four novel molecules identified by iTRAQ were also validated for their expression in rat tissues. These include Tenascin N and Coronin 1a by Immunohistochemistry and showed sequential upregulation. Trichohyalin and Thrombospondin 2 were validated by real time PCR. Trichohyalin was sequentially down regulated while Thrombospondin 2 was sequentially upregulated. The expression of Tenasin N and Coronin1a were evaluated in human tongue tissues (normal (n= 14), Leukoplakia (n=

10) and Tumor (n=32)) to determine if the observations from the rat model are also valid in human tongue.

4. Bioinformatics by GO analysis

Bioinformatics analysis was carried out to classify proteins based on subcellular localization and biological function using Gene Ontology (GO) annotations.

These results will be discussed at length in thesis.

5. Conclusions and Future perspective

This is the most extensive quantitative proteomic study in rat model of 4NQO induced oral carcinogenesis carried out to date. Through this model several known proteins like vimentin, fascin, transglutaminase3, periostin and cornulin were identified thereby supporting the use of the model for evaluating markers for different steps of the carcinogenesis process. The model has also enabled the identification of novel molecules like Tenascin N, Coronin1a, Trichohyalin, and Thrombospondin2. Using this model, it has been possible to show sequential alterations in expression pattern during rat tongue carcinogenesis. Furthermore, the observations could also be extrapolated from the rat model data to human system indicating the fact that this model has potential to be used for biomarker discovery for human oral cancer. The clinical utility of the novel proteins will be now evaluated on a large scale on human tissues of SCC of tongue at different stages i.e. from T1 to T4, and leukoplakia of tongue with the ultimate aim of establishing these proteins as predictive markers for human oral cancer.

References :

1. Sankaranarayanan, R.; Black, R.; Swaminathan, R.; Parkin, D., An overview of cancer survival in developing countries. *IARC scientific publications* **1998**, 135-157.
2. Hunter, K. D.; Parkinson, E. K.; Harrison, P. R., Profiling early head and neck cancer. *Nature reviews cancer* **2005,** 5, (2), 127-135.
3. Kanojia, D.; Sawant, S. S.; Borges, A. M.; Ingle, A. D.; Vaidya, M. M., Alterations in keratins and associated proteins during 4-Nitroquinoline-1-oxide induced rat oral carcinogenesis. *Journal of carcinogenesis* **2012,** 11, (1), 14.
4. Kanojia, D.; Vaidya, M. M., 4-Nitroquinoline-1-oxide induced experimental oral carcinogenesis. *Oral oncology* **2006,** 42, (7), 655-667.
5. McDonald, W. H.; Yates, J. R., Shotgun proteomics and biomarker discovery. *Disease markers* **2002,** 18, (2), 99-105.
6. Pawar, H.; Kashyap, M. K.; Sahasrabuddhe, N. A.; Renuse, S.; Harsha, H.; Kumar, P.; Sharma, J.; Kandasamy, K.; Marimuthu, A.; Nair, B., Quantitative tissue proteomics of esophageal squamous cell carcinoma for novel biomarker discovery. *Cancer biology & therapy* **2011,** 12, (6), 510-522.
7. Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970,** 227, (5259), 680-685.
8. Fulzele, A.; Malgundkar, S. A.; Govekar, R. B.; Patil, A.; Kane, S. V.; Chaturvedi, P.; D'Cruz, A. K.; Zingde, S. M., Proteomic profile of keratins in cancer of the gingivo buccal complex: Consolidating insights for clinical applications. *Journal of proteomics* **2013,** 91, 242-258.
9. Schmittgen, T. D.; Livak, K. J., Analyzing real-time PCR data by the comparative CT method. *Nature protocols* **2008,** 3, (6), 1101-1108.
10. Alves, P. M.; Godoy, G. P.; Gomes, D. Q.; Medeiros, A. M.; de Souza, L. B.; da Silveira, E. J.; Vasconcelos, M. G.; Queiroz, L. M., Significance of galectins-1, -3, -4 and -7 in the progression of squamous cell carcinoma of the tongue. *Pathol Res Pract* **2011,** 207, (4), 236-40.

Publications in Refereed Journal:

Accepted: Bihari Lal Soni, Arivusudar Marimuthu, Harsh Pawar, Sharada S. Sawant, Anita Borges, Ranganathan Kannan, Pandey, A., Arvind D. Ingle, Harsha, H. C., and Vaidya M.M. "Quantitative proteomic analysis of different stages of rat lingual carcinogenesis" Accepted for publication in Clinical Communications-Oncology.

Signature of Student:

Date: 16.04.2019

Doctoral Committee:

Sr. N	Name	Designation	Signature	Date
1	Dr. Vinay Kumar	Chairman		17/04/2014
2	Dr. Milind Vaidya	Convener		16.04.2019
3	Dr. G.B. Maru	Member		16.04.2014
5	Dr. Rukmini Govekar	Member		16/04/14.
6	Dr. Anita Borges	Invitee		18/04/2014

Forwarded through

Dr. S.V. Chiplunkar

Director, ACTREC &

Chairperson, Academics and Training Programme,

ACTREC Dr. S. V. Chiplunkar
Director
Advanced Centre for Treatment, Research & Education in Cancer (ACTREC)
Tata Memorial Centre

Dr. K.S. Sharma

Director Academics

Tata Memorial Centre.

Prof. K.S. Sharma
DIRECTOR - ACADEMICS, TMC
Mumbai - 400 012

Abbreviations

2-DE: 2-dimensional gel electrophoresis

4NQO: 4-Nitroquinoline 1-oxide

cDNA: complementary DNA

CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

DIGE: Differential in gel electrophoresis

DMBA: 7,12-dimethylbenz[α]anthracene

DTT: Dithiothreitol

FDR: False discovery rate

HPV: Human papilloma virus

HRP: Horseradish peroxidase

ICAT: Isotope-coded affinity tags

IEC: Isoelectric cell

IEF: Isoelectric focusing

iTRAQ: Isobaric tags for relative and absolute quantitation

LC-MS: Liquid chromatography–mass spectrometry

MALDI: Matrix-assisted laser desorption/ionization

MMTS: Methyl methanethiosulfonate

NEPHGE: Non-equilibrium pH gel electrophoresis

OSCC: Oral squamous cell carcinoma

OSMF: Oral submucous fibrosis

Q RT-PCR: Quantitative real-time polymerase chain reaction

SCX: Strong cation exchange

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SILAC: Stable isotope labeling by/with amino acids in cell culture

TBST: Tris-Buffered Saline and Tween 20

TCEP: Tris(2-carboxyethyl)phosphine

TEMED: Tetramethylethylenediamine

TFA: Trifluoroacetic acid

TOF: Time of flight

List of Tables

List of Figures

CHAPTER 1
INTRODUCTION

Every year 400,000 new cases of oral cavity and pharynx cancer occur worldwide and more than 50 % of which occur in India. Each year over 200,000 people die of the disease, and over a third of these deaths occur in India[1]. The most commonly involved sites of tumor development in the Indian population are buccal mucosa and tongue[2]. The major risk factors for oral cancer are chewing tobacco either alone or with allied products and alcohol consumption. Precancerous lesions of leukoplakia and sub mucous fibrosis are also prevalent in India due to these habits[3].

Advances in surgery, radiation and chemotherapy have not changed the survival rates[4]. The clinical staging of oral cancer has limited prognostic importance as the patients with comparable stages respond differently to the same therapy. Several studies have focused on defining tumor-specific molecular markers that can either detect cancer at an early stage or can predict patient's outcome [4, 5]. However, clinicopathological factors and molecular biomarkers that could identify patients at early stage or patients at highest risk of recurrence/ lymph node metastasis are still undefined [6].

At present there is paucity of sensitive and specific early diagnostic and prognostic markers of OSCC. In human system it is not possible to get all the stages of oral carcinogenesis and tissue size is also a major limitation. Cancer progression is multistage development process and involves accumulation of genetic lesions resulting into alterations in cell proliferation and differentiation pathways[7]. During carcinogenesis many biochemical pathways involved in development, differentiation, proliferation, apoptosis, cell signaling, cell cycle, angiogenesis etc. get altered [8]. Experimental chemical carcinogenesis is being widely used to investigate the process of carcinogenesis. Rodent models like mouse, rats and Hamsters using chemical carcinogens such as 4NQO, DMBA etc. are being routinely used to study oral carcinogenesis process [9].

There is a need to devise critical tools for the early detection of OSCC and the monitoring of disease progression. In addition, the identification of therapeutic targets is an attractive strategy to further relieve the burden of OSCC. Among these tools, validated biomarkers are viewed as the most important tool[10]. Therefore there is a critical need to discover new specific and sensitive biomarkers in OSCC.

Proteomics is a promising approach in the identification of proteins which may be used as markers for early detection of cancers and prediction of regional lymph nodal metastasis [11]. It has been successfully employed in studies of various tumors, tissues and body fluids. Many studies on oral cancer patients led to identification of possible biomarkers for early diagnosis/ prognosis. Development of oral biomarkers by using genomics and proteomics approaches have been reviewed earlier by R. Ralhan [10]. Various proteomics platforms have been used to identify the biomarkers for early diagnosis and prognosis of Oral cancer[5, 12-15].

In spite of the fact that a large number of molecules have been identified as potential early diagnostic and prognostic markers for oral cancer, none of them has reached the clinics. In order to sequentially dissect the molecular events during different stages of oral carcinogenesis, proteomic analysis on samples obtained at sequential stages of rat lingual carcinogenesis was carried out. We used iTRAQ-LC-MS system for precise detection of differences in protein profile at various stages of lingual carcinogenesis.

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.

Authors	Country	Year	No. of patients	Malignant Transformation (%)	Observation periods (Years)	Reference
Silverman *et al.*	India	1976	4762	0.13	2	[45]
Gupta *et al.*	India	1980	360	0.3	1–10 (7)	[46]
Mehta *et al.*	India	1972	117	0.8	10	[47]
Gupta *et al.*	India	1980	410	2.2	1–10 (8)	[46]
Einhorn *et al.*	Sweden	1967	782	4.0	1–20	[48]
Kramer *et al.*	England	1969	187	4.8	1–16	[49]
Bánócy	Hungary	1977	670	6.0	1–30	[50]
Lind	Norway	1987	157	8.9	6	[51]
Gangadharan *et al.*	England	1971	626	10.0	1–19	[52]
Schepman *et al.*	Holland	1997	166	12.0	6M–17 (2.7)	[53]
Silverman *et al.*	USA	1984	257	17.5	6M–39 (7.2)	[54]

Table 2.1: Malignant transformation potential of Leukoplakia (modified and adopted from T. Amagasa et. al. 2006)

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 cholanthrene (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].

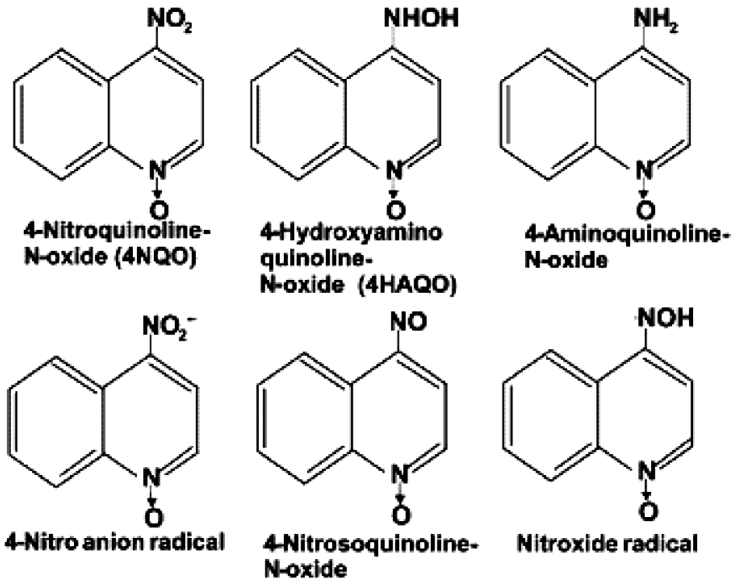


Figure 2.1: Structure of 4NQO and its metabolites (Koontongkaew *et. al.*, 2000).[65]

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 multi-factorial. 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 cysteinyl 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 (^{13}C or ^{15}N) 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 ^{13}C or ^{15}N 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].

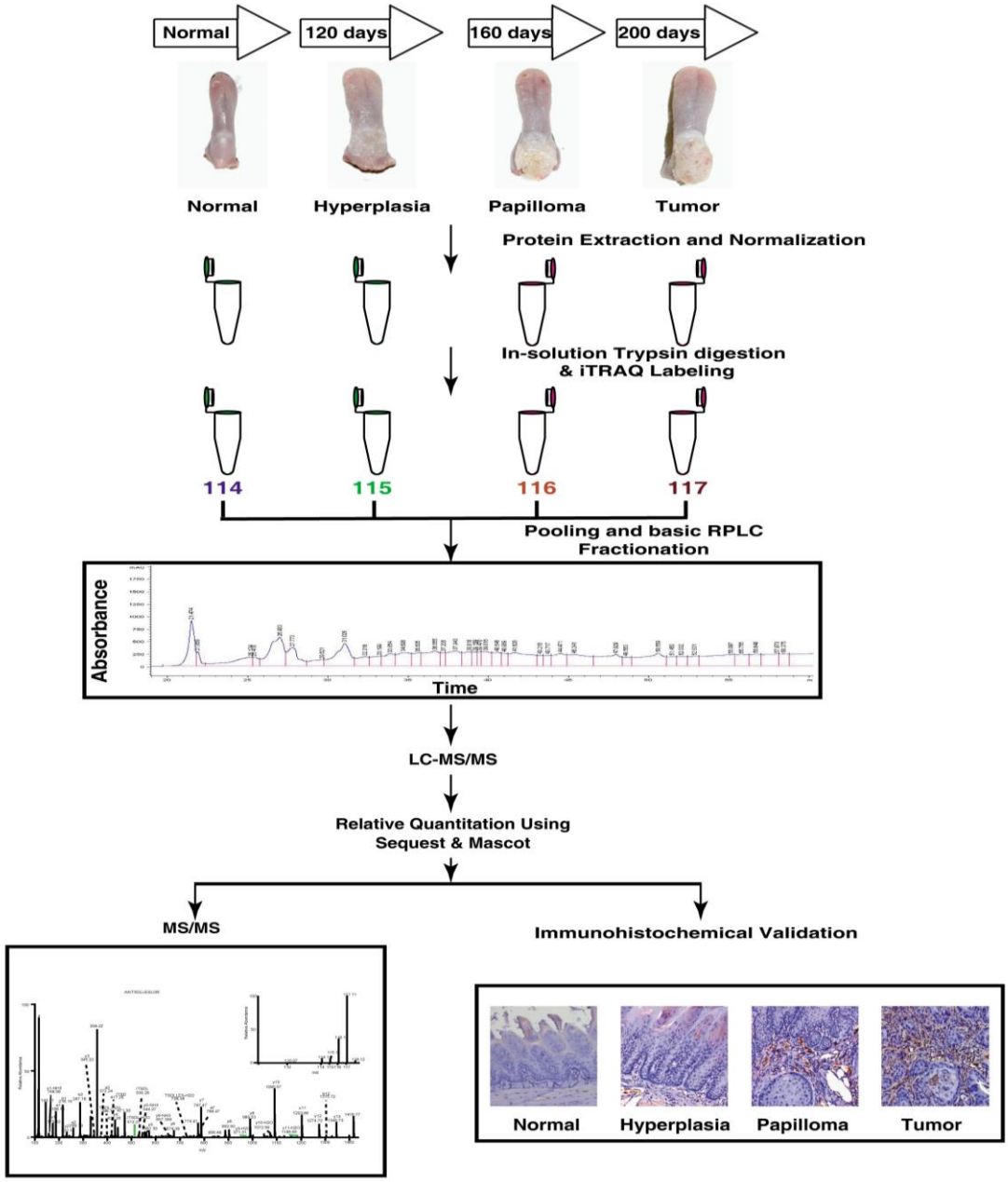


Figure 2.2 The iTRAQ reagent strategy for quantitative proteomics: Protein lysate is made from different tissues and then digested with MALDI grade trypsin to obtain the peptides. Each set of peptides is labeled with respective reporter ions. The labeled peptides are pooled, separated using cation exchange 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 Norwey 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.

CHAPTER 3
OBJECTIVES

OBJECTIVES:

The objectives of the study were as follows:

1. Establishment of rat lingual cancer model induced by carcinogen (4NQO)
2. Identification of differentially expressed proteins at different stages of lingual cancer development in rat model using quantitative proteomics
3. Validation of results obtained in quantitative proteomic study
4. Correlate the data with human samples.

CHAPTER 4
MATERIALS AND METHODS

3.1 Establishment of animal model:

3.1.1 Standard animal house conditions

Standard laboratory diet was prepared in ACTREC animal house. Pellets were made with 41% Cracked wheat, 50% Roasted Bengal gram powder, 5% Casein powder, 1% Refined oil, 1% Skimmed milk powder, 0.5% Yeast powder and 0.5% Common salt. The moisture content of the diet was 17.47%, Fat 4%, Protein 21.6%, Crude Fiber 0.95%, Carbohydrates 57.2% and ash was 3.38%. The total caloric content was 3330 Kcal. Standard conditions for maintenance of rats were 22 + 2^{o}C, 45 + with 10-12 hr dark and light cycle. Drinking water was passed through Aquaguard for UV sterilization. The cages were changed each day for removal of fecal matter and replaced with sterile cages along with bedding.

3.1.2 4NQO treatment to the animals:

All experimental protocols involving animals were approved by the institutional animal ethics committee and conformed to procedures described in the guiding principles for the use of laboratory animals. Forty five days old male Sprague Dawley rats, weighing about 150-200 g were used. Animals were fed standard diet and water *ad libitum*. All rats were allowed access to the drinking water, which was replaced thrice a week with freshly prepared solution. Bottles of drinking water with 4NQO were shielded with black paint to prevent light exposure. Animals were randomized and grouped in three groups: Untreated group (n=40), acetone (vehicle) treated (n=40), and 4NQO treated (n=56). Each group was further sub divided into 4 sub-groups and treated for 80, 120, 160 and 200 days respectively. For 4NQO treatment animals were distributed into 4 groups (8 animals for 80 days, 12 animals each for 120 and 160 days and 24 animals for 200 days). 4NQO was dissolved in acetone and finally given to the animals at 30 ppm concentration in normal drinking water. After each time point, animals were fed with normal drinking water for another 15 days to get the stable changes. Animals were sacrificed by CO_2 inhalation followed by cervical dislocation. Tongue tissues were dissected, and one piece was fixed in 10% buffered formalin for histology and

immunostaining, and rest of the piece was snap frozen in liquid nitrogen for RNA/protein isolation.

Time point (days)	**80**	**120**	**160**	**200**
Untreated controls	**4**	**12**	**12**	**12**
4NQO* treated	**8**	**12**	**12**	**24**
Acetone control	**4**	**12**	**12**	**12**
Rats utilized for each time point	**16**	**36**	**36**	**48**

Table 3.1: Different time points and the number of rats utilized for development of different stages of oral carcinogenesis

3.2 Histology

Reagents: Buffered formalin (10 % formalin, 0.025 M sodium dihydrogen phosphate and 0.046 M disodium hydrogen phosphate in distilled water), Poly-L-Lysine (0.01% Poly-L-Lysine in milli Q water), Xylene, Alcohol, Paraffin, Haematoxylin and Eosin stain.

Protocol: After dissection tissues were immediately fixed into buffered formalin and incubated overnight at RT. Next day excess formalin was removed by placing the tissue in tap water. This was followed by dehydration of tissues gradually from 70-80-95-100% ethanol for 5 min each. Tissues were given two changes of xylene (30 min each) followed by treatment of xylene and paraffin (1:1) for 1h. Tissues were paraffinized in fresh paraffin for 2 h twice and embedded in paraffin blocks. Tissues embedded in paraffin blocks were cut into 5 µm thick sections, which were placed on poly-l-lysine coated slides. Hematoxylin and Eosin staining was performed and slides were mounted using mounting agent.

3.3. Differential Proteomics:

3.3.1. Differential proteomics by 2 Dimensional gel Electrophoresis:

3.3.1.1 Cell lysate preparation:

Reagents: Urea lysis buffer (8M Urea, 2M Thio urea, 2% CHAPS, 50mM DTT).

Protocol: Approximately 30 mg of epithelial tissue from the rat tongue was pulverized in liquid nitrogen by mortar and pestle. The powdered tissue was reconstituted in Urea lysis buffer and sonicated using ultrsonicator on ice. Each sonication cycle was of 20 seconds of pulsing at 50% output with intermittent gap of 45 seconds; this cycle was repeated 3 times. Subsequently the cell lysate was centrifuged at 14000 rpm for 10 min at 4^0C. Supernatant was transferred into fresh eppendorf tube and total protein content was measured using RC DC kit.

3.3.1.2 Protein estimation by RC DC Kit:

Reagents: RC Reagent I, II, A, B, Reagent A' (5 µl of DC Reagent S was added to 250 µl of DC Reagent A), 1mg/ml BSA.

Protocol: BSA protein standards (5, 10, 15, 20 and 25µg) and the samples (3µl) to be estimated were added in the 1.7 ml microfuge tubes and the volume was made up to 25 µl with distilled water. RC Reagent I (125 µl) was added in each tube, vortexed and incubated for 1min at RT. RC Reagent II (125 µl) was added in each tube and vortexed. The tubes were then centrifuged at 15,000 x g for 20 min. Supernatant was discarded by inverting the tubes on clean, absorbent tissue paper. The liquid was drained completely from the tubes. Reagent A' (127 µl) was then added to each microfuge tube and vortexed. The tubes were incubated at room temperature for 5min, or until precipitate was completely dissolved. The tubes were vortexed and 1 ml of DC Reagent B was added to each tube and vortexed immediately. The tubes were incubated at RT for 15 min and absorbance was read at 750 nm.

3.3.1.3 Isoelectic Focusing:

Reagents: Rehydration buffer (8M Urea, 2M Thiourea, 2% CHAPS, 1% DTT, 0.2% ampholytes, 0.0002% Bromophenol blue), Equilibration buffer I (6M Urea, 0.375M Tris HCl pH 8.8, 2% SDS, 20 % Glycerol, 2% DTT), Equilibration buffer II (6M Urea, 0.375M Tris HCl pH 8.8, 2% SDS, 20 % Glycerol, 2.5% Iodoacetamide)

Protocol: Isoelectic focusing was performed as per manufacturer's instructions (Bio-Rad). For each sample (normal or tumor) 200µg of protein was used for Isoelectic focusing. 17 cm IPG-Strips (pI range 3-10 or 4-7, Bio-Rad) were rehydrated in rehydration buffer containg cell lysate, overnight. After rehydration, Isoelectic focusing was performed for 60,000 Vh using Biorad Protean IEF cell. The voltage was stepped up using automated program as described in **Table 3.2**. After IEF, strips were equilibrated for 15 min with Equilibration buffer I and Equilibration buffer II respectively. Each strip was washed in milliQ water and placed on to 12% SDS PAGE and resolved on constant 100 V overnight for second dimension as per Laemmli protocol. [105] The strips were overlaid with 1% low melting agarose gel.

Steps	***Start voltage***	***End voltage***	***Time (min)***	***Final volt Hours***	***Condition***
Step 1	0	250	30	-	Linear
Step 2	250	8000	150	-	Linear
Step 3	8000	10,000	120	-	Linear
Step 4	10,000	10,000	-	60,000 Vh	Rapid

Table 3.2: Protocol for first dimension isoelectric focusing in Bio-Rad Protean cell. The temperature was set at 20ºC and maximum current was set at 50 µA/strip.

3.3.1.4 SDS Poly Acrylamide Gel Electrophoresis:

Reagents: PAGE sample buffer (62.5mM Tris HCl (pH 6.8), 25% Glycerol w/v, 2% SDS, 0.5% Bromophenol blue), 30% Acrylamide (29.2 Acrylamide and 0.8% Bis Acrylamide), 1.5M Tris HCl (pH 8.8), 1 M Tris HCl (pH 6.8), 10% SDS, 10% APS, TEMED.

Protocol: The samples were dissolved in PAGE sample buffer and were separated on 10-12% SDS PAGE depending on the molecular weight of the proteins being analyzed with 3.9% stacking gel. The composition of SDS PAGE is as follows:

Component	10% Separating gel in ml	12% Stacking gel in ml	3.9% Stacking gel in ml
30% Acrylamide	3.3	4	0.33
1.5M Tris HCl pH 8.8	2.5	2.5	-
1 M Tris HCl pH 6.8	-	-	0.25
Distilled water	4	3.3	1.4
10% SDS	0.1	0.1	0.02
10% APS	0.1	0.1	0.02
TEMED	0.004	0.004	0.002
Total	10	10	2

Table 3.3: Composition of SDS PAGE

3.3.1.5 Silver Staining of 2DE gel:

After second dimension gels were fixed in fixative containing 50% methanol and 5% glacial acetic acid for 2h. Gels were processed for silver staining as per following protocol.

3.3.1.5. A. Silver staining of gels:

Reagents:

Solution 1: 0.02 % Sodium thiosulphate, **Solution 2:** 0.2 % Silver nitrate, 0.075 % formaldehyde (75 µl of formaldehyde was added at the time of staining). **Solution 3:** 2 % Sodium carbonate, 0.05 % formaldehyde Note: 50 µl of formaldehyde was added at the time of staining. **Stop solution:** 10 % Acetic acid.

Protocol: After electrophoresis, the gel was placed in 500 ml destainer in a washed plastic container for 1 h. The gel was washed with 500 ml deionized water for 30 min on a shaker at room temperature. The washing was repeated three times. After washing, the gel was incubated with 100 ml Solution 1 for 1min on the shaker followed by a quick wash with deionized water. The gel was then incubated with 500 ml Solution 2 for 20 min on the shaker followed by a quick wash with deionized water. Gel was then placed in 500 ml Solution 3 on a shaker until silver stained spots could be seen clearly. The staining was stopped by adding 500 ml Stop solution.

3.3.1.6 Mass Spectrometry

Reagents for Mass-spectrometry:

For mass spectrometry, the plastic ware used was not autoclaved. Buffer composition for destaining of silver stained gel pieces: i) Ammonium bicarbonate (50 mM NH_4HCO_3), ii) Potassium ferricyanide ($K_3[Fe(CN)_6]$), iii) Sodium thioslphate ($Na_2S_2O_3$)

Trypsin for in-gel digestion:

10 ng/µl: 20 µg trypsin powder was dissolved in 2 ml 25 mM ammonium bicarbonate to make a 10 ng/µl solution of Trypsin and kept in 100 µl aliquots at -20 °C for further use.

Extraction buffer for extraction of peptides:

50 % Acetonitrile and 1 % Trifluoro acetic acid (TFA): 500 µl of 100 % acetonitrile, 495 µl of deionized water and 5µl of TFA were mixed.

Reconstitution buffer:

50 % Acetonitrile and 0.1% TFA: 500 µl of 100 % acetonitrile, 499 µl of deionized water and 1 µl of TFA were mixed.

Protocol: Differential spots from normal Vs SCC gels were picked up and processed for mass spectrometry (MALDI TOF-TOF) as per Shevchenko et al.[106] Briefly silver stained gel plugs were destained with 100 µl of destaining solution (30 mM potassium ferricyanide/100 mM sodium thiosulfate mixed 1:1 v/v). After thorough rinsing with water, the gels were dehydrated in 100% acetonitrile which was removed by drying in a speed-vac. The proteins in the spots were then trypsinized overnight with 20 ng/µl trypsin in 25 mM ammonium bicarbonate in water and the peptides were recovered by extraction with 50% ACN and 1% TFA. Tryptic protein digests were reconstituted in 10% ACN with 0.1% TFA solvent before subjecting them to mass spectrometry analysis. Peptide mixtures were analyzed by MALDI-TOF-MS using a Reflex III mass spectrometer (Bruker Daltonics) operating in positive ion reflector mode. The resulting MS data was analysed using Flex analysis 3.0 (Brucker Daltonik, Germany) software and was acquired using Biotools 2.1 software (Brucker Daltonik,Germany). Five most intense peaks for protein identity obtained in MS analysis were subjected to MS/MS. The MS peaklist and MS/MS ions of the chosen

peptides were searched against SwissProt database version 2012_08 onwards using MASCOT search engine for protein ID with precursor tolerance of 100 ppm for MS and fragment tolerance of 1 Da for MS/MS analysis. A mass tolerance of 100 ppm and 0-1 miss cleavage site were allowed, oxidation of methionine residues was considered as variable modification, and carbamido-methylation of cysteines as fixed modification. The search was restricted to *Rattus norvegicus* proteins.

Shifting from traditional 2DE to iTRAQ LC-MS/MS:

Due to limitations in proper resolution in 2DE gel and limited identity of proteins by MALDI-TOF-TOF we adopted iTRAQ-LC-MS/MS based quantitative proteomics technology for improved proteome coverage.

3.4 Differential proteomics using iTRAQ technology:

3.4.1 Protein isolation, digestion and iTRAQ Labeling.

Reagents:0.5 % SDS, iTRAQ reagents, TCEP(tris (2-corboxyethyl) phosphine), MMTS (methanethiosulphonate), Trypsin

Protocol: Approximately 30 mg of epithelial tissue from the rat tongue was pulverized in liquid nitrogen by mortar and pestle. The powdered tissue was lysed in 0.5% SDS and sonicated using ultrsonicator on ice. Each sonication cycle was of 20 seconds of pulsing at 50% output with intermittent gap of 45 seconds; this cycle was repeated 3 times. Subsequently the cell lysate was centrifuged at 14000 rpm for 10 min at 4^0C. Supernatant was transferred in to fresh eppendorf tube and total protein content was measured using Lowry's method. Total protein (100µg) from each pool representing control (n=10), hyperplasia (n=5), papilloma (n=5) and carcinoma (n=5) was used for iTRAQ labeling. Labeling was carried out as per manufacturer's instructions. Briefly, proteins were subjected to reduction using 2µl of TCEP at 60^oC for 1 h and alkylated with cystein blocking reagent, MMTS for 10' at room temperature. They were then digested with sequencing grade trypsin (Promega, Madison, WI)

(1:20) at 37^{o}C for 16 h. The peptide digest from each sample type was subjected to iTRAQ labeling. Normal, hyperplasia, papilloma and carcinoma samples were labeled with reporter ion of m/z of 114, 115, 116 and 117 respectively. Labeled samples were then pooled and subjected to strong cationic exchange chromatography.

3.4.2 Protein Fractionation using strong cation-exchanger:

Reagents: Solvent A (10mM of KH_2PO_4, 20% acetonitrile, pH 2.8), Farmic acid, Solvent B(350mM KCl, 10mM KH_2PO_4, 20% Acetonitrile pH 2.8)

Protocol: Pooled labeled samples were diluted with solvent A. The diluted samples were acidified by adding 2µl of formic acid. Acidified samples were manually injected on to 200 µl bed volume of a strong cation exchange chromatography column (polyLC Inc.). Peptides were loaded on the column at a flow rate of 250µl per minute followed by washing for 20 minutes. Using a gradient of 30' from 8% Solvent B to 50% Solvent B, peptides were eluted into 50 fractions. The fractions of similar absorbance were pooled and a total of 23 fractions were obtained. Subsequently, the peptides were cleaned up using C18 zip tips. Prior to LC-MS/MS analysis, the peptide fractions were dried and stored at -20^{o} C.

3.4.3 LC-MS/MS and spectra analysis:

Reagent: Acetonitrile

Protocol: LC-MS/MS analysis of the iTRAQ labeled peptides was carried out using LTQ-Orbitrap Velos mass spectrometer which is interfaced with Agilent's 1200 Series nanoflow LC system. The chromatographic capillary columns used were packed with Magic C18 AQ (Michrom Bioresources, 5 µm particle size, pore size100Å) reversed phase material in 100% acetonitrile at a pressure of 1000 psi. The peptides were passed on to a trap column (75 µm x 2 cm) at a flow rate of 5 µl/min followed by separation on an analytical column (75 µm x 10 cm) at a flow rate of 300 nl/min. The peptides were then eluted using a linear gradient of 7-30% acetonitrile over 50'. Mass spectrometry analysis was performed in a data dependent

manner with full scans acquired using Orbitrap mass analyzer at a mass resolution of 60,000 at 400 m/z. For each cycle, twenty most intense precursor ions from a survey scan were selected for MS/MS and detected at a mass resolution of 15,000 at m/z 400. The fragmentation was carried out using higher-energy collision dissociation as the activation method with 40% normalized collision energy. The ions selected for fragmentation were excluded for 30 sec. The automatic gain control for full FT MS was set to 1 million ions and for FT MS/MS was set to 0.1 million ions with a maximum time of accumulation of 750 ms and 100 ms, respectively. For accurate mass measurements, the lock mass option was enabled. Internal calibration was carried out using the Polydimethylcyclosiloxane (m/z, 445.12) ion.

3.4.4 Data analysis:

The raw files obtained from LC-MS/MS analysis were searched using Sequest and Mascot algorithms. The searches were submitted through Proteome Discoverer (Version 1.3.0.339) software (Thermo Fisher Scientific). NCBI RefSeq 49 rat protein database (n= 25,317). Oxidation of methionine, iTRAQ 4-plex modification at peptide N-terminus and Lysine (K) were selected as variable modifications and methylthio of cysteine as a fixed modification. MS and MS/MS tolerance were set to 20 ppm and 0.1 Da, respectively. One missed cleavage was allowed. False discovery rate (FDR) was calculated using a decoy database. Peptide spectrum matches (PSMs) at 1% FDR were used for protein identification and quantitation. Relative quantification of proteins was done on the basis of relative intensity of tagged ion (115, 116 and 117 for hyperplasia, papilloma and carcinoma respectively) with respect to normal (114 for Control). Relative value >2 was considered as up regulated while <0.5 was considered as down regulated.

3.5. Collection of human oral tumors and premalignant tissues

This study was approved by the Human Ethics Committees of the respective Institutional Review Boards. Informed consent was obtained from the patients before enrolling them in this study. The tongue tumor tissues (n=34) were collected from Tata Memorial Hospital (TMH), Mumbai, India at the time of surgery. In 14 of the cases, the adjoining histologically normal tissue was also collected. 10 paraffin embedded blocks of the biopsies collected from leukoplakia of tongue were obtained from Ragas Dental College, Chennai, India and Nair Dental Hospital, Mumbai, India.

3.6 Validation of proteomics out come by IHC/Western blot/Real Time PCR

3.6.1 Different antibodies used in the study:

Following antibodies were used in the study:

Antibody	Dilution	Clone	Company	Catalog no.
Vimentin	1:400	V9 clone, Mouse monoclonal	Sigma	V 6630
Transglutaminase 3	1:8000	Mouse monoclonal	SantaCruz	sc-101366
Periostin	1:50	Rabbit polyclonal	SantaCruz	sc 49480
Coronin 1a	1:1000	Rabbit polyclonal	Covance	PRB-5002-100
Tenascin N	1:100	Rabbit polyclonal	HPA	HPA-026764
Fascin	1:200	Mouse monoclonal	Thermo Fischer	MA1-20912

Table 3.4: List of antibodies used in the study

3.6.2 Immunohistochemistry using Vectastain universal elite ABC Kit

Reagents: Xylene, Alcohol, Methanol, TBS pH 7.2 (0.05M Tris, 0.8% NaCl), 0.1M Citrate buffer, 0.08% DAB with 3% H_2O_2 in TBS.

Protocol: The tissue sections were deparafinised by keeping the slides in xylene twice for 15 min each. The slides were then treated with xylene and ethanol mixed in 1:1 ratio, and this step was repeated once. The sections were then dehydrated by keeping the slides in 100 % ethanol. Further, the tissues were again treated with 100% methanol for 20 min. For endogenous peroxidase inactivation tissues were treated with 3% hydrogen peroxide in methanol for 30 min and washed with distilled water. The antigens were retrieved with citrate buffer (pH 6.0), using microwave treatment at full power for 3min followed by 5min followed by 5 min. The slides were then allowed to come at RT for 1 h. Nonspecific antigenic sites were blocked with pre-immune serum (horse serum diluted 1:100) for 1 h at RT. Sections were then incubated with the respective primary antibody overnight at 4ºC. Antibodies were diluted in 0.05M Tris-buffered saline pH 7.2 (TBS). This was followed by incubation with secondary biotinylated antibody (dilution 1:50) for 1 h at RT and then with avidin–biotin–peroxidase complex (dilution 1:1:50) (1 h at RT). After each step, sections were washed with 0.05 M Tris-buffered saline pH 7.2. Peroxidase activity was visualized by keeping the slides in a substrate solution containing 0.08% diaminobenzidene and 3% H_2O_2. Counterstaining was performed with Mayer's haematoxylin (0.5 % haematoxylin in distilled water). In each slide, sections for which primary antibody replaced with PBS served as negative controls of the assay.

3.6.3Western Blot[107]:

Reagents: Transfer Buffer (190mM Glycine, 20% methanol, 0.05%SDS, 25mM Tris base), Tris-buffered saline (150mM NaCl, 10mM Tris HCl pH 8.0), Tris-buffered saline Tween (TBST) (0.1% Tween (v/v), 150mM NaCl, 10mM Tris HCl pH 8.0), Ponceau Staining solution (0.2% ponceau in 5% acetic acid),Blocking buffer (3% BSA in TBS), Antibody dilutions were made in 0.5% BSA in TBS, ECL+ Kit from (GE Healthcare).

Protocol: After SDS-PAGE, the gel was placed in transfer buffer. PVDF membrane was activated in methanol for 10 seconds, and was placed in transfer buffer. Sandwich of gel and membrane was prepared and wet electro-blotting was carried out at 100V for 1 h. Transfer of protein was visualized using Ponceau-S staining for 20 seconds, and later rinsed with MilliQ water. Ponceau-S stain was completely removed by washing the blot with 1X TBST. The blot was then placed in blocking solution (3% BSA in MilliQ) and incubated for 1 h at RT on a rocker. After blocking the blot was incubated with diluted antibody for 1h at RT on the rocker. The blot was later washed thrice with TBST for 15 min each on the rocker. The blot was incubated with horseradish peroxidase (HRPO) conjugated secondary antibody for 1 hr at RT on the rocker. The secondary antibody solution was removed and the blot was washed thrice with TBST for 15 min each on the rocker. Excess buffer was drained and the blot was covered with enhanced chemiluminescence solution (ECL+) for 5 min. The ECL+ solution was prepared according to the manufacturer's protocol. Excess solution was drained and the blot was wrapped in saran wrap. The wrapped membrane was placed in a cassette with protein side facing upwards. An X-rayfilm was placed over it and incubated for various time intervals depending on the signal strength. The signal was visualized after developing the X-ray film in a developing machine.

3.6.4 RNA isolation and qRT-PCR

Where ever antibody was not available we performed qRT-PCR for validation. Total cellular RNA was extracted from the tissue by Tri-reagent (Sigma-Aldrich, USA) as per manufacturer's protocol. RNA was estimated by measuring absorbance at 260 nm and 280 nm using nanodrop (ND-1000 Spectrophotometer, Wilmington, USA). cDNA synthesis was carried out as per the manufacturer's protocol (Fermentas, Thermo Scientific, Waltham, MA) and the obtained cDNA was used as template for qRT-PCR. Master Mix SYBR Green (Applied Biosystems, Bedford, MA) was used with 5nM of forward and reverse primers

(**Table 3.5)**. Real-time quantitative PCR was performed with the ABI PRISM7700 Sequence Detection System. Beta actin gene was used as endogenous control. All amplifications were done in triplicate. Results are expressed as relative gene expression using the $2^{-\Delta\Delta Ct}$ method[108].

No	Oligo Name	5'<Sequence>3'	Length
1	Rattus Cornulin_F	CTCACGAAGCAGGAGCTGAA	20
2	Rattus Cornulin_R	AGGATCATGGGGCTTCACTA	20
3	Rattus Beta actin_F	ACCCGCGAGTACAACCTTCTT	21
4	Rattus Beta actin_R	TATCGTCATCCATGGCGAACTGG	23
5	Rattus Trichohyalin_F	TGATGGAGCATCGCTTAGCA	20
6	Rattus Trichohyalin_R	TCCGGATCATGTGGTTTCTGA	21
7	Rattus Thbs2_F	TCGCCGATGGTTTCGATGAG	20
8	Rattus Thbs2_R	TAGTCATCGTCCCGGTCAGT	20

Table 3.5. Primer sequences used in Quantitative Real Time-PCR

3.7 Bioinformatics analysis of proteomics Data:

In order to perform bioinformatics analysis, all identified and differentially expressed proteins in tumors were subjected to GO analysis[109]. To fetch the gene ontology information for the identified protein in our study we used a computational approach using in house customized python scripts to search for gene ontology information (biological process, and sub cellular localizations) from the gene ontology database. This was performed using the protein accession numbers for the identified proteins from the total rat proteome as well as differentially expressed proteins in tumors. This resulted in the distribution of proteins according to various biological processes in which these proteins were involved.

CHAPTER 5
RESULTS

4.1 Development of sequential stages of rat oral carcinogenesis:

4.1.1 Standardization of 4NQO dose for rat lingual carcinogenesis:

4NQO was used as a carcinogen to induce the lingual carcinogenesis and was given to animals in normal drinking water. For standardization of 4NQO dose, we initially gave two concentrations of 4NQO i.e. 30 ppm and 50ppm for 200 days respectively to two groups of rats. Rats receiving 50 ppm concentration of 4NQO showed higher toxicity and mortality (5 animals out of 8 died before completion of 200days) in comparison to 30 ppm 4NQO receiving rats (only 3 died out of 8 animals). Therefore we continued with 30 ppm of 4NQO treatment in drinking water to obtain sequential stages of rat tongue carcinogenesis.

4.1.2 Gross morphological alterations after milliQ (untreated control), acetone (Vehicle control) and 4NQO treatment:

In order to isolate different stages of carcinogenesis, SD rats were treated with milliQ, acetone (vehicle) and 30ppm 4NQO for 80, 120, 160 and 200 days respectively. All the animals were carefully observed every alternate day and changes in the oral cavity were assessed. Lingual tissues treated with either milliQ (untreated control) or acetone (vehicle control) did not show any alterations at any time point **(Figure 4.1A1).** We also did not see any change till 80 days of treatment with 4NQO. Lingual tissues treated for 120 days with 4NQO demonstrated white lesions on posterior dorsal tongue **(Figure 4.1A2).** After 160 days of 4NQO treatment, non-homogenous exophytic growth in the posterior dorsal tongue **(Figure 4.1A3)** was observed whereas after 200 days homogenous and uniform exophytic growth was seen **(Figure 4.1A4).** 4NQO treated animals became weak and irritable after 160-180 days and were difficult to handle. Some of these animals showed toxicity and died before their respective end point was reached.

4.1.2 Histopathological analysis of tongue epithelium at different time points:

Histopathological analysis of untreated, acetone control and 80 days treated tissues did not show any alterations in histology of posterior dorsal tongue epithelium **(Figure 4.1B1).**

Seven out of 12, 4NQO treated animals at the end of 120 days treatment exhibited hyperplastic changes **(Figure 4.1B2)** whereas remaining 5 animals showed atypical papillomas. Similarly, 5 of the 160 days treated animals showed papillomas **(Figure 4.1 B3)** in the tongue while 2 animals showed hyperplasia (2/12) and tongue tumor (2/12). At the end of 200 days, 10/24 animals demonstrated uniform well differentiated SCC **(Figure 4.1B4)** while 5/24 animals demonstrated a mixed histology of papilloma and well differentiated carcinoma. Three out of 24 animals demonstrated uniform papilloma. The histopathological analysis of all the 4NQO treated animals is tabulated in **Table Number 4.1**.

Group	Histopathological analysis of lingual tissue treated with 4NQO.				
	Normal (No change)	Hyperplasia/ Atypical Hyperplasia			SCC
			Atypical papilloma/papilloma	Papilloma + SCC	
80 days (n=8)	8	-	-	-	-
120 days(n=12)	-	7	5	0	0
160 days(n=12)	3*	2	5	0	2
200 days (n=24)	6*	0	5	3	10

*=animals died during experiment

Table 4.1 Incidence of lesions in tongue of 4-nitroquinoline 1-oxide (4NQO) treated rats for the development of oral carcinogenesis model as assessed by histopathology.

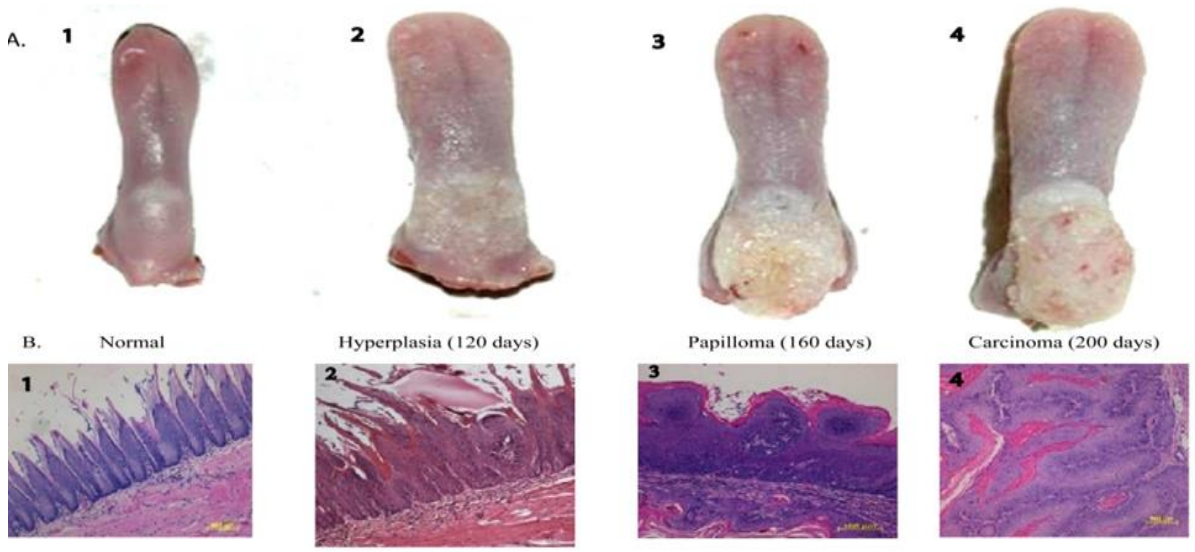


Figure 4.1: Alterations in the tongue after 4NQO treatment A. Morphological alterations after 4 NQO treatment (B) photomicrograph of Hematoxylin and Eosin staining of different stages of rat lingual carcinogenesis (1) vehicle treated tongue, tongue treated for (2) 120 days (hyperplasia), (3) 160 days (papilloma) and (4) 200 days (carcinoma) with 4NQO respectively. (Magnification 100 X)

4.2 Establishment of differential protein expression profile of normal vs. SCC of rat tongue by two dimensional gel electrophoresis followed by MALDI-TOF-TOF:

In order to obtain differential protein expression pattern, two dimensional gel electrophoresis followed by MALDI-TOF-TOF was performed initially. 2-DE was performed on wider range of pH strips i.e. 3-10 pH strips to get more differential protein spots. However, clear identity of only five differentially expressed protein spots could be obtained using this method. The differential expression of the proteins was identified by visual inspection in three different pairs of SCC vs. normal tissues (**Appendix figure A1)**. These differential protein spots were galectin 7, keratin 6A, Transglutaminase 3, fatty acid binding protein 5 and serum albumin precursor protein **Figure 4.2A.** Some important characteristics of identified proteins are given in **Appendix Table A1.** Among the identified differential protein spots galectin 7 and transglutaminase 3 were down regulated in the SCC while keratin 6A, fatty acid binding rotein 5 and serum albumin precursor protein were up regulated in SCC in comparison to normal tissues **(Figure 4.2B).**

In order to have better resolution of protein spots we also used narrow range pH strips i.e. 4-7 pH strips. However, we were not able to get good resolution/separation of proteins on 2DE gels **(Appendix figure A2**). One of the possible reasons could be that, the squamous epithelial cells are rich in keratins and this abundance of keratin masks the separation of low abundance proteins.

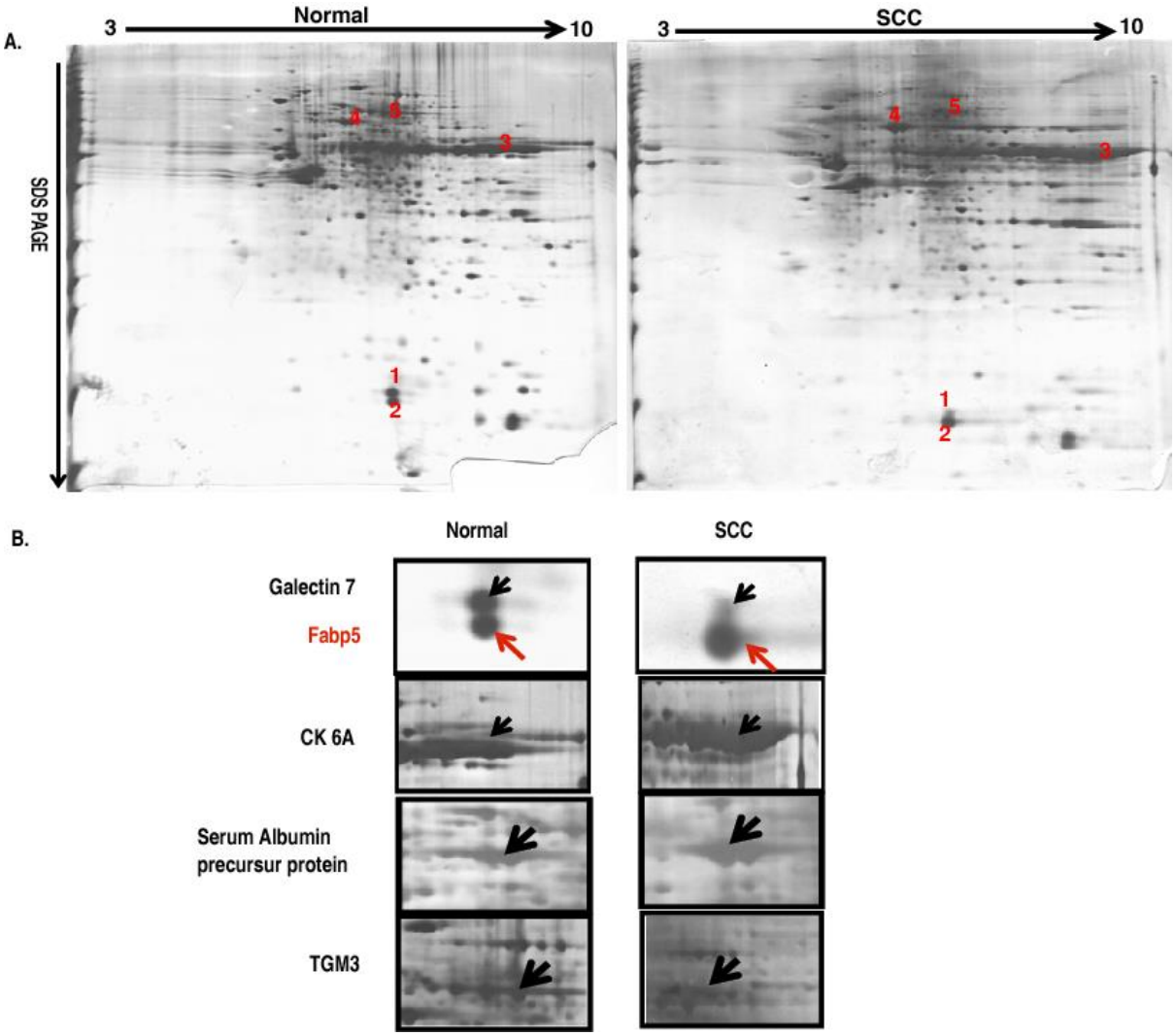


Figure 4.2 Two dimensional gel electrophoresis based differential proteomics of normal and SCC samples A. Representative 2-DE gel. 200 µg of proteins from normal and SCC samples was subjected to first dimension IEF (pI range 3-10) and then separated on second dimension SDS PAGE (12%). B. Differential protein spots. Their identity was established by MALDI-TOF/TOF. Spot 1 is identified as galectin 7, spot 2 as fatty acid binding protein 5 (Fabp 5), spot 3 as keratin 6A (CK 6A), spot 4 as serum albumin precursor protein and spot 5 as transglutaminase 3(TGM3).

4.3 Validation of galectin 7 by Immunohistochemistry and Western blot:

Because of contradictory reports on galectin 7 expression and function in human cancer we only validated the alterations in galectin 7 expression pattern at various stages of rat lingual carcinogenesis by Immunohistochemistry and western blot.

Histologically normal and hyperplasia tissues showed expression of galectin 7 in all layers of epithelia (basal and suprabasal) which was localized in cytoplasm, membrane as well as nucleus. In papillomatous lesions and SCC decreased expression of galectin 7 was noticed. **(Figure 4.3 A)** In order to quantify the expression levels of galectin 7 in these tissues, western blot analysis was performed. No change in galectin 7 level was noticed in histologically normal and hyperplastic tissues while galectin 7 level was decreased in papillomas and SCC tissues. Beta actin was used as loading control **(Figure 4.3 B).**

To explore larger protein repertoire, we adapted the gel free and labeling method i.e. iTRAQ method to study the differential proteomics.

4.4 Study of differential proteomics by isotope tagged relative and absolute quantitation (iTRAQ).

An iTRAQ-based differential proteomic analysis was carried out by labeling peptides obtained from post-trypsin digestion of proteins isolated from different stages of rat lingual carcinogenesis, followed by LC-MS/MS analysis. Peptides from different stages i.e. normal, hyperplasia, papilloma and carcinoma were successfully labeled with 114, 115, 116 and 117 respectively. The data from a total of 77,988 MS/MS spectras generated by LC-MS/MS analysis of 23 SCX fractions were searched against rat RefSeq 49 data base using SEQUEST and MASCOT search algorithm. A False discovery rate (FDR) cut off of 1% was applied to eliminate false positive identification.

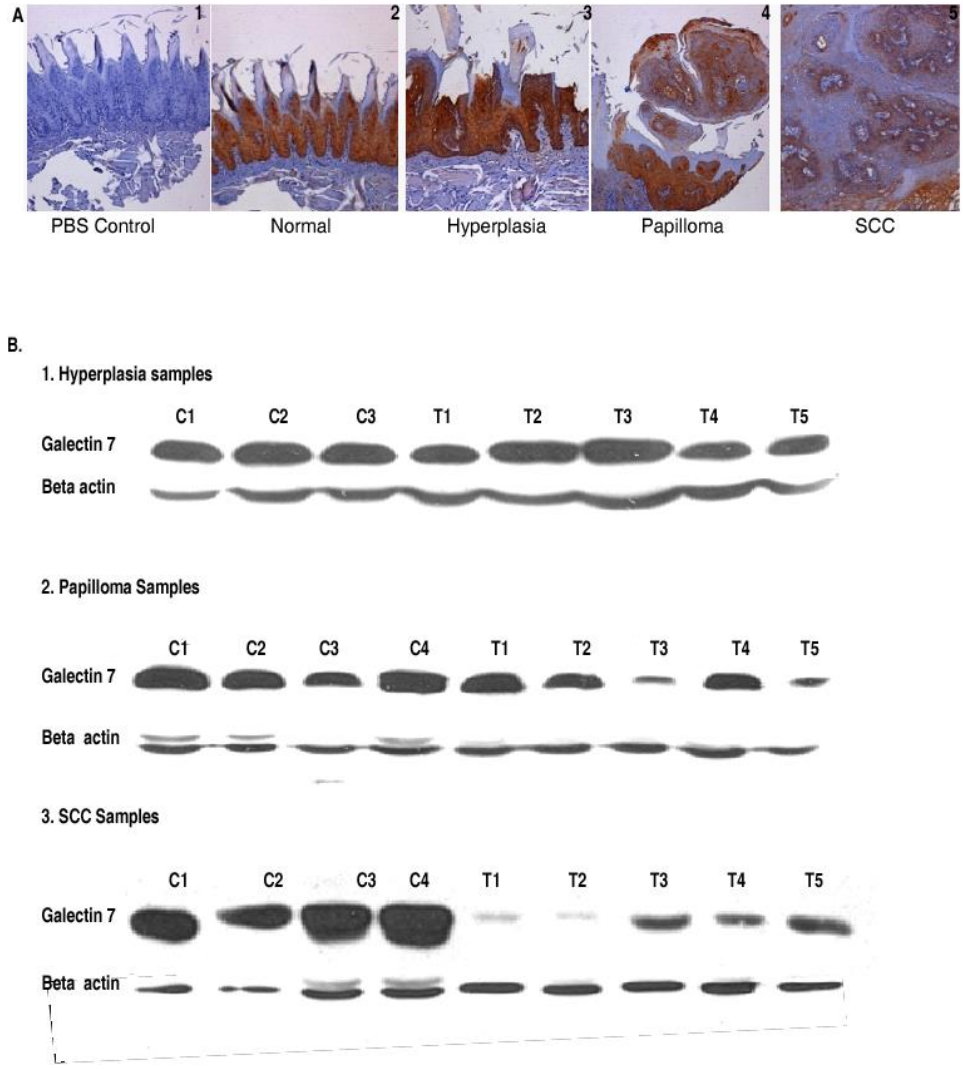


Figure 4.3 Immunohistochemical analysis of Galectin 7 during 4NQO induced tongue carcinogenesis. A. In Normal tissues (2), galectin 7 is detected as cytoplasmic, membranous and nuclear staining in all the layers of stratified epithelium (basal, suprabasal and sub epithelial layers of tongue tissues). In hyperplastic tissues (3) staining pattern is similar to the normal tongue tissues. In papillomatous area (4) galectin 7 shows reduced staining while its staining is reduced further in squamous cell carcinoma (5). Figure A (1) represents antibody staining control i.e. primary antibody is replaced with PBS buffer. B. Western blot analysis showing galectin 7expression in hyperplasia (1), papilloma (2) and SCC samples (3). Galectin 7 is seen to be down regulated in SCC. C represents control tissues while T represents the tongue tissues treated with 4NQO.

4.5 Quantitative analysis of mass spectrometry data:

Differentially expressed proteins were quantified based on the iTRAQ ratio of the peptides for respective protein. Quantitative analysis using SEQUEST and MASCOT search algorithm led to the identification of 2223 proteins. Out of these, 415 proteins were found to be differentially expressed in SCC, 333 proteins in papillomas and 109 proteins in hyperplasia. Among the 415 differentially expressed proteins in tumors, 194 proteins were up regulated while 221 proteins were down regulated. In papillomas 155 proteins were up regulated while 178 proteins were down regulated. Among 109 differential proteins of hyperplasia, 35 proteins were upregulated while 74 proteins were down regulated. Number of differentially expressed proteins at different stages of rat oral carcinogenesis has been shown in **Table 4.2.**

Stage	No. of upregulated proteins	No. of downregulated proteins	Total
Hyperplasia	35	74	109
Papilloma	155	178	333
Squamous cell carcinoma	194	221	415
Total	384	473	817

Table 4.2. Number of differentially expressed proteins in this study

Among the differentially expressed proteins, 5 proteins were sequentially upregulated while 10 proteins were sequentially down regulated from hyperplasia to SCC. Similarly, sequentially up or down regulation of 62 and 51 proteins respectively was observed from papilloma to carcinoma tissues. We also observed sequential up regulation of 154 proteins and sequential downregulation of 170 proteins from normal to SCC tissues. A partial list of sequentially altered proteins at different stages is shown in **Appendix table No. A2.**

We have found alterations in the expression profile of several previously reported proteins e.g. vimentin, fascin as well as some novel proteins e.g. Tenascin N, coronin 1a, Trichohyalin and thrombospondin 2 during rat tongue carcinogenesis. We have validated some of the known candidate proteins whose differential expression in human oral carcinomas has been previously reported. These include vimentin (*Vim*), fascin (*Fscn1)*, transglutaminase 3 (*Tgm3*), periostin (*Postn*) and cornulin (*Crnn*).

4.6 Validation of known upregulated proteins identified in rat lingual carcinogenesis

4.6.1 Vimentin:

We observed sequential increase in Vimentin expression at different stages of rat oral carcinogenesis in our proteomics analysis **(Figure 4.4A)**. We noted a 2 fold up regulation of vimentin in SCCs 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. We noticed increased suprabasal and cytoplasmic expression of vimentin in papillomas and carcinomas as compared to normal tissues.

4.6.2 Fascin *(Fscn1):*

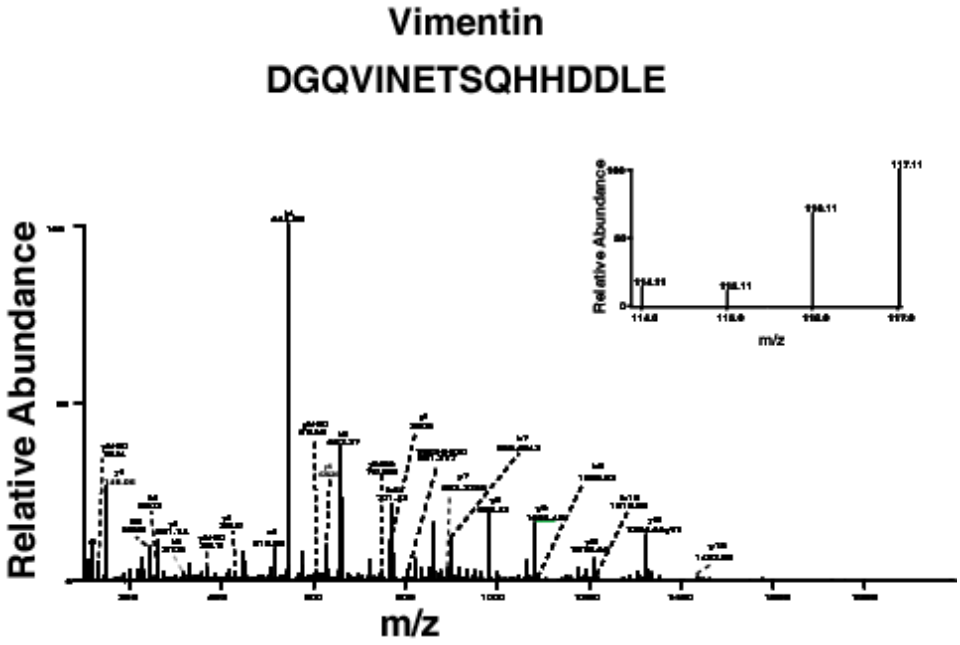
Our proteomics study suggests it's sequential up regulation during the process of carcinogenesis. We found upregulation to 3 fold in SCCs as compared to normal tissues (**Figure 4.5A**). IHC studies on rat tongue at different stages revealed that fascin expression

was not detectable in the vehicle treated group while weak cytoplasmic staining was observed in the basal layer of hyperplastic tissues. Furthermore, strong cytoplasmic, and suprabasal staining was seen in papilloma and carcinoma tissues, respectively (**Figure 4.5B**).

4.6.3 Periostin *(Postn)*:

Proteomics data demonstrated its sequential up regulation during rat tongue carcinogenesis. We noted 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 tumor tissues showed periostin expression only in the stromal region (**Figure 4.6B**).

A.



B.

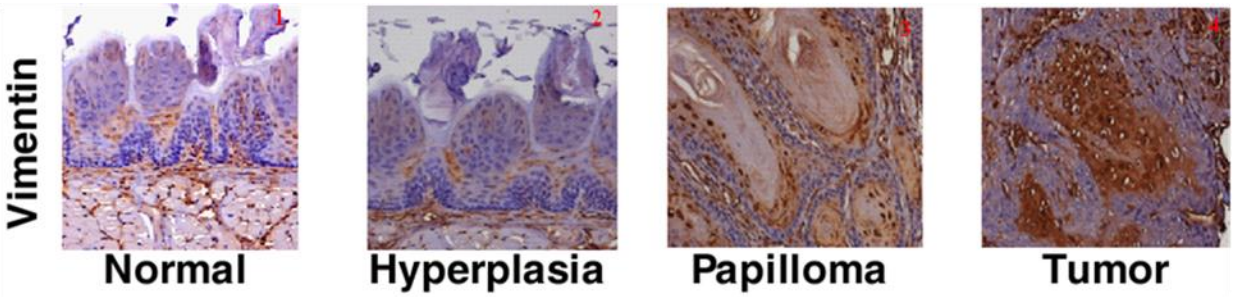
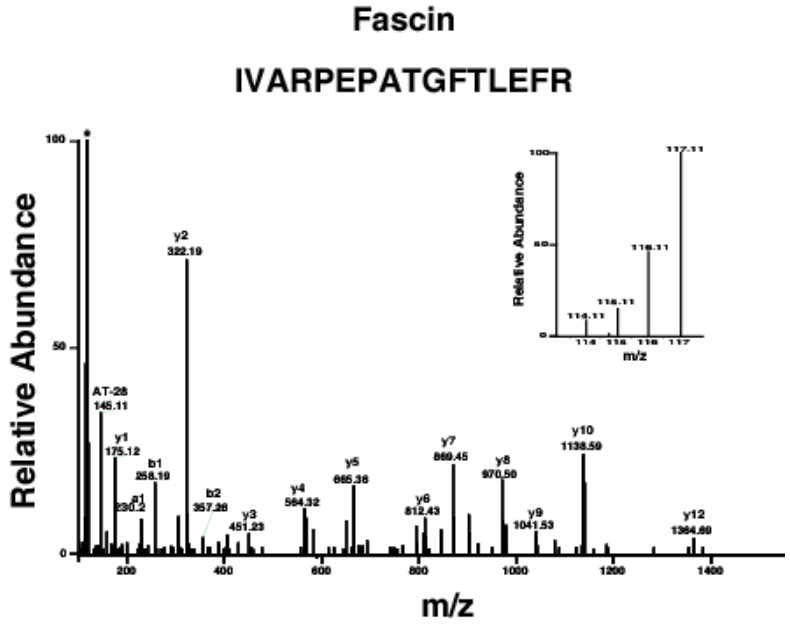


Figure 4.4. MS/MS spectra of Vimentin and validation of Vimentin by IHC on rat tongue tissues: A). MS/MS spectra of peptide (DGQVINETSQHHDDE) representing Vimentin. The inset showing the reporter ions used for quantitation suggests sequential increase in Vimentin across the stages of rat oral carcinogenesis (B). Representative photomicrographs showing immunohistochemical staining of Vimentin at different stages of rat lingual carcinogenesis (magnification ×200). (1) Vehicle treated tissue, (2) tongue treated for 120 days (hyperplasia), (3) 160 days (papilloma) and (4) 200 days (SCC) with 4NQO respectively. Vimentin immunostaining is not seen in normal tissues while seen in suprabasal layers of hyperplastic tissues. Further high expression of Vimentin is observed in papilloma and SCC tissues. Note: Sequential increase in Vimentin staining across the stages of rat oral carcinogenesis.

A.



B.

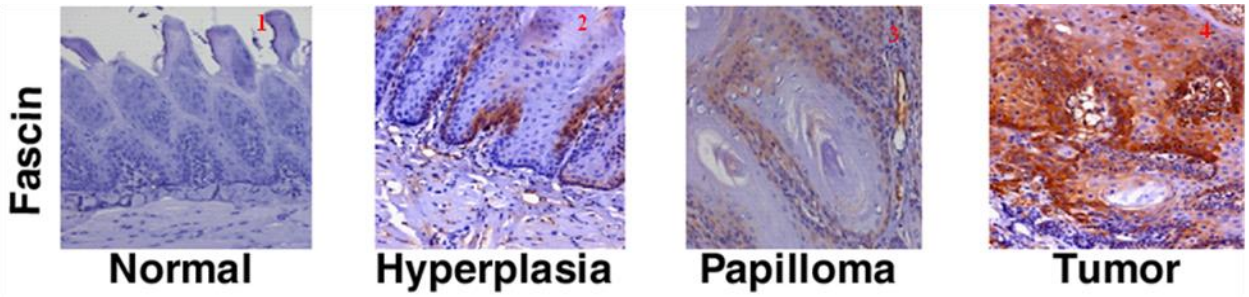
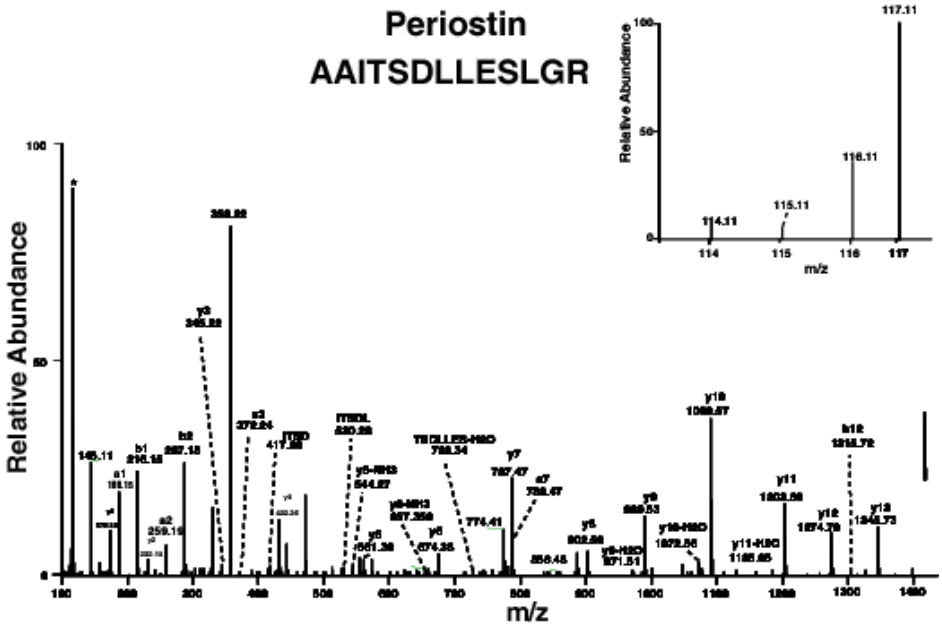


Figure 4.5. MS/MS spectra of Fascin and validation of Fascin by IHC on rat tongue tissues: A). MS/MS spectra of peptide (IVARPEPATGFTLEFR) representing Fascin. The inset showing the reporter ions used for quantitation suggests sequential increase in Fascin across the stages of rat oral carcinogenesis. (B) Representative photomicrographs showing immunohistochemical staining of Fascin at different stages of rat lingual carcinogenesis (magnification ×200) (1) Vehicle treated tissue, (2) tongue treated for 120 days (hyperplasia), (3) 160 days (papilloma) and (4) 200 days (SCC) with 4NQO respectively. Fascin immunostaining is not seen in normal tissues while is seen in suprabasal layers of hyperplastic tissues. Further high expression of Fascin is observed in papilloma and SCC tissues.

A.



B.

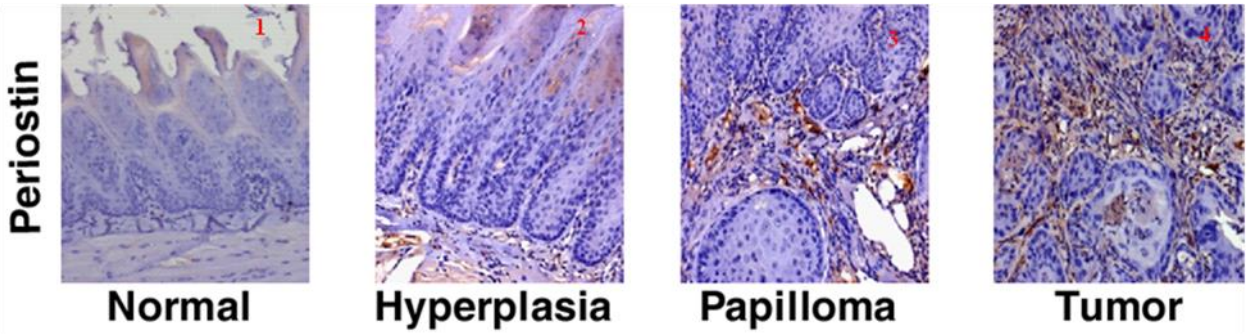


Figure 4.6. MS/MS spectra of Periostin and validation of Periostin by IHC on rat tongue tissues: A). MS/MS spectra of peptide (AAITSDLLESLGR) representing Periostin. The inset showing the reporter ions used for quantitation suggests sequential increase in Periostin across the stages of rat oral carcinogenesis. (B) Representative photomicrographs showing immunohistochemical staining of Periostin at different stages of rat lingual carcinogenesis (magnification ×200) (1) Vehicle treated tissue, (2) tongue treated for 120 days (hyperplasia), (3) 160 days (papilloma) and (4) 200 days (SCC) with 4NQO respectively. Periostin staining is not observed in epithelial layers of normal and hyperplastic tissues while it is present in the stromal part of the papillomatous and SCC tissues.

4.7 Validation of known downregulated proteins identified in rat lingual carcinogenesis

4.7.1 Transglutaminase 3 (*Tgm3*):

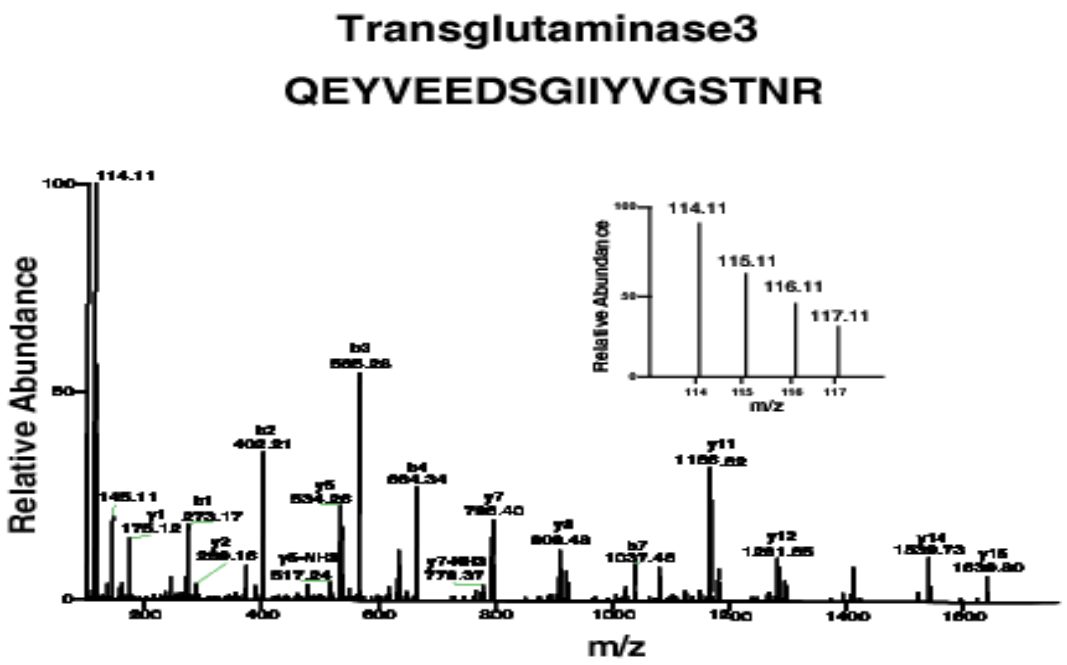
We obtained sequential down regulation of Tgm3 in our proteomics study. We noted a ~ 6 fold down regulation in SCCs as compared to normal tissues (**Figure 4.7A**). Validation by immunohistochemistry indicates its strong cytoplasmic and suprabasal expression in normal tongue tissues. While its cytoplasmic expression was sequentially down regulated during the process of tumorogenesis (**Figure 4.7B**).

4.7.2 Cornulin (*Crnn*):

Because of unavailability of specific antibody for rat cornulin, 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. Proteomics data revealed its 14 fold down regulation in tumors as compared to normal. Real time data revealed that cornulin down regulation is an early event in carcinogenesis (**Figure 4.8B**).

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. 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. We have validated some upregulated/down regulated novel proteins either in rat tissues and/or both rat and human tissues. Out of several novel proteins whose sequential up or down regulation was seen in our iTRAQ analysis, we selected four proteins for validation whose up or downregulation has been shown in other human cancers or during terminal differentiation.

A.



B.

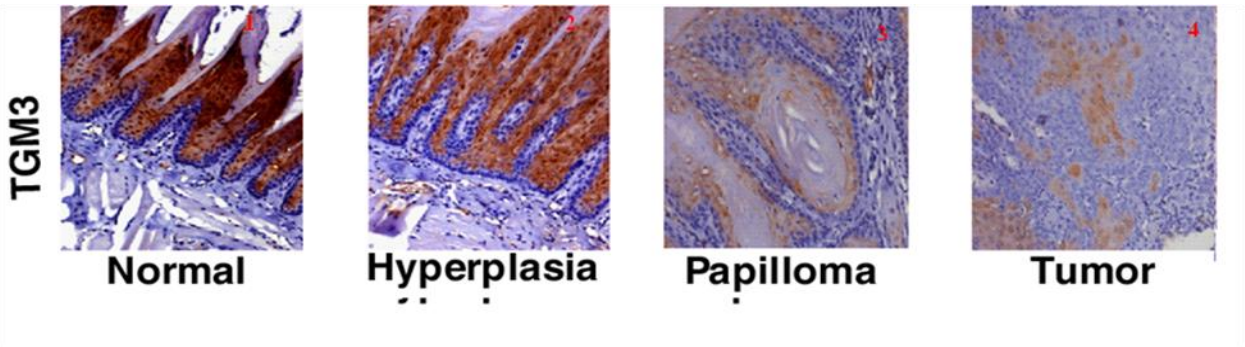
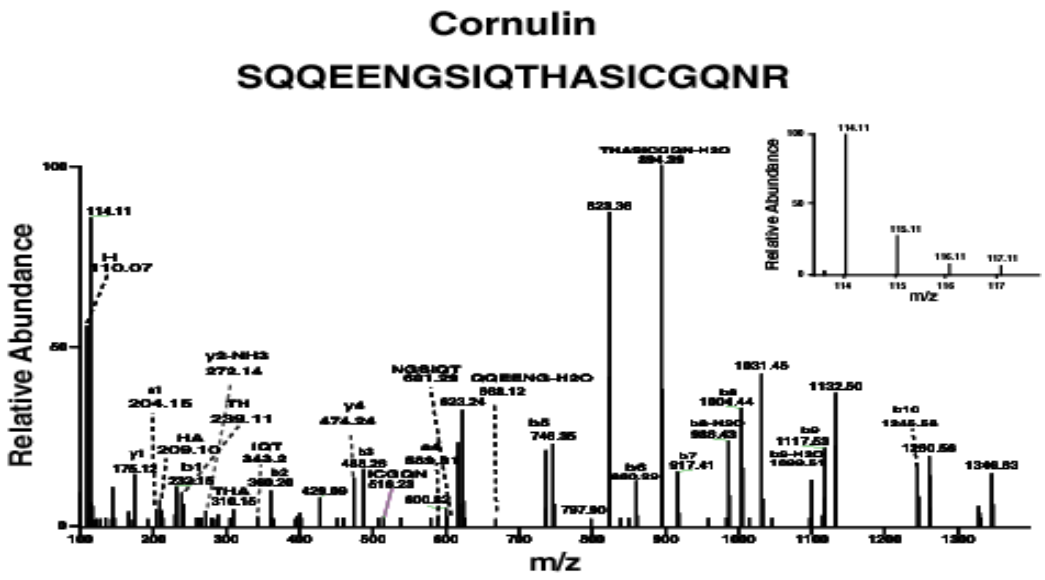


Figure 4.7. MS/MS spectra of Transglutaminase 3 and validation of Transglutaminase 3 by IHC on rat tongue tissues: (A). MS/MS spectra of peptide (QEYVEEDSGIIYVGSTNR) representing Transglutaminase 3. The inset showing the reporter ions used for quantitation suggests sequential down regulation of Transglutaminase 3 across the stages of rat oral carcinogenesis (B). Representative photomicrographs showing immunohistochemical staining of Transglutaminase 3 at different stages of rat lingual carcinogenesis (magnification ×200) (1) Vehicle treated tissue, (2) tongue treated for 120 days (hyperplasia), (3) 160 days (papilloma) and (4) 200 days (SCC) with 4NQO respectively. Strong staining of Transglutaminase 3 is seen in suprabasal layers of normal (1) and hyperplastic tissues (2) while reduced staining is observed in papillomatous and SCC tissues.

A.



B.

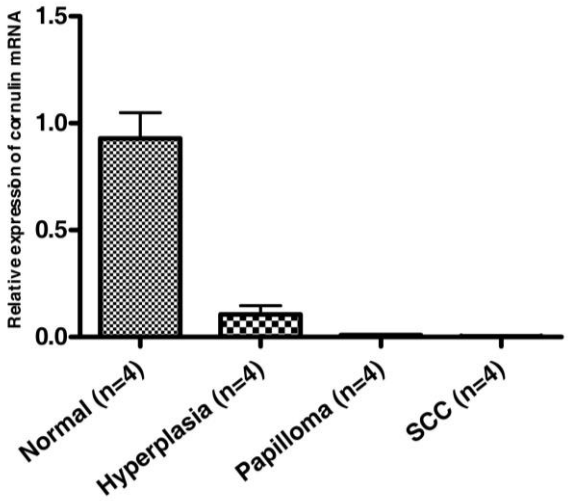


Figure 4.8: MS/MS spectra of Cornulin and Real time PCR validation of cornulin expression during different stages of lingual carcinogenesis. A. MS/MS spectra of peptide from representative of cornulin (SQQEENGSIQTHASICGQNR). Inset shows the reporter ions used for quantitation. . Down regulation of Cornulin was indicated. **B**. Relative cornulin mRNA expression during rat lingual carcinogenesis. Cornulin mRNA is sequentially downregulated during rat lingual carcinogenesis. Note: Cornulin down regulation is an early event during process of rat oral carcinogenesis.

4.8 Validation of Trichohyalin and thrombospondin 2 novel proteins in rat tongue tumerogenesis

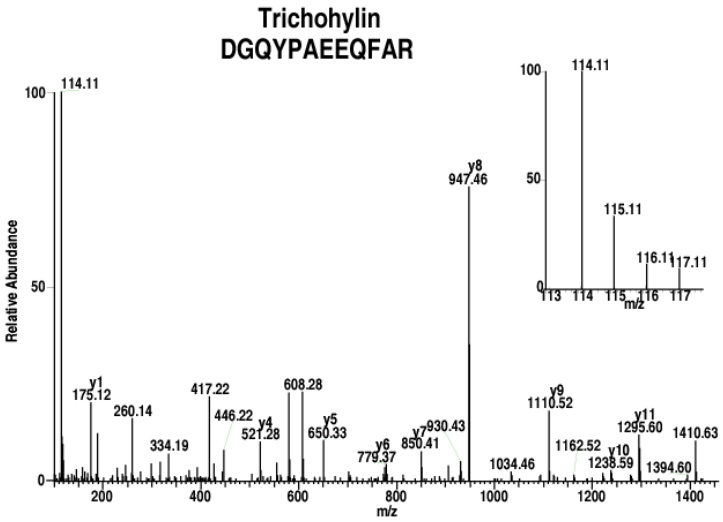
4.8.1 Trichohyalin

Our proteomics study revealed sequential down regulation of Trichohyalin in the process of rat oral carcinogenesis. We noted 14 fold downregulation of Trichohyalin in SCC as compared to normal tissues (**Figure 4.9A**). Because of unavailability of suitable antibody against rat we validated Trichohyalin expression profile by qRT-PCR. Quantitative real time PCR on rat tissues confirmed the proteomics finding (**Figure 4.9B**).

4.8.2 Thrombospondin 2

Thrombospondin 2 (Thsb2) was found to be sequentially up regulated across the stages of rat oral carcinogenesis in proteomics study. We noted a 7-fold up regulation of Thrombospondin 2 in SCC as compared to normal tissues(**Figure 4.10A**). Because of unavailability of specific antibody against rat we validated thrombospondin 2 by qRT-PCR. Validation on rat tissues by real time PCR confirmed the proteomics findings (**Figure 4.10B**).

A.



B

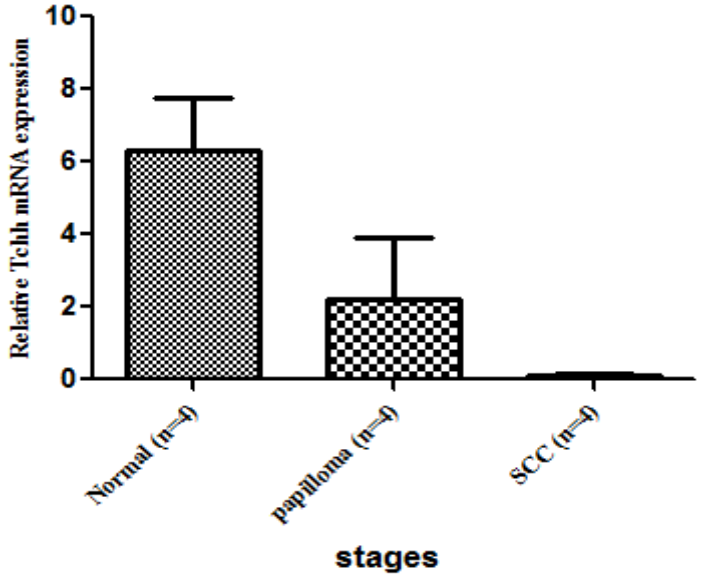
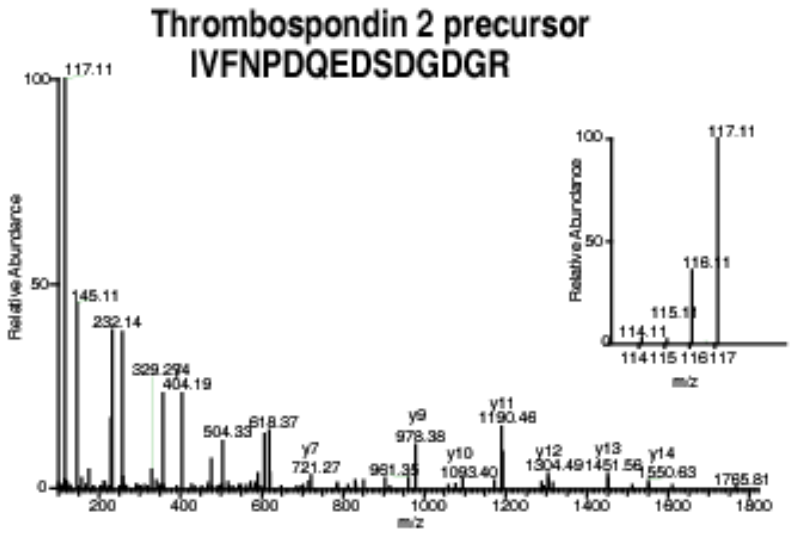


Figure 4.9: MS/MS spectra of Trichohyalin and Real time PCR validation of Trichohyalin expression during different stages of lingual carcinogenesis. MS/MS spectra of peptide from representative of Trichohyalin (DGQYPAEEQFAR). The inset shows the reporter ions used for quantitation. **B.** Relative Trichohyalin mRNA expression during rat lingual carcinogenesis. Note: Sequential down regulation of Trichohyalin during process of rat oral carcinogenesis.

.

A.



B

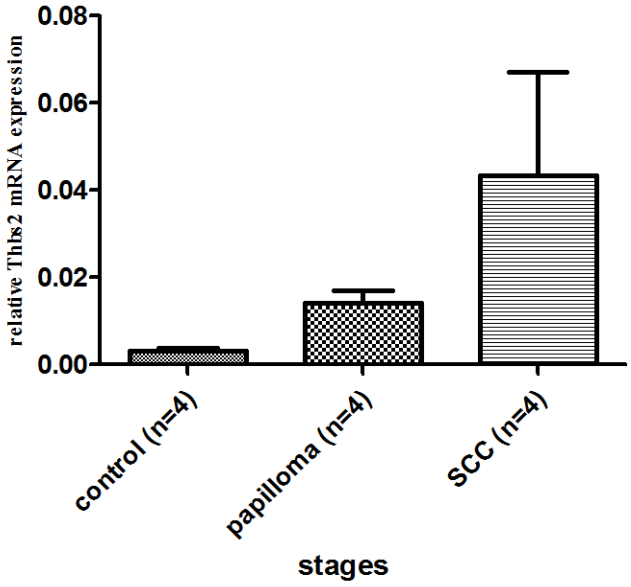


Figure 4.10: MS/MS spectra of Thrombospondin 2 and Real time validation of Thrombospondin 2 expression during different stages of lingual carcinogenesis. MS/MS spectra of peptide from representative of Thrombospondin 2 (IVFNPDQEDSDGDGR). The inset shows the reporter ions used for quantitation. **B.** Relative Thrombospondin 2 mRNA expression during rat lingual carcinogenesis. Note: Sequential up regulation of Thrombospondin 2 during process of rat oral carcinogenesis.

4.9 Validation of Tenascin N and Coronin 1a, novel proteins in rat and human tongue tumerogenesis

4.9.1 Tenascin N (*Tnn*):

Our rat proteomics data demonstrated that tenascin N (*Tnn)* was sequentially up regulated across the stages of rat lingual carcinogenesis. It was found to be upregulated by 2.5 fold in SCCs as compared to normal tissues (**Figure 4.11A**). To validate our proteomics results we performed immunohistochemistry on rat tissues (**Figure 4.11B**). Tenascin N expression was not seen in the vehicle treated rat tissues (control groups) while hyperplastic tissues showed weak cytoplasmic staining in keratinized layer of epithelium. Tenascin N expression was confined to keratinized layer also in papillomas and carcinomas. Carcinomas showed higher expression of tenascin N as compared to papillomas and hyperplastic tissues. We further validated tenascin 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 cells while its basal cell expression was weak. Strong cytoplasmic staining was detected in tumor cells.

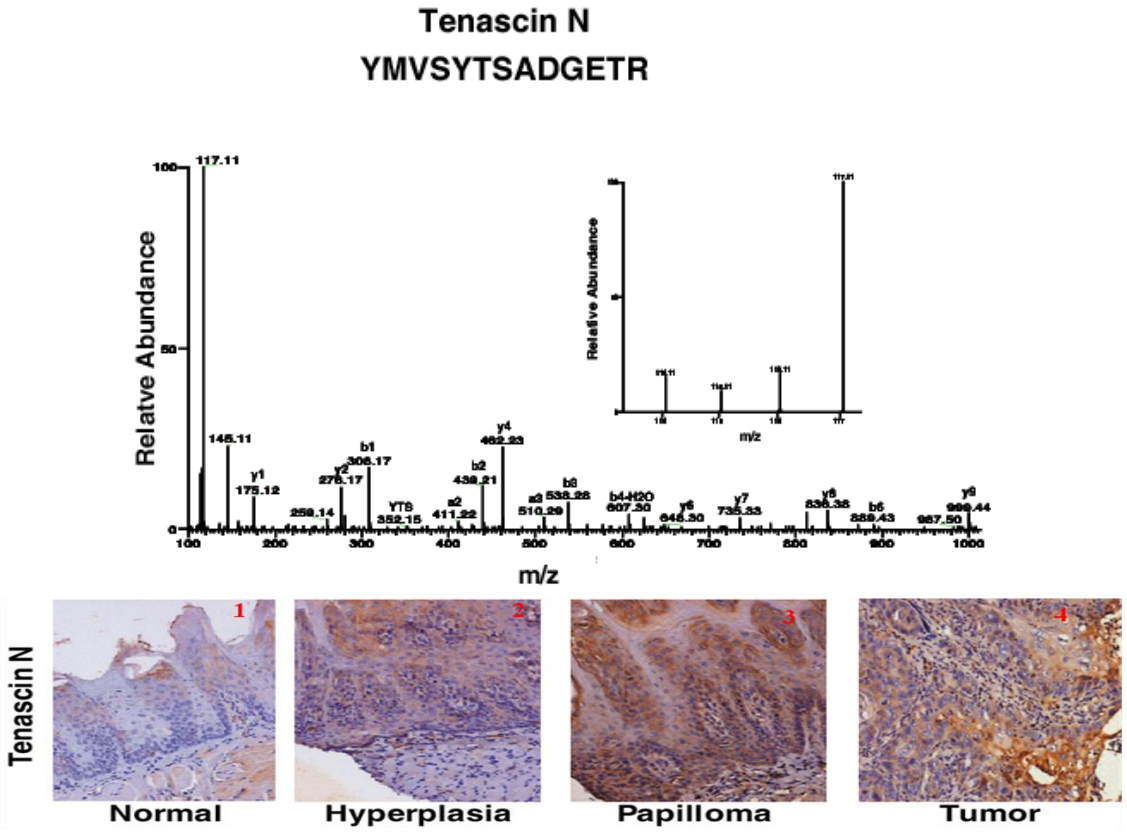
4.9.2 Coronin 1A:

Results of our iTRAQ analysis showed sequential up regulation of coronin1a at different stages of rat oral carcinogenesis (**Figure 4.12A**). 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 Coronin 1a is exclusively expressed by hematopoietic cells, normal or abnormal epithelial cells did not stained for coronin1a while infiltrating dendritic cells or hematopoietic cells were stained. Examination of papilloma and SCC tissues reveled more infiltrating hematopoietic cells in tumor than papilloma thus

giving more intense staining of Coronin 1a in tumors than in papillomas. Furthermore extrapolation to human tongue tissues showed that infiltrating hematopoietic cells were more in SCC in comparison to normal and leukoplakia tissues (**Figure 4.12C)**.

A.

B.



C.

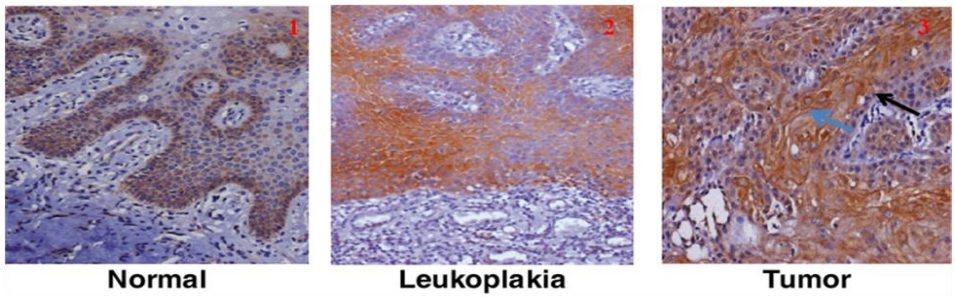


Figure 4.11. MS/MS spectra of Tenascin N and validation of Tenascin N by Immunohistochemistry in rat and human tissues. A. MS/MS spectra of peptide from representative of Tenascin N (YMVSYTSADGETR). Inset shows the reporter ions used for quantitation. **B.** Representative photomicrographs showing Immunohistochemical staining of Tenascin N during rat lingual carcinogenesis (magnification ×200) Note: Weak Tenascin N staining is present in keratinized layers of hyperplastic and papillomatous tissues while increased staining is present in SCC tissues. **C.** Representative photomicrographs showing Immunohistochemical detection of Tenascin N in human normal (1), leukoplakia (2) and tumor of tongue tissues (3). Arrows indicate the weak expression of Tenascin N in basal layer (black) of tumor while increased expression of Tenascin N in differentiated layers (blue) (magnification ×200)

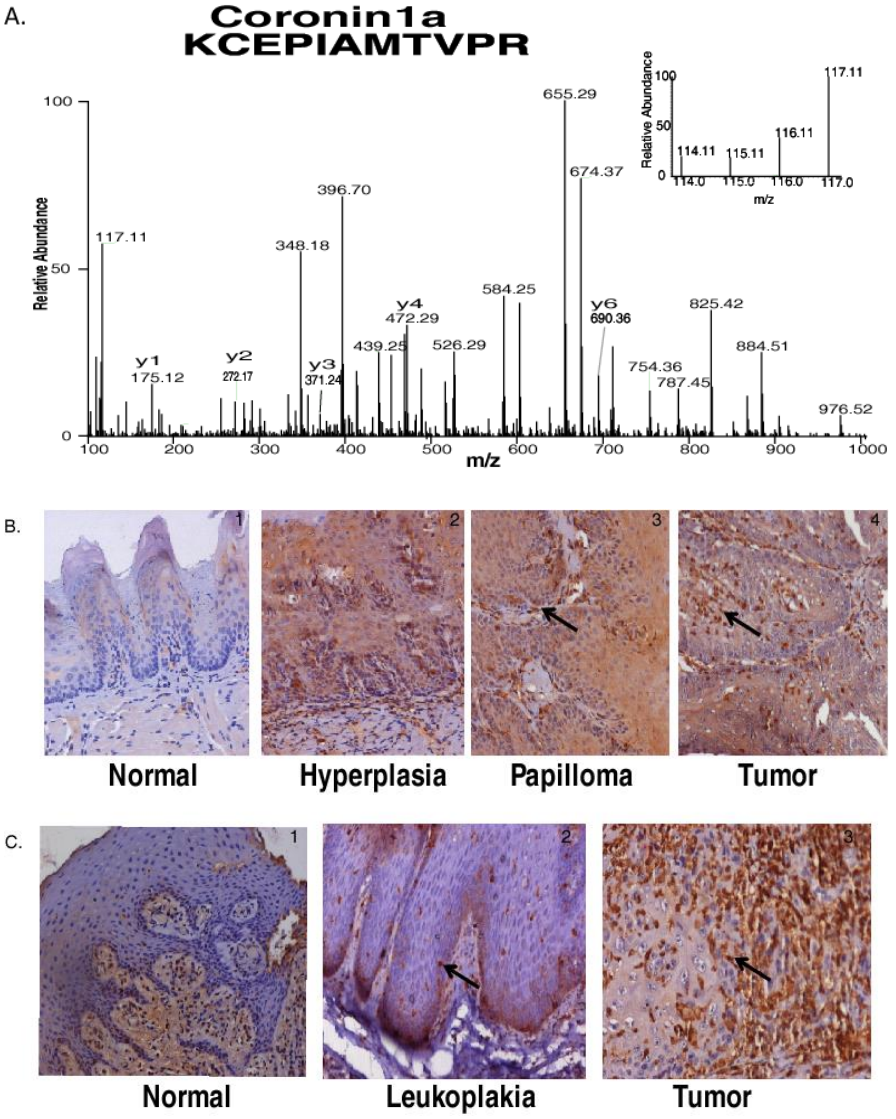


Figure 4.12. MS/MS spectra of Coronin 1A and validation of coronin 1A by Immunohistochemistry in rat and human tissues. A. MS/MS spectra of peptide from representative of Coronin1A (KCEPIAMTVPR). The inset shows the reporter ions used for quantitation. **B.** Representative photomicrographs showing Immunohistochemical detection labeling of Coronin 1A during rat lingual carcinogenesis (magnification ×200) Note: Coronin 1A staining is not seen in the epithelial layers of normal, hyperplastic, papillomatous or SCC tissues but seen only in the infiltrating hematopoietic cells **C.** Representative photomicrographs showing Immunohistochemical detection of coronin 1a in human normal (1), leukoplakia (2) and tumor of tongue tissues (3). (magnification ×200) Note: Coronin 1A expression is detected in infiltrating lymphocytes and not in epithelial layers.

4.10 Bioinformatics analysis by GO analysis:

Bioinformatics analysis was carried out to classify proteins based on sub cellular localization and biological function. We carried out classification based on Gene Ontology (GO) annotations. The distribution of proteins identified in our study based on sub cellular localization and biological process is shown in **(Figure 4.13A)** and **(Figure 4.13B)** respectively. All proteins identified in the current iTRAQ-based analysis of rat lingual carcinogenesis were categorized on the basis of primary sub cellular locations **(Figure 4.13A)** which resulted in 1,835 proteins (83%) being localized to one of the sub cellular compartments. Additionally, proteins were also classified on the basis of biological processes (e.g. cell signaling and communication). This resulted in the identification of 1,786 proteins (80%) which were grouped into one of biological processes **(Figure 4.13B).** The majority of the grouped proteins play a role in cellular metabolism, protein synthesis, degradation and transport.

Further we subjected differentially expressed proteins of tumors for gene ontology based classification. The up and down regulated proteins were classified based on their gene ontology annotations. Out of 194 upregulated proteins, 157 proteins belong to one of the biological processes (cell cycle, immune response etc.) **(Figure 4.14A).** Further analysis identified sub cellular localization of 157 proteins (cytoplasm, membrane, mitochondria etc.) **(Figure 4.14B).** Similarly, out of 221 down regulated proteins, 158 proteins belong to one of the biological processes **(Figure 4.15A).** Further analysis identified sub cellular localization of 167 proteins **(Figure 4.15B).**

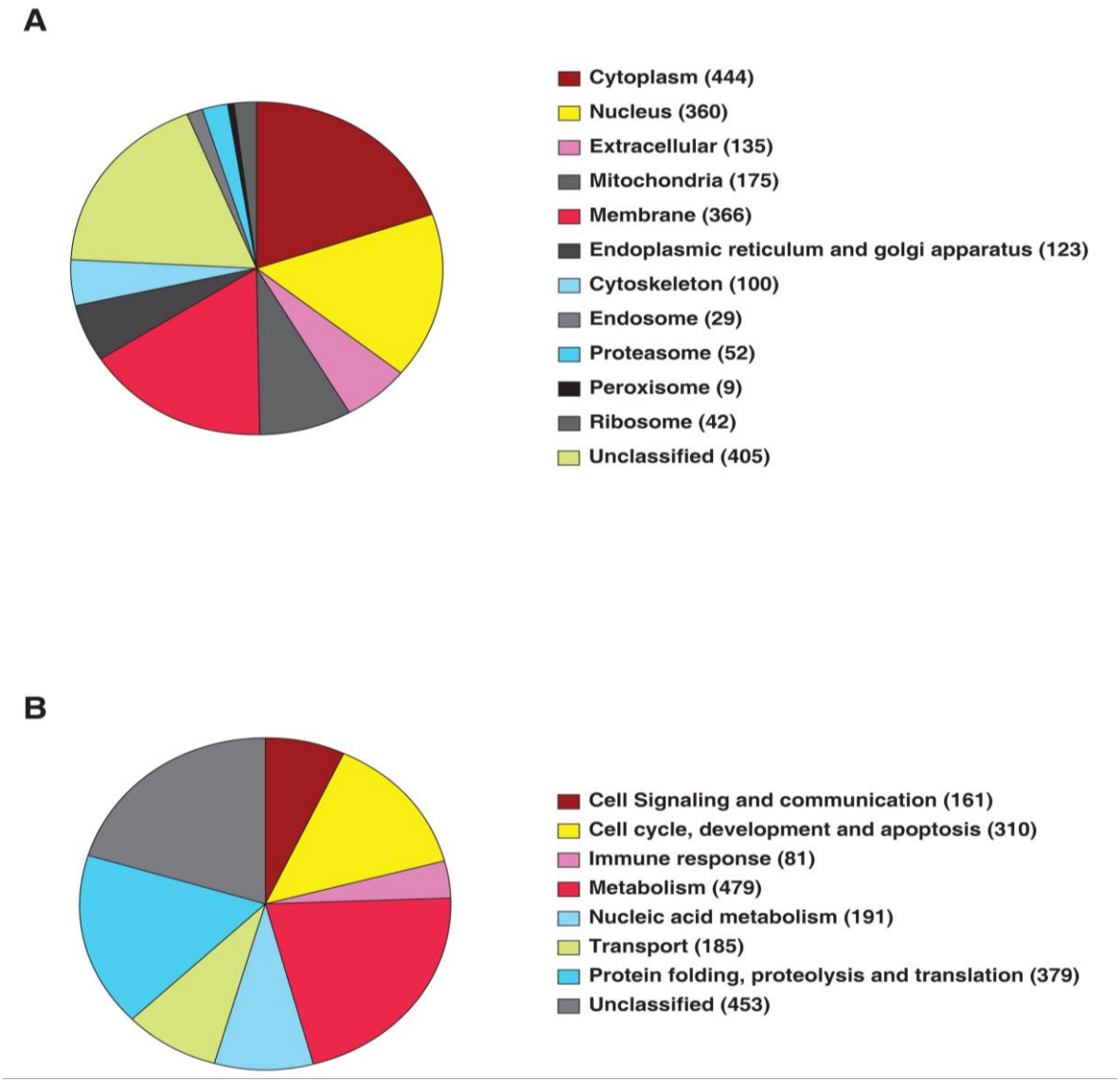


Figure 4.13: Classification of proteins by gene ontology based on their cellular localization and biological process

A) Distribution of proteins based on their Cellular localization using gene ontology classifier
B) Distribution of proteins based on their biological processes using gene ontology classifier

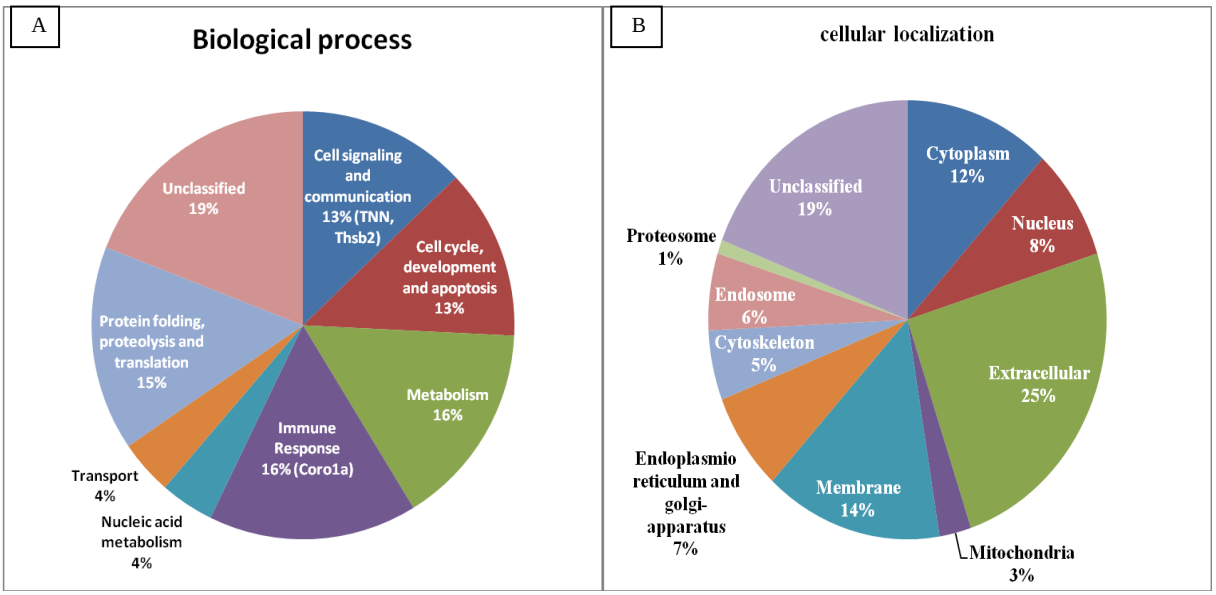


Figure 4.14: Classification of up regulated proteins in SCC by gene ontology based on their cellular localization and biological process

A) Distribution of up regulated proteins based on their biological processes using gene ontology classifier B) Distribution of up regulated proteins based on their Cellular localization using gene ontology classifier

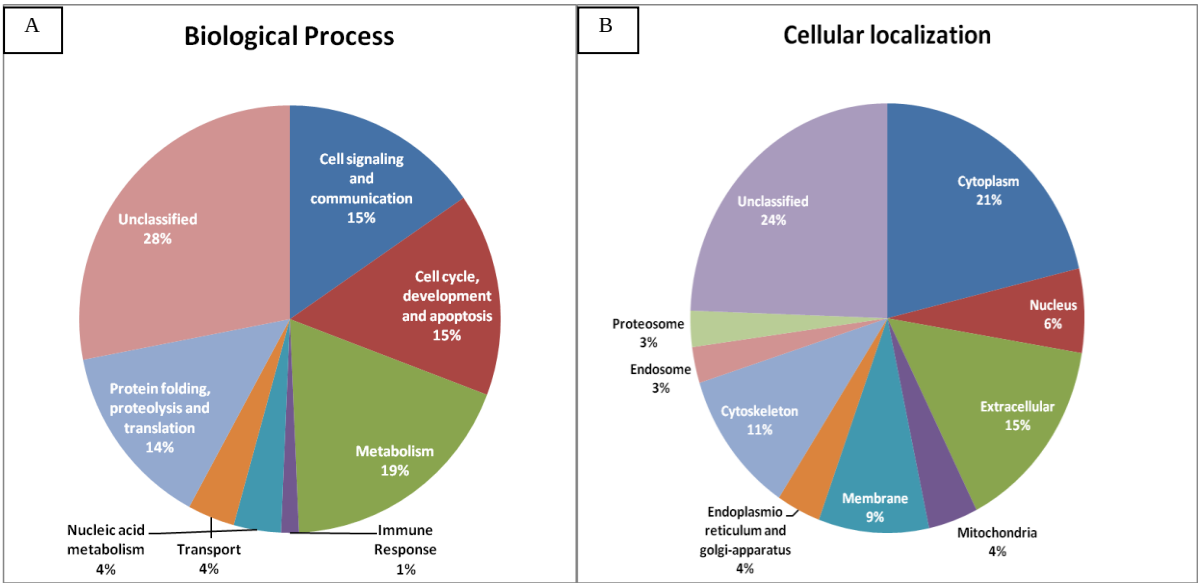


Figure 4.15: Classification of down regulated proteins in SCC by gene ontology based on their cellular localization and biological process

A) Distribution of down regulated proteins based on their biological processes using gene ontology classifier B) Distribution of down regulated proteins based on their Cellular localization using gene ontology classifier

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 12^{th} 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 veraity 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 humen, galectin 7 was found to be up regulated 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 togather 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 vey 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] reveled 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 3and 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[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[164]. They regulate cell proliferation induced by rac1 redox-dependent Signaling. TSP-2 inhibits tumor growth and angiogenesis of human squamous cell carcinomas[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)**.

Tenascin N:

Tenascin is a high molecular weight extracellular matrix glycoprotein. Its expression was detected during embryogenesis, wound healing and neoplastic processes[167]. Tenascin 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 tenascin 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)**. Tenascin 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. Tenascin N expression was also confined to keratinized layer in papillomas and carcinomas. Carcinomas showed higher expression of tenascin N as compared to papillomas and hyperplastic tissues. We further validated tenascin 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 (C*oro1a*)

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].

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 ($p27^{kip1}$) 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.

CHAPTER 7

SUMMARY AND CONCLUSION

Summary

1. A rat model of lingual carcinogenesis was established where all the stages of oral carcinogenesis viz. hyperplasia, papilloma and carcinoma were obtained after 120, 160 and 200 days of 4NQO treatment in drinking water respectively.
2. Differential proteomics study by MALDI-TOF-TOF resulted in identification of five differential protein spots which include upregulated proteins as, fatty acid binding protein 5, serum albumin and Keratin 6a while down regulated proteins galectin 7 and transglutaminase 3.
3. Validation of galectin 7 by IHC and western blot, in rat tongue tissues showed that galectin 7 was sequentially down regulated across the stages of carcinogenesis.
4. Differential proteomics study by iTRAQ strategy resulted in identification of 2223 proteins. Out of these, 415 proteins were found to be differentially expressed in tumors, 333 in papillomas and 109 in hyperplasia.
5. Among the 415 differentially expressed proteins in tumors 194 were up regulated while 221 were down regulated. In papillomas 155 proteins were up regulated while 178 were down regulated. Among 109 differential proteins of hyperplasia, 35 proteins were upregulated while 74 were down regulated.
6. Among the differentially expressed proteins, 5 were sequentially upregulated while 10 were sequentially down regulated from hyperplasia to SCC. Similarly, sequentially up or down regulation of 62 and 51 proteins respectively was observed from papilloma to carcinoma tissues. We also observed sequential up regulation of 154 proteins while sequential downregulation of 170 proteins from normal to SCC.
7. IHC validation of Vimentin, Fascin and Periostin confirmed the sequential up regulation while it confirmed sequential down regulation of TGM3 across stages of rat tongue carcinogenesis.

8. Validation of cornulin by real time PCR revealed that cornulin was sequentially down regulated in the rat tongue carcinogenesis. Its down regulation was an early event in the carcinogenesis process.
9. Our proteomics data demonstrated sequential up regulation of TNN, CORO1A and THBS2 and downregulation of TCHH.
10. Sequential up and down regulation of THBS2 and TCHH respectively were confirmed by real time PCR across the stages of rat oral carcinogenesis.
11. Sequential up regulation of TNN and CORO 1A was confirmed by IHC in both rat and human tissues.
12. Bioinformatics studies based Gene Ontology on proteins identified in iTRAQ studies revealed that 83 % of proteins identified were grouped in sub cellular compartment while 80% of identified proteins were grouped into one of biological process.

Conclusion:

This is the most extensive quantitative proteomic study in rat model of 4NQO-induced oral carcinogenesis carried out until date. 2DE followed by MALDI-TOF-TOF led to identification of only a small number of differential proteins as compared to ITRAQ-LC MS/MS, indicating that iTRAQ LC MS/MS is superior method for identification of differentially expressed proteins. We successfully validated several known proteins like VIM, FSCN1, TGM3, POSTN and CRNN and novel molecules like, TCHH, THBS2, TNN, and CORO 1A, based on our proteomics findings. Using this model, we are able to show sequential alterations in expression pattern during rat tongue carcinogenesis. Furthermore, we are also able to extrapolate our rat model data to human system indicating the fact that this model has potential to be used for biomarker discovery in human oral cancer.

CHAPTER 8
BIBLIOGRAPHY

1. Ferlay J SI, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F.: **GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]**. 2012.
2. Sankaranarayanan R, Black RJ, Swaminathan R, Parkin DM: **An overview of cancer survival in developing countries**. *IARC scientific publications* 1998(145):135-173.
3. Hunter KD, Parkinson EK, Harrison PR: **Profiling early head and neck cancer**. *Nat Rev Cancer* 2005, **5**(2):127-135.
4. Lippman SM, Hong WK: **Molecular markers of the risk of oral cancer**. *New England Journal of Medicine* 2001, **344**(17):1323-1326.
5. Hu S, Arellano M, Boontheung P, Wang J, Zhou H, Jiang J, Elashoff D, Wei R, Loo JA, Wong DT: **Salivary proteomics for oral cancer biomarker discovery**. *Clinical Cancer Research* 2008, **14**(19):6246-6252.
6. Schliephake H: **Prognostic relevance of molecular markers of oral cancer—a review**. *International journal of oral and maxillofacial surgery* 2003, **32**(3):233-245.
7. Hanahan D, Weinberg RA: **The hallmarks of cancer**. *cell* 2000, **100**(1):57-70.
8. Tysnes BB, Bjerkvig R: **Cancer initiation and progression: involvement of stem cells and the microenvironment**. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer* 2007, **1775**(2):283-297.
9. Kanojia D, Vaidya MM: **4-Nitroquinoline-1-oxide induced experimental oral carcinogenesis**. *Oral oncology* 2006, **42**(7):655-667.
10. Ralhan R: **Diagnostic potential of genomic and proteomic signatures in oral cancer**. *INTERNATIONAL JOURNAL OF HUMAN GENETICS* 2007, **7**(1):57.
11. Chaurand P, Sanders ME, Jensen RA, Caprioli RM: **Proteomics in diagnostic pathology: profiling and imaging proteins directly in tissue sections**. *The American journal of pathology* 2004, **165**(4):1057-1068.
12. Ralhan R, DeSouza LV, Matta A, Chandra Tripathi S, Ghanny S, DattaGupta S, Thakar A, Chauhan SS, Siu KM: **iTRAQ-multidimensional liquid chromatography and tandem mass spectrometry-based identification of potential biomarkers of oral epithelial dysplasia and novel networks between inflammation and premalignancy**. *Journal of proteome research* 2008, **8**(1):300-309.
13. Macabeo-Ong M, Jordan RC, Hardt M, Hall S, Dixon S, Lim S, Schmidt BL: **Proteomic discovery of markers of metastatic oral SCC**. *Proceedings of the American Association for Cancer Research* 2006, **2006**(2):A19.
14. Lai C-H, Chang N-W, Lin C-F, Lin C-D, Lin Y-J, Wan L, Sheu JJ-C, Chen S-Y, Huang Y-P, Sing Y-T: **Proteomics-based identification of haptoglobin as a novel plasma biomarker in oral squamous cell carcinoma**. *Clinica Chimica Acta* 2010, **411**(13):984-991.
15. Thiel UJ, Feltens R, Adryan B, Gieringer R, Brochhausen C, Schuon R, Fillies T, Grus F, Mann WJ, Brieger J: **Analysis of differentially expressed proteins in oral squamous cell carcinoma by MALDI- TOF MS**. *Journal of Oral Pathology & Medicine* 2011, **40**(5):369-379.
16. La Vecchia C, Tavani A, Franceschi S, Levi F, Corrao G, Negri E: **Epidemiology and prevention of oral cancer**. *Oral oncology* 1997, **33**(5):302-312.

17. Kujan O, Glenny AM, Duxbury J, Thakker N, Sloan P: **Evaluation of screening strategies for improving oral cancer mortality: a Cochrane systematic review**. *Journal of dental education* 2005, **69**(2):255-265.
18. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A: **Global cancer statistics, 2012**. *CA Cancer J Clin* 2015, **65**(2):87-108.
19. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM: **Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008**. *Int J Cancer* 2010, **127**(12):2893-2917.
20. Scully C: **Challenges in predicting which oral mucosal potentially malignant disease will progress to neoplasia**. *Oral diseases* 2014, **20**(1):1-5.
21. Sundaram S: **Tobacco Amid Controversy**. *Facts For You* 2003.
22. Murthy N, Mathew A: **Cancer epidemiology, prevention and control**. *CURRENT SCIENCE-BANGALORE*- 2004, **86**(4):518-527.
23. Warnakulasuriya K, Ralhan R: **Clinical, pathological, cellular and molecular lesions caused by oral smokeless tobacco–a review**. *Journal of oral pathology & medicine* 2007, **36**(2):63-77.
24. Notani PN, Jayant K: **Role of diet in upper aerodigestive tract cancers**. 1987.
25. Murthy N, Mathew A: **Cancer epidemiology, prevention and control**. *CURRENT SCIENCE-BANGALORE*- 2004, **86**(4):518-527.
26. Seitz HK, Stickel F: **Molecular mechanisms of alcohol-mediated carcinogenesis**. *Nature reviews cancer* 2007, **7**(8):599-612.
27. Pöschl G, Seitz HK: **Alcohol and cancer**. *Alcohol and Alcoholism* 2004, **39**(3):155-165.
28. Maier H, Sennewald E, Heller G, Weidauer H: **Chronic alcohol consumption--the key risk factor for pharyngeal cancer**. *Otolaryngology--head and neck surgery: official journal of American Academy of Otolaryngology-Head and Neck Surgery* 1994, **110**(2):168.
29. Seitz HK, Stickel F, Homann N: **Pathogenetic mechanisms of upper aerodigestive tract cancer in alcoholics**. *International journal of cancer* 2004, **108**(4):483-487.
30. Ha PK, Califano JA: **The role of human papillomavirus in oral carcinogenesis**. *Critical Reviews in Oral Biology & Medicine* 2004, **15**(4):188-196.
31. Marshall JR, Boyle P: **Nutrition and oral cancer**. *Cancer causes and control* 1996, **7**(1):101-111.
32. Parkin DM, Muir C, Whelan S, Gao Y, Ferlay J, Powell J: **Cancer incidence in five continents, volume VI**: International Agency for Research on Cancer; 1992.
33. Marshall J, Graham S, Mettlin C, Shedd D, Swanson M: **Diet in the epidemiology of oral cancer**. 1981.
34. Sinha R, Anderson D, McDonald S, Greenwald P: **Cancer risk and diet in India**. *Journal of postgraduate medicine* 2003, **49**(3):222.
35. Aggarwal BB, Kumar A, Bharti AC: **Anticancer potential of curcumin: preclinical and clinical studies**. *Anticancer Res* 2003, **23**(1A):363-398.
36. Shklar G: **Oral leukoplakia**. *New England Journal of Medicine* 1986, **315**(24):1544-1546.
37. Neville BW, Day TA: **Oral cancer and precancerous lesions**. *CA: a cancer journal for clinicians* 2009, **52**(4):195-215.
38. Shafer WG, Waldron CA: **Erythroplakia of the oral cavity**. *Cancer* 1975, **36**(3):1021-1028.
39. Sharma A, BIALYNICKI-BIRULA R, Schwartz RA, Janniger CK: **Lichen Planus: An Update and Review**. *Cutis* 2012, **90**(1):17-23.

40. Georgakopoulou EA, Achtari MD, Achtaris M, Foukas PG, Kotsinas A: **Oral Lichen Planus as a Preneoplastic Inflammatory Model**. *Journal of Biomedicine and Biotechnology* 2012, **2012**.
41. Murti P, Bhonsle R, Gupta P, Daftary D, Pindborg J, Mehta FS: **Etiology of oral submucous fibrosis with special reference to the role of areca nut chewing**. *Journal of Oral Pathology & Medicine* 1995, **24**(4):145-152.
42. Tilakaratne W, Klinikowski M, Saku T, Peters T, Warnakulasuriya S: **Oral submucous fibrosis: review on aetiology and pathogenesis**. *Oral oncology* 2006, **42**(6):561-568.
43. Amagasa T, Yamashiro M, Ishikawa H: **Oral leukoplakia related to malignant transformation**. *Oral Science International* 2006, **3**(2):45-55.
44. Amagasa T, Yamashiro M, Uzawa N: **Oral premalignant lesions: from a clinical perspective**. *International Journal of Clinical Oncology* 2011, **16**(1):5-14.
45. Silverman S, Bhargava K, Mani N, Smith LW, Malaowalla A: **Malignant transformation and natural history of oral leukoplakia in 57,518 industrial workers of Gujarat, India**. *Cancer* 1976, **38**(4):1790-1795.
46. Gupta P, Mehta FS, Daftary D, Pindborg J, Bhonsle R, Jalnawalla P, Sinor P, Pitkar V, Murti P, Irani R: **Incidence rates of oral cancer and natural history of oral precancerous lesions in a 10-year follow-up study of Indian villagers**. *Community dentistry and oral epidemiology* 1980, **8**(6):287-333.
47. Mehta FS, Shroff B, Gupta P, Daftary D: **Oral leukoplakia in relation to tobacco habits: A ten-year follow-up study of Bombay policemen**. *Oral Surgery, Oral Medicine, Oral Pathology* 1972, **34**(3):426-433.
48. Einhorn J, Wersäll J: **Incidence of oral carcinoma in patients with leukoplakia of the oral mucosa**. *Cancer* 1967, **20**(12):2189-2193.
49. Kramer I: **Precancerous conditions of the oral mucosa. A computer-aided study**. *Annals of the Royal College of Surgeons of England* 1969, **45**(6):340.
50. Bánóczy J: **Follow-up studies in oral leukoplakia**. *Journal of maxillofacial surgery* 1977, **5**:69-75.
51. LIND PO: **Malignant transformation in oral leukoplakia**. *European Journal of Oral Sciences* 1987, **95**(6):449-455.
52. Gangadharan P, Paymaster J: **Leukoplakia—An Epidemiologic Study of 1504 Cases Observed at the Tata Memorial Hospital, Bombay, India**. *British Journal of Cancer* 1971, **25**(4):657.
53. Schepman K, Van der Meij E, Smeele L, Van der Waal I: **Malignant transformation of oral leukoplakia: a follow-up study of a hospital-based population of 166 patients with oral leukoplakia from The Netherlands**. *Oral oncology* 1998, **34**(4):270-275.
54. Gorsky M, Ms FL: **Oral leukoplakia and malignant transformation. A follow-up study of 257 patients**. *Cancer* 1984, **53**(3):563-568.
55. Frese KK, Tuveson DA: **Maximizing mouse cancer models**. *Nature reviews cancer* 2007, **7**(9):654-658.
56. Eveson J: **Animal models of infra-oral chemical carcinogenesis: A review**. *Journal of Oral Pathology & Medicine* 1981, **10**(3):129-146.
57. Salley JJ: **Experimental carcinogenesis in the cheek pouch of the Syrian hamster**. *Journal of dental research* 1954, **33**(2):253-262.
58. Nauta JM, Roodenburg JL, Nikkels PG, Witjes MJ, Vermey A: **Epithelial dysplasia and squamous cell carcinoma of the Wistar rat palatal mucosa: 4NQO model**. *Head & neck* 1996, **18**(5):441-449.

59. MacDonald DG: **Comparison of epithelial dysplasia in hamster cheek pouch carcinogenesis and human oral mucosa**. *Journal of Oral Pathology & Medicine* 1981, **10**(3):186-191.
60. Nauta J, Roodenburg J, Nikkels P, Witjes M, Vermey A: **Comparison of epithelial dysplasia—the 4NQO rat palate model and human oral mucosa**. *International journal of oral and maxillofacial surgery* 1995, **24**(1):53-58.
61. Kim S: **Animal models of cancer in the head and neck region**. *Clinical and experimental otorhinolaryngology* 2009, **2**(2):55-60.
62. John S, Thiebach L, Frie C, Mokkapati S, Bechtel M, Nischt R, Rosser-Davies S, Paulsson M, Smyth N: **Epidermal Transglutaminase (TGase 3) Is Required for Proper Hair Development, but Not the Formation of the Epidermal Barrier**. *PloS one* 2012, **7**(4):e34252.
63. Benson AM: **Conversion of 4-nitroquinoline 1-oxide (4NQO) to 4-hydroxyaminoquinoline 1-oxide by a dicumarol-resistant hepatic 4NQO nitroreductase in rats and mice**. *Biochemical pharmacology* 1993, **46**(7):1217-1221.
64. Friedberg EC, Walker GC, Siede W: **DNA repair and mutagenesis**: American Society for Microbiology (ASM); 1995.
65. Koontongkaew S, Chareonkitkajorn L, Chanvitan A, Leelakriangsak M, Amornphimoltham P: **Alterations of p53, pRb, cyclin D< sub> 1</sub> and cdk4 in human oral and pharyngeal squamous cell carcinomas**. *Oral oncology* 2000, **36**(4):334-339.
66. Aronson J: **Biomarkers and surrogate endpoints**. *British journal of clinical pharmacology* 2005, **59**(5):491-494.
67. Seibert V, Ebert MP, Buschmann T: **Advances in clinical cancer proteomics: SELDI-ToF-mass spectrometry and biomarker discovery**. *Briefings in functional genomics & proteomics* 2005, **4**(1):16-26.
68. Wilkins MR, Sanchez J-C, Gooley AA, Appel RD, Humphery-Smith I, Hochstrasser DF, Williams KL: **Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it**. *Biotechnology and genetic engineering reviews* 1996, **13**(1):19-50.
69. Aebersold R, Goodlett DR: **Mass spectrometry in proteomics**. *Proteomics* 2001, **3**:5.
70. Ilyin SE, Belkowski SM, Plata-Salamán CR: **Biomarker discovery and validation: technologies and integrative approaches**. *TRENDS in Biotechnology* 2004, **22**(8):411-416.
71. O'Farrell PH: **High resolution two-dimensional electrophoresis of proteins**. *Journal of Biological Chemistry* 1975, **250**(10):4007-4021.
72. Derosa C, Furusato B, Shaheduzzaman S, Srikantan V, Wang Z, Chen Y, Siefert M, Ravindranath L, Young D, Nau M: **Elevated osteonectin/SPARC expression in primary prostate cancer predicts metastatic progression**. *Prostate cancer and prostatic diseases* 2011, **15**(2):150-156.
73. Schulze WX, Usadel B: **Quantitation in mass-spectrometry-based proteomics**. *Annual review of plant biology* 2010, **61**:491-516.
74. O'Farrell PZ, Goodman HM, O'Farrell PH: **High resolution two-dimensional electrophoresis of basic as well as acidic proteins**. *cell* 1977, **12**(4):1133-1142.
75. Walker JM: **The protein protocols handbook**, vol. 1996: Springer; 1996.
76. Abdallah C, Dumas-Gaudot E, Renaut J, Sergeant K: **Gel-based and gel-free quantitative proteomics approaches at a glance**. *International journal of plant genomics* 2012, **2012**.

77. Van den Bergh G, Arckens L: **Fluorescent two-dimensional difference gel electrophoresis unveils the potential of gel-based proteomics**. *Current opinion in biotechnology* 2004, **15**(1):38-43.
78. Bijian K, Mlynarek AM, Balys RL, Jie S, Xu Y, Hier MP, Black MJ, Di Falco MR, LaBoissiere S, Alaoui-Jamali MA: **Serum Proteomic Approach for the Identification of Serum Biomarkers Contributed by Oral Squamous Cell Carcinoma and Host Tissue Microenvironment†**. *Journal of proteome research* 2009, **8**(5):2173-2185.
79. Manadas B, Mendes VM, English J, Dunn MJ: **Peptide fractionation in proteomics approaches**. *Expert review of proteomics* 2010, **7**(5):655-663.
80. Abdallah C, Dumas-Gaudot E, Renaut J, Sergeant K: **Gel-based and gel-free quantitative proteomics approaches at a glance**. *International journal of plant genomics* 2012, **2012**.
81. Fournier ML, Gilmore JM, Martin-Brown SA, Washburn MP: **Multidimensional separations-based shotgun proteomics**. *Chemical reviews* 2007, **107**(8):3654-3686.
82. Zhao Y, Lee W-NP, Xiao GG: **Quantitative proteomics and biomarker discovery in human cancer**. *Expert review of proteomics* 2009, **6**(2):115.
83. Ong S-E, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M: **Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics**. *Molecular & cellular proteomics* 2002, **1**(5):376-386.
84. Liang S, Xu Z, Xu X, Zhao X, Huang C, Wei Y: **Quantitative proteomics for cancer biomarker discovery**. *Combinatorial Chemistry & High Throughput Screening* 2012, **15**(3):221-231.
85. Wiese S, Reidegeld KA, Meyer HE, Warscheid B: **Protein labeling by iTRAQ: a new tool for quantitative mass spectrometry in proteome research**. *Proteomics* 2006, **7**(3):340-350.
86. Hu S, Wong DT: **Oral cancer proteomics**. *Current opinion in molecular therapeutics* 2007, **9**(5):467.
87. Hatakeyama H, Kondo T, Fujii K, Nakanishi Y, Kato H, Fukuda S, Hirohashi S: **Protein clusters associated with carcinogenesis, histological differentiation and nodal metastasis in esophageal cancer**. *Proteomics* 2006, **6**(23):6300-6316.
88. Nishimori T, Tomonaga T, Matsushita K, Oh-Ishi M, Kodera Y, Maeda T, Nomura F, Matsubara H, Shimada H, Ochiai T: **Proteomic analysis of primary esophageal squamous cell carcinoma reveals downregulation of a cell adhesion protein, periplakin**. *Proteomics* 2006, **6**(3):1011-1018.
89. Zhang L-Y, Ying W-T, Mao Y-S, He H-Z, Liu Y, Wang H-X, Liu F, Wang K, Zhang D-C, Wang Y: **Loss of clusterin both in serum and tissue correlates with the tumorigenesis of esophageal squamous cell carcinoma via proteomics approaches**. *World Journal of Gastroenterology* 2003, **9**(4):650-654.
90. Contu SS, Contu PC, Damin DC, Fagundes RB, Bevilacqua F, Rosa AS, Prolla JC, Moreira LF: **pRB expression in esophageal mucosa of individuals at high risk for squamous cell carcinoma of the esophagus**. *World Journal of Gastroenterology* 2007, **13**(11):1728.
91. Qi Y, Chiu JF, Wang L, Kwong DL, He QY: **Comparative proteomic analysis of esophageal squamous cell carcinoma**. *Proteomics* 2005, **5**(11):2960-2971.
92. Schoenherr RM, Whiteaker JR, Zhao L, Ivey RG, Trute M, Kennedy J, Voytovich UJ, Yan P, Lin C, Paulovich AG: **Multiplexed quantification of estrogen receptor and HER2/Neu in tissue and cell lysates by peptide immunoaffinity enrichment mass spectrometry**. *Proteomics* 2012, **12**(8):1253-1260.

93. Indovina P, Marcelli E, Maranta P, Tarro G: **Lung cancer proteomics: recent advances in biomarker discovery**. *International journal of proteomics* 2011, **2011**.
94. Sun Y, Mi W, Cai J, Ying W, Liu F, Lu H, Qiao Y, Jia W, Bi X, Lu N: **Quantitative proteomic signature of liver cancer cells: tissue transglutaminase 2 could be a novel protein candidate of human hepatocellular carcinoma**. *Journal of proteome research* 2008, **7**(9):3847-3859.
95. Sun Y, Zang Z, Xu X, Zhang Z, Zhong L, Zan W, Zhao Y, Sun L: **Differential proteomics identification of HSP90 as potential serum biomarker in hepatocellular carcinoma by two-dimensional electrophoresis and mass spectrometry**. *International journal of molecular sciences* 2010, **11**(4):1423-1433.
96. Wu R, Zhao X, Wang Z, Zhou M, Chen Q: **Novel molecular events in oral carcinogenesis via integrative approaches**. *Journal of dental research* 2011, **90**(5):561-572.
97. Herrmann PC, Liotta LA, Petricoin Iii EF: **Cancer proteomics: the state of the art**. *Disease markers* 2001, **17**(2):49-58.
98. Hayashi E, Kuramitsu Y, Fujimoto M, Zhang X, Tanaka T, Uchida K, Fukuda T, Furumoto H, Ueyama Y, Nakamura K: **Proteomic profiling of differential display analysis for human oral squamous cell carcinoma: 14-3-3 σ Protein is upregulated in human oral squamous cell carcinoma and dependent on the differentiation level**. *PROTEOMICS-Clinical Applications* 2009, **3**(11):1338-1347.
99. Govekar RB, D'Cruz AK, Alok Pathak K, Agarwal J, Dinshaw KA, Chinoy RF, Gadewal N, Kannan S, Sirdeshmukh R, Sundaram CS: **Proteomic profiling of cancer of the gingivo-buccal complex: Identification of new differentially expressed markers**. *PROTEOMICS-Clinical Applications* 2009, **3**(12):1451-1462.
100. Matta A, DeSouza LV, Shukla NK, Gupta SD, Ralhan R, Siu KM: **Prognostic Significance of Head-and-Neck Cancer Biomarkers Previously Discovered and Identified Using iTRAQ-Labeling and Multidimensional Liquid Chromatography− Tandem Mass Spectrometry**. *Journal of proteome research* 2008, **7**(5):2078-2087.
101. Chang K-P, Yu J-S, Chien K-Y, Lee C-W, Liang Y, Liao C-T, Yen T-C, Lee L-Y, Huang L-L, Liu S-C: **Identification of PRDX4 and P4HA2 as metastasis-associated proteins in oral cavity squamous cell carcinoma by comparative tissue proteomics of microdissected specimens using iTRAQ technology**. *Journal of proteome research* 2011, **10**(11):4935-4947.
102. Tung C-L, Lin S-T, Chou H-C, Chen Y-W, Lin H-C, Tung C-L, Huang K-J, Chen Y-J, Lee Y-R, Chan H-L: **Proteomics-based identification of plasma biomarkers in oral squamous cell carcinoma**. *Journal of pharmaceutical and biomedical analysis* 2013, **75**:7-17.
103. ROMAN E, LUNDE MLS, MIRON T, WARNAKULASAURIYA S, JOHANNESSEN AC, VASSTRAND EN, IBRAHIM SO: **Analysis of Protein Expression Profile of Oral Squamous Cell Carcinoma by MALDI-TOF-MS**. *Anticancer Research* 2013, **33**(3):837-845.
104. Chanthammachat P, Promwikorn W, Pruegsanusak K, Roytrakul S, Srisomsap C, Chokchaichamnankit D, Svasti J, Boonyaphiphat P, K S, Thongsuksai P: **Comparative proteomic analysis of oral squamous cell carcinoma and adjacent non-tumour tissue from Thailand**. *Arch Oral Biol* 2013, **58**(11):1677-1685.
105. Laemmli U: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4 Nature 227: 680–685**. *Find this article online* 1970.

106. Shevchenko A, Wilm M, Vorm O, Mann M: **Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels**. *Analytical chemistry* 1996, **68**(5):850-858.
107. Towbin H, Staehelin T, Gordon J: **Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications**. *Proceedings of the National Academy of Sciences* 1979, **76**(9):4350-4354.
108. Schmittgen TD, Livak KJ: **Analyzing real-time PCR data by the comparative CT method**. *Nature protocols* 2008, **3**(6):1101-1108.
109. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT: **Gene Ontology: tool for the unification of biology**. *Nature genetics* 2000, **25**(1):25-29.
110. Herzig M, Christofori G: **Recent advances in cancer research: mouse models of tumorigenesis**. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer* 2002, **1602**(2):97-113.
111. Minicucci E, Ribeiro D, Da Silva G, Pardini M, Montovani J, Salvadori D: **The role of the TP53 gene during rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide**. *Experimental and Toxicologic Pathology* 2011, **63**(5):483-489.
112. Tanaka T, Ishigamori R: **Understanding carcinogenesis for fighting oral cancer**. *Journal of oncology* 2011, **2011**.
113. Kanojia D, Sawant SS, Borges AM, Ingle AD, Vaidya MM: **Alterations in keratins and associated proteins during 4-Nitroquinoline-1-oxide induced rat oral carcinogenesis**. *Journal of carcinogenesis* 2012, **11**(1):14.
114. Ribeiro DA, Fracalossi ACC, Gomes TS, Oshima CT: **COX-2 expression during 4-nitroquinoline 1-oxide induced rat tongue carcinogenesis**. *Basic and Applied Pathology* 2009, **2**(1):9-14.
115. Booth D: **A relationship found between intra-oral sites of 4NQO reductase activity and chemical carcinogenesis**. *Cell Proliferation* 1990, **23**(4):331-340.
116. Ribeiro DA, Kitakawa D, Domingues MAC, Cabral LAG, Marques MEA, Salvadori DMF: **Survivin and inducible nitric oxide synthase production during 4NQO-induced rat tongue carcinogenesis: a possible relationship**. *Experimental and molecular pathology* 2007, **83**(1):131-137.
117. Niwa S, Ueno S, Shirasu R: **Alteration of pRb expression in the development of rat tongue carcinoma induced by 4-nitroquinoline 1-oxide**. *Oral oncology* 2001, **37**(7):579-585.
118. Jayam R: **Oral field cancerization-A review**. *Journal of Indian Academy of Oral Medicine and Radiology* 2010, **22**(4):201-205.
119. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R: **The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells**. *Cell* 1982, **31**(1):11-24.
120. Vaidya MM, Kanojia D: **Keratins: markers of cell differentiation or regulators of cell differentiation?** *J Biosci* 2007, **32**(4):629-634.
121. SESTERHENN AM, MANDIC R, DÜNNE AA, WERNER JA: **Cytokeratins 6 and 16 are frequently expressed in head and neck squamous cell carcinoma cell lines and fresh biopsies**. *Anticancer research* 2005, **25**(4):2675-2680.
122. Hamada T, Tsuruta D, Fukuda S, Ishii N, Teye K, Numata S, Dainichi T, Karashima T, Ohata C, Furumura M: **How do keratinizing disorders and blistering disorders overlap?** *Experimental dermatology* 2013, **22**(2):83-87.
123. Vogel Hertzel A, Bernlohr DA: **The mammalian fatty acid-binding protein multigene family: molecular and genetic insights into function**. *Trends in Endocrinology & Metabolism* 2000, **11**(5):175-180.

124. Madsen P, Rasmussen HH, Leffers H, Honoré B, Celis JE: **Molecular cloning and expression of a novel keratinocyte protein (psoriasis-associated fatty acid-binding protein [PA-FABP]) that is highly up-regulated in psoriatic skin and that shares similarity to fatty acid-binding proteins**. *Journal of investigative dermatology* 1992, **99**(3):299-305.
125. Melle C, Ernst G, Winkler R, Schimmel B, Klussmann JP, Wittekindt C, Guntinas-Lichius O, von Eggeling F: **Proteomic analysis of human papillomavirus-related oral squamous cell carcinoma: Identification of thioredoxin and epidermal-fatty acid binding protein as upregulated protein markers in microdissected tumor tissue**. *Proteomics* 2009, **9**(8):2193-2201.
126. Fang LY, Wong TY, Chiang WF, Chen YL: **Fatty-acid-binding protein 5 promotes cell proliferation and invasion in oral squamous cell carcinoma**. *Journal of Oral Pathology & Medicine* 2010, **39**(4):342-348.
127. Kondoh N, Imazeki N, Ohkura S, Arai M, Yamazaki Y, Shindoh M, Hitomi K, Takahashi M, Ishikawa T, Matsumura K: **Suppression of Transglutaminase 3 gene in oral squamous cell carcinoma cells**. *Proceedings of the American Association for Cancer Research* 2006, **2006**(1):965.
128. Ohkura S, Kondoh N, Hada A, Arai M, Yamazaki Y, Sindoh M, Takahashi M, Matsumoto I, Yamamoto M: **Differential expression of the keratin-4,-13,-14,-17 and transglutaminase 3 genes during the development of oral squamous cell carcinoma from leukoplakia**. *Oral oncology* 2005, **41**(6):607-613.
129. Méndez E, Fan W, Choi P, Agoff SN, Whipple M, Farwell DG, Futran ND, Weymuller EA, Zhao LP, Chen C: **Tumor-specific genetic expression profile of metastatic oral squamous cell carcinoma**. *Head & neck* 2007, **29**(9):803-814.
130. Choi P, Jordan CD, Mendez E, Houck J, Yueh B, Farwell DG, Futran N, Chen C: **Examination of oral cancer biomarkers by tissue microarray analysis**. *Archives of Otolaryngology—Head & Neck Surgery* 2008, **134**(5):539.
131. Turhani D, Krapfenbauer K, Thurnher D, Langen H, Fountoulakis M: **Identification of differentially expressed, tumor-associated proteins in oral squamous cell carcinoma by proteomic analysis**. *Electrophoresis* 2006, **27**(7):1417-1423.
132. Yang R-Y, Rabinovich GA, Liu F-T: **Galectins: structure, function and therapeutic potential**. *Expert Rev Mol Med* 2008, **10**(3).
133. Baba M, Wada J, Eguchi J, Hashimoto I, Okada T, Yasuhara A, Shikata K, Kanwar YS, Makino H: **Galectin-9 Inhibits Glomerular Hypertrophy in db/db Diabetic Mice via Cell-Cycle–Dependent Mechanisms**. *Journal of the American Society of Nephrology* 2005, **16**(11):3222-3234.
134. Perillo N, Marcus ME, Baum LG: **Galectins: versatile modulators of cell adhesion, cell proliferation, and cell death**. *Journal of Molecular Medicine* 1998, **76**(6):402-412.
135. Alves PM, Godoy GP, Gomes DQ, Medeiros AMC, de Souza LB, da Silveira EJ, Vasconcelos MG, Queiroz LM: **Significance of galectins-1,-3,-4 and-7 in the progression of squamous cell carcinoma of the tongue**. *Pathology-Research and Practice* 2011, **207**(4):236-240.
136. Zhu X, Ding M, Yu M-L, Feng M-X, Tan L-J, Zhao F-K: **Identification of galectin-7 as a potential biomarker for esophageal squamous cell carcinoma by proteomic analysis**. *BMC cancer* 2010, **10**(1):290.
137. Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B: **A model for p53-induced apoptosis**. *Nature* 1997, **389**(6648):300-305.

138. Nakahara Y, Shintani S, Mihara M, Matsumura T, Hamakawa H: **High frequency methylation of p16INK4A gene during 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis**. *Oncology reports* 2004, **12**(1):101-106.
139. Kim S-J, Hwang J-A, Ro JY, Lee Y-S, Chun K-H: **Galectin-7 is epigenetically-regulated tumor suppressor in gastric cancer**. *Oncotarget* 2013, **4**(9):1461.
140. Chevalier F: **Review Highlights on the capacities of" Gel-based" proteomics**. *Proteome Science* 2010, **8**(23).
141. Mendez MG, Kojima SI, Goldman RD: **Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition**. *The FASEB Journal* 2010, **24**(6):1838-1851.
142. Lo W-Y, Tsai M-H, Tsai Y, Hua C-H, Tsai F-J, Huang S-Y, Tsai C-H, Lai C-C: **Identification of over-expressed proteins in oral squamous cell carcinoma (OSCC) patients by clinical proteomic analysis**. *Clinica Chimica Acta* 2007, **376**(1):101-107.
143. Vechio AMCD, Giudice FS, Sperandio FF, Mantesso A, Junior P, dos Santos D: **Vimentin expression and the influence of Matrigel in cell lines of head and neck squamous cell carcinoma**. *Brazilian Oral Research* 2011, **25**(3):235-240.
144. Kokkinos MI, Wafai R, Wong MK, Newgreen DF, Thompson EW, Waltham M: **Vimentin and epithelial-mesenchymal transition in human breast cancer–observations in vitro and in vivo**. *Cells Tissues Organs* 2007, **185**(1-3):191-203.
145. Sawant SS, Vaidya MM, Chaukar DA, Alam H, Dmello C, Gangadaran P, Kannan S, Kane S, Dange PP, Dey N: **Clinical significance of aberrant vimentin expression in oral premalignant lesions and carcinomas**. *Oral diseases* 2013.
146. Chaw S, Abdul Majeed A, Dalley A, Chan A, Stein S, Farah C: **Epithelial to mesenchymal transition (EMT) biomarkers–E-cadherin, beta-catenin, APC and Vimentin–in oral squamous cell carcinogenesis and transformation**. *Oral oncology* 2012, **48**(10):997-1006.
147. Yamashiro S, Yamakita Y, Ono S, Matsumura F: **Fascin, an actin-bundling protein, induces membrane protrusions and increases cell motility of epithelial cells**. *Molecular biology of the cell* 1998, **9**(5):993-1006.
148. Darnel AD, Behmoaram E, Vollmer RT, Corcos J, Bijian K, Sircar K, Su J, Jiao J, Alaoui-Jamali MA, Bismar TA: **Fascin regulates prostate cancer cell invasion and is associated with metastasis and biochemical failure in prostate cancer**. *Clinical Cancer Research* 2009, **15**(4):1376-1383.
149. Alam H, Bhate A, Gangadaran P, Sawant S, Salot S, Sehgal L, Dange P, Chaukar D, D'cruz A, Kannanl S: **Fascin overexpression promotes neoplastic progression in oral squamous cell carcinoma**. *BMC cancer* 2012, **12**(1):32.
150. Chi L-M, Lee C-W, Chang K-P, Hao S-P, Lee H-M, Liang Y, Hsueh C, Yu C-J, Lee I-N, Chang Y-J: **Enhanced interferon signaling pathway in oral cancer revealed by quantitative proteome analysis of microdissected specimens using 16O/18O labeling and integrated two-dimensional LC-ESI-MALDI tandem MS**. *Molecular & cellular proteomics* 2009, **8**(7):1453-1474.
151. Bhatt AN, Mathur R, Farooque A, Verma A, Dwarakanath B: **Cancer biomarkers-Current perspectives**. 2010.
152. Hamilton DW: **Functional role of periostin in development and wound repair: implications for connective tissue disease**. *Journal of cell communication and signaling* 2008, **2**(1):9-17.
153. Kudo Y, Ogawa I, Kitajima S, Kitagawa M, Kawai H, Gaffney PM, Miyauchi M, Takata T: **Periostin promotes invasion and anchorage-independent growth in the**

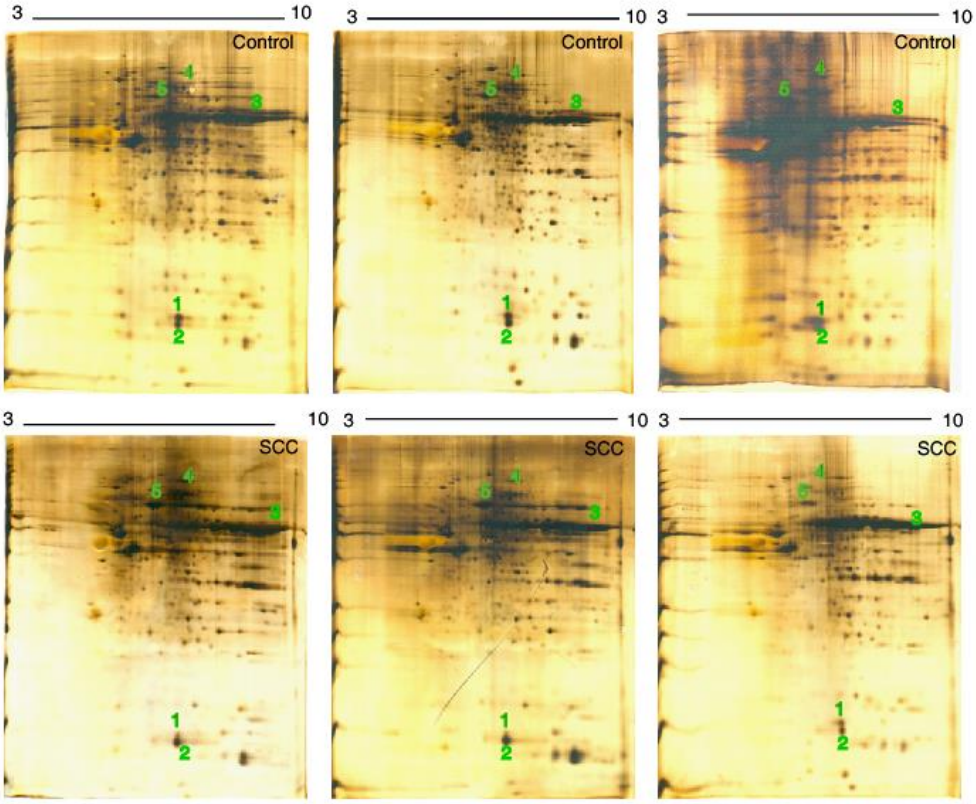
metastatic process of head and neck cancer. *Cancer research* 2006, **66**(14):6928-6935.
154. Kashyap MK, Marimuthu A, Peri S, Kumar GSS, Jacob HKC, Prasad TSK, Mahmood R, Kumar K, Kumar MV, Meltzer SJ: **Overexpression of periostin and lumican in esophageal squamous cell carcinoma**. *Cancers* 2010, **2**(1):133-142.
155. Siriwardena B, Kudo Y, Ogawa I, Kitagawa M, Kitajima S, Hatano H, Tilakaratne W, Miyauchi M, Takata T: **Periostin is frequently overexpressed and enhances invasion and angiogenesis in oral cancer**. *British Journal of Cancer* 2006, **95**(10):1396-1403.
156. Kyutoku M, Taniyama Y, Katsuragi N, Shimizu H, Kunugiza Y, Iekushi K, Koibuchi N, Sanada F, Oshita Y, Morishita R: **Role of periostin in cancer progression and metastasis: inhibition of breast cancer progression and metastasis by anti-periostin antibody in a murine model**. *International journal of molecular medicine* 2011, **28**(2):181-186.
157. Greenberg CS, Birckbichler PJ, Rice RH: **Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues**. *The FASEB Journal* 1991, **5**(15):3071-3077.
158. Gonzalez HE, Gujrati M, Frederick M, Henderson Y, Arumugam J, Spring PW, Mitsudo K, Kim H-W, Clayman GL: **Identification of 9 genes differentially expressed in head and neck squamous cell carcinoma**. *Archives of Otolaryngology—Head & Neck Surgery* 2003, **129**(7):754.
159. Contzler R, Favre B, Huber M, Hohl D: **Cornulin, a new member of the "fused gene" family, is expressed during epidermal differentiation**. *Journal of investigative dermatology* 2005, **124**(5):990-997.
160. Pawar H, Maharudraiah J, Kashyap MK, Sharma J, Srikanth SM, Choudhary R, Chavan S, Sathe G, Manju HC, Kumar KVV: **Downregulation of cornulin in esophageal squamous cell carcinoma**. *Acta Histochemica* 2012.
161. Imai FL, Uzawa K, Nimura Y, Moriya T, Imai MA, Shiiba M, Bukawa H, Yokoe H, Tanzawa H: **< i> Chromosome 1 open reading frame 10</i>(< i> C1orf10</i>) gene is frequently down-regulated and inhibits cell proliferation in oral squamous cell carcinoma**. *The international journal of biochemistry & cell biology* 2005, **37**(8):1641-1655.
162. Lieden A, Ekelund E, Kuo IC, Kockum I, Huang CH, Mallbris L, Lee S, Seng L, Chin G, Wahlgren CF: **Cornulin, a marker of late epidermal differentiation, is down-regulated in eczema**. *Allergy* 2009, **64**(2):304-311.
163. Schaaij-Visser T, Bremmer JF, Braakhuis BJ, Heck AJ, Slijper M, van der Waal I, Brakenhoff RH: **Evaluation of cornulin, keratin 4, keratin 13 expression and grade of dysplasia for predicting malignant progression of oral leukoplakia**. *Oral oncology* 2010, **46**(2):123-127.
164. Risher WC, Eroglu C: **Thrombospondins as key regulators of synaptogenesis in the central nervous system**. *Matrix Biology* 2012, **31**(3):170-177.
165. Lopes N, Gregg D, Vasudevan S, Hassanain H, Goldschmidt-Clermont P, Kovacic H: **Thrombospondin 2 regulates cell proliferation induced by Rac1 redox-dependent signaling**. *Molecular and cellular biology* 2003, **23**(15):5401-5408.
166. O'Guin WM, Sun T-T, Manabe M: **Interaction of trichohyalin with intermediate filaments: Three immunologically defined stages of trichohyalin maturation**. *Journal of investigative dermatology* 1992, **98**(1).
167. Mukaratirwa S, Nederbragt H: **Tenascin and proteoglycans: the role of tenascin and proteoglycans in canine tumours**. *Research in veterinary science* 2002, **73**(1):1-8.

168. Neidhardt J, Fehr S, Kutsche M, Löhler J, Schachner M: **Tenascin-N: characterization of a novel member of the tenascin family that mediates neurite repulsion from hippocampal explants**. *Molecular and Cellular Neuroscience* 2003, **23**(2):193-209.
169. Sun Y, Shang Y, Ren G, Zhou L, Feng B, Li K, Deng L, Liang J, Lu Y, Wang X: **Coronin3 regulates gastric cancer invasion and metastasis by interacting with Arp2**. *Cancer biology & therapy* 2014, **15**(9):0--1.
170. Arandjelovic S, Wickramarachchi D, Hemmers S, Leming SS, Kono DH, Mowen KA: **Mast cell function is not altered by Coronin-1A deficiency**. *Journal of leukocyte biology* 2010, **88**(4):737-745.
171. Kim DH, Bae J, Lee JW, Kim SY, Kim YH, Bae JY, Yi JK, Yu MH, Noh DY, Lee C: **Proteomic analysis of breast cancer tissue reveals upregulation of actin-remodeling proteins and its relevance to cancer invasiveness**. *PROTEOMICS-Clinical Applications* 2009, **3**(1):30-40.
172. Sekhon BS: **Matrix metalloproteinases-an overview**. *Res Rep Biol* 2010, **1**:1-20.
173. Fanjul-Fernández M, Folgueras AR, Cabrera S, López-Otín C: **Matrix metalloproteinases: evolution, gene regulation and functional analysis in mouse models**. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 2010, **1803**(1):3-19.
174. Vilen S-T, Salo T, Sorsa T, Nyberg P: **Fluctuating roles of matrix metalloproteinase-9 in oral squamous cell carcinoma**. *The Scientific World Journal* 2013, **2013**.
175. Yiu GK, Chan WY, Ng S-W, Chan PS, Cheung KK, Berkowitz RS, Mok SC: **SPARC (secreted protein acidic and rich in cysteine) induces apoptosis in ovarian cancer cells**. *The American journal of pathology* 2001, **159**(2):609-622.
176. Aquino G, Sabatino R, Cantile M, Aversa C, Ionna F, Botti G, La Mantia E, Collina F, Malzone G, Pannone G: **Expression Analysis of SPARC/Osteonectin in Oral Squamous Cell Carcinoma Patients: From Saliva to Surgical Specimen**. *BioMed research international* 2013, **2013**.
177. Bellahcene A, Castronovo V: **Increased expression of osteonectin and osteopontin, two bone matrix proteins, in human breast cancer**. *The American journal of pathology* 1995, **146**(1):95.
178. Yang E, Kang HJ, Koh KH, Rhee H, Kim NK, Kim H: **Frequent inactivation of SPARC by promoter hypermethylation in colon cancers**. *International journal of cancer* 2007, **121**(3):567-575.
179. Chlenski A, Liu S, Crawford SE, Volpert OV, DeVries GH, Evangelista A, Yang Q, Salwen HR, Farrer R, Bray J: **SPARC is a key Schwannian-derived inhibitor controlling neuroblastoma tumor angiogenesis**. *Cancer research* 2002, **62**(24):7357-7363.
180. Puolakkainen PA, Brekken RA, Muneer S, Sage EH: **Enhanced Growth of Pancreatic Tumors in SPARC-Null Mice Is Associated With Decreased Deposition of Extracellular Matrix and Reduced Tumor Cell Apoptosis**. *Molecular Cancer Research* 2004, **2**(4):215-224.
181. Said N, Frierson HF, Sanchez-Carbayo M, Brekken RA, Theodorescu D: **Loss of SPARC in bladder cancer enhances carcinogenesis and progression**. *The Journal of clinical investigation* 2013, **123**(2):751-766.
182. DiMartino J, Lacayo N, Varadi M, Li L, Saraiya C, Ravindranath Y, Yu R, Sikic B, Raimondi S, Dahl G: **Low or absent SPARC expression in acute myeloid leukemia with**

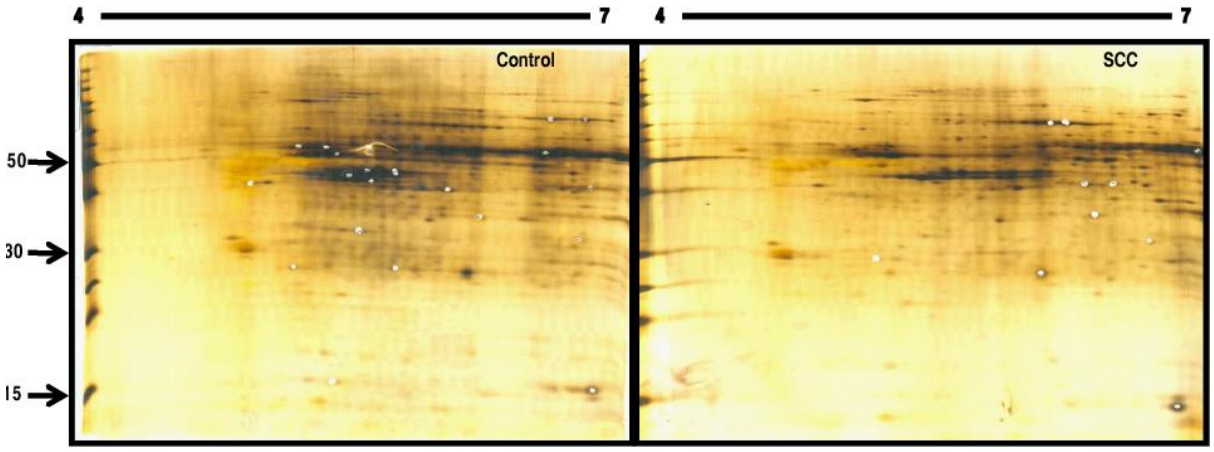
MLL rearrangements is associated with sensitivity to growth inhibition by exogenous SPARC protein. *Leukemia* 2006, **20**(3):426-432.
183. Tait JF, Smith C, Frankenberry DA, Miao CH, Adler DA, Disteche CM: **Chromosomal mapping of the human annexin IV (ANX4) gene**. *Genomics* 1992, **12**(2):313-318.
184. Crosby KC, Postma M, Hink MA, Zeelenberg CH, Adjobo-Hermans MJ, Gadella TW: **Quantitative analysis of self-association and mobility of annexin A4 at the plasma membrane**. *Biophys J* 2013, **104**(9):1875-1885.
185. Naso MF, Liang B, Huang CC, Song XY, Shahied-Arruda L, Belkowski SM, D'Andrea MR, Polkovitch DA, Lawrence DR, Griswold DE *et al*: **Dermokine: an extensively differentially spliced gene expressed in epithelial cells**. *J Invest Dermatol* 2007, **127**(7):1622-1631.
186. Hasegawa M, Higashi K, Yokoyama C, Yamamoto F, Tachibana T, Matsushita T, Hamaguchi Y, Saito K, Fujimoto M, Takehara K: **Altered expression of dermokine in skin disorders**. *Journal of the European Academy of Dermatology and Venereology* 2013, **27**(7):867-875.
187. Tagi T, Matsui T, Kikuchi S, Hoshi S, Ochiai T, Kokuba Y, Kinoshita-Ida Y, Kisumi-Hayashi F, Morimoto K, Imai T *et al*: **Dermokine as a novel biomarker for early-stage colorectal cancer**. *J Gastroenterol* 2010, **45**(12):1201-1211.
188. Kishi T, Grass L, Soosaipillai A, Shimizu-Okabe C, Diamandis EP: **Human kallikrein 8: immunoassay development and identification in tissue extracts and biological fluids**. *Clinical chemistry* 2003, **49**(1):87-96.
189. Yousef G, Yacoub G, Polymeris M-E, Popalis C, Soosaipillai A, Diamandis E: **Kallikrein gene downregulation in breast cancer**. *British Journal of Cancer* 2004, **90**(1):167-172.
190. Jin H, Nagai N, Shigemasa K, Gu L, Tanimoto H, Yunokawa M, Ohama K, Kudo Y, O'Brien TJ: **Expression of tumor-associated differentially expressed Gene-14 (TADG-14/KLK8) and its protein hK8 in uterine endometria and endometrial carcinomas**. *Tumor Biology* 2006, **27**(5):274-282.
191. Darling MR, Tsai S, Jackson-Boeters L, Daley TD, Diamandis EP: **Human kallikrein 8 expression in salivary gland tumors**. *Head and neck pathology* 2008, **2**(3):169-174.
192. Ikeda W, Nakanishi H, Miyoshi J, Mandai K, Ishizaki H, Tanaka M, Togawa A, Takahashi K, Nishioka H, Yoshida H *et al*: **Afadin: A key molecule essential for structural organization of cell-cell junctions of polarized epithelia during embryogenesis**. *J Cell Biol* 1999, **146**(5):1117-1132.
193. Vogl T, Gharibyan AL, Morozova-Roche LA: **Pro-inflammatory S100A8 and S100A9 proteins: self-assembly into multifunctional native and amyloid complexes**. *International journal of molecular sciences* 2012, **13**(3):2893-2917.
194. Gebhardt C, Németh J, Angel P, Hess J: **S100A8 and S100A9 in inflammation and cancer**. *Biochemical pharmacology* 2006, **72**(11):1622-1631.
195. Ding L, Wang Z, Yan J, Yang X, Liu A, Qiu W, Zhu J, Han J, Zhang H, Lin J: **Human four-and-a-half LIM family members suppress tumor cell growth through a TGF-β–like signaling pathway**. *The Journal of clinical investigation* 2009, **119**(2):349-361.
196. Koike K, Kasamatsu A, Iyoda M, Saito Y, Kouzu Y, Koike H, Sakamoto Y, Ogawara K, Tanzawa H, Uzawa K: **High prevalence of epigenetic inactivation of the human four and a half LIM domains 1 gene in human oral cancer**. *International journal of oncology* 2013, **42**(1):141-150.

197. Nickeleit I, Zender S, Kossatz U, Malek NP: **p27kip1: a target for tumor therapies**. *Cell Div* 2007, **2**:13.
198. Harada K, O Hoque M, Bando T, Yoshida H, Sato M: **Overexpression of p27Kip1 induces growth arrest and apoptosis in an oral cancer cell line**. *Oral oncology* 2002, **38**(7):730-736.

CHAPTER 9
APPENDIX



Appendix Figure A1: **Two dimensional gel electrophoresis of different control tissues and rat tongue tumors**. 200µg of sample is used for each tissue. 3-10 pH strips were used for IEF. Differential spots identified are numbered. Spot (1) represents galectin 7; spot (2) fatty acid binding protein 5, spot (3) Keratin 6a, spot (4) Transglutaminase 3 and spot (5) serum albumin precursor protein



Appendix Figure A2: **Two Dimensional gel electrophoresis of control and rat SCC samples using 4-7 pH strips.** Note: Improper spot formation due to horizontal streaking in the gel.

Appendix Table A1: 2DE-PMF data of the spots from the 2DE gels from Normal and SCC tissue samples																
*Gel piece No	Protein Name	Accession No.	PMF							MS/MS						
			Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	expect	Match	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerance (Da)
1	LEG7_RAT		58	15333	4.2	44	100	0.012	4	K.TVIGDDEYLHFHHR.M	0.01	25	25	10	1(1)	1
										R.GQPFEVLIITTEEGFK.T	-	-				
										R.GTGIPFQR.G	-	-				
										R.FHVNLLCGEEQEADAALHFNPR.L	-	-				
2	FABP5_RAT	gi|1706754	68	15335	32	45	100	0.0012	5	R.LVESHGFEDYMK.E	4.3e-008	89	246	31	4(3)	1
										K.ELGVGLALR.K	-	-				
										K.TETVCTFTDGALVQHQK.W	2.2e-006	69				
										K.MVVECVMNNAICTR.V	4.6e-008	88				
										K.MVVECVMNNAICTR.V (oxidation)	1.7	12				
3	K2C6A_RAT		89	59555	22	22%	100	9.2e-006	9	R.AVCGGAGFGSR.S	-	-	196	12	5(5)	1
										R.ISIGGGSCGIGGGYGGR.F	-	-				
										K.FASFIDK.V	3.8e-005	54				
										R.QGLETLFEQYINDLR.K	-	-				
										K.ADSLTDEINFLR.A	1.9e-005	55				
										R.ALYEAELSQMQTHISDTSVVLSMDNNR.S	3.4e-005	46				
										K.YEELQITAGR.H	1e-005	58				
										K.QIANLQAAIAEAEQR.G	4.6e-006	60				

										K.EYQDLMNVK.L	-	-				
5	TGM3_RAT	gi|157822549	**109**	77638		17	100	9.9e-08	9	K.FSSQDFIVR.R	20	**43**	**43**	5	4(1)	1
										R.GQPWEVILLCNR.S	**0.001**					
										R.SLESGDNLNFIVSTGPQPSESAR.T	**-**	-				
										R.QEYVEEDSGIIYVGSTNR.I	-	-				
										K.GSDSVWNFHVWNEGWFVR.T	-	-				
										R.ITWIYNNR.D	-	-				
										K.IAYSQYDR.Y	-	-				
										R.DVILDNPTLTLEVLDQAQLR.K	2	-				
										R.FEIFPTR.I	**0.37**	-				
4	ALBU_RAT	gi|158138568	**74**	70710	61	14	100	0.0003	7	K.GLVLIAFSQYLQK.C	-	-	185	11	5(3)	1
										K.CPYEEHIK.L	-	-				
										R.FPNAEFAEITK.L	**1.5e-007**	75				
										K.DVFLGTFLYEYSR.R	**4.2e-010**	98				
										K.APQVSTPTLVEAAR.N	**0.31**	12				
										R.LPCVEDYLSAILNR.L	**6.6e-005**	47				
										R.RPCFSALTVDETYVPK.E	**0.99**	3				

Abbreviations: IC; Intensity Coverage, SC; Sequence Coverage, M; Match, PMF; Peptide Mass Fingerprint, MS; Mass Spectrometry

Appendix Table A2: List of sequentially altered proteins during rat oral carcinogenesis

Sequentially up regulated proteins from Hyperplasia (5)

Accession	Description	Gene Symbol	Gene IDs	Coverage	Unique Peptides	PSMs	115/114	116/114	117/114	MW [kDa]	calc. pI
109494445	PREDICTED: stefin A1 like 1-like [Rattus norvegicus]	LOC684499	689230	37.11	2	14	2.90	4.47	5.76	11.1	6.30
109494457	PREDICTED: stefin A3-like isoform 1 [Rattus norvegicus]	LOC684525	684525	30.10	2	7	2.78	2.04	3.23	11.8	5.54
57528407	leucine-rich alpha-2-glycoprotein [Rattus norvegicus]	Lrg1	367455	7.53	2	4	2.37	2.32	5.06	36.3	7.23
56090431	serpin B9 [Rattus norvegicus]	Serpinb9	361241	12.03	3	7	2.15	5.16	5.17	42.3	5.86
218156285	complement factor B [Rattus norvegicus]	Cfb	294257	4.85	3	4	2.08	2.30	2.71	85.3	6.96
Sequentially Down regulated proteins from Hyperplasia (10)											
109467091	**PREDICTED: cornulin [Rattus norvegicus]**	**Crnn**	**295186**	**19.05**	**6**	**17**	**0.26**	**0.10**	**0.08**	**58.2**	**6.02**
57012430	keratin, type I cuticular Ha5 [Rattus norvegicus]	Krt35	287697	12.53	1	54	0.26	0.06	0.04	50.6	5.01
149944672	lysozyme g-like protein 1 precursor [Rattus norvegicus]	Lyg1	100910070	19.29	4	30	0.28	0.11	0.11	21.9	9.14
6981610	seminal vesicle secretory protein 4 [Rattus norvegicus]	Svs4	100909594	15.32	1	1	0.30	0.06	0.05	11.9	9.04
6981146	L-lactate dehydrogenase B chain [Rattus norvegicus]	Ldhb	24534	21.26	4	24	0.32	0.16	0.16	36.6	6.05
56847624	keratin, type I cytoskeletal 23 [Rattus norvegicus]	Krt23	287678	7.58	1	39	0.36	0.13	0.13	48.1	6.05
57012366	keratin, type II cuticular Hb4 [Rattus norvegicus]	Krt84	315320	29.98	18	269	0.38	0.09	0.07	61.2	7.84
48040390	LIM domain only protein 7 [Rattus norvegicus]	Lmo7	361084	1.97	2	3	0.38	0.30	0.17	195.5	6.60
293345493	**PREDICTED: trichohyalin [Rattus norvegicus]**	**Tchh**	**310588**	**21.55**	**34**	**368**	**0.45**	**0.10**	**0.07**	**205.1**	**5.78**
293342784	PREDICTED: enhancer of polycomb homolog 1-like [Rattus norvegicus]	LOC100362678	100362678	1.57	1	1	0.49	0.13	0.10	84.6	8.57
Sequentially up regulated proteins from Papilloma (62)											
28212254	insulin-like growth factor 2 mRNA-binding protein 1 [Rattus norvegicus]	Igf2bp1	303477	2.25	1	2	1.29	12.81	16.26	63.4	9.20
56090431	serpin B9 [Rattus norvegicus]	Serpinb9	361241	12.03	3	7	2.15	5.16	5.17	42.3	5.86
281332082	thrombospondin 2 precursor [Rattus norvegicus]	Thbs2	292406	2.47	2	3	0.65	4.71	6.88	129.6	4.82
16758364	protein S100-A9 [Rattus norvegicus]	S100a9	94195	34.51	4	90	1.55	4.47	5.26	13.2	7.94
109494445	PREDICTED: stefin A1 like 1-like [Rattus norvegicus]	LOC684499	689230	37.11	2	14	2.90	4.47	5.76	11.1	6.30
20301952	solute carrier family 2, facilitated glucose transporter member 1 [Rattus norvegicus]	Slc2a1	24778	8.54	4	11	1.51	4.15	5.38	53.9	8.72
157817107	grancalcin [Rattus norvegicus]	Gca	295647	3.18	1	2	0.81	4.14	6.03	24.6	5.07
158262001	cathelicidin antimicrobial peptide [Rattus norvegicus]	Camp	316010	34.29	5	22	1.33	3.70	7.26	19.7	8.13
71043724	proteasome subunit beta type-10 [Rattus norvegicus]	Psmb10	291983	8.79	2	7	1.94	3.61	6.17	29.0	6.64
31543514	legumain precursor [Rattus norvegicus]	Lgmn	63865	3.91	1	4	1.47	3.44	4.88	49.4	6.58
18426834	**coronin-1A [Rattus norvegicus]**	**Coro1a**	**155151**	**12.80**	**5**	**21**	**1.50**	**3.42**	**6.50**	**51.0**	**6.48**

51854235	rano class II histocompatibility antigen, B-1 beta chain precursor [Rattus norvegicus]	RT1-Bb	309622	6.08	1	2	1.86	3.39	6.15	30.0	7.85
40254742	neutrophil cytosol factor 1 [Rattus norvegicus]	Ncf1	114553	2.31	1	2	1.51	3.35	6.44	44.7	9.17
281485600	protein S100-A8 [Rattus norvegicus]	S100a8	116547	33.71	3	120	1.40	3.27	3.81	10.2	6.05
157823473	eosinophil peroxidase [Rattus norvegicus]	Epx	303414	2.52	1	8	1.25	3.12	6.94	81.2	10.14
16758986	protein S100-A6 [Rattus norvegicus]	S100a6	85247	24.72	3	8	1.69	3.11	3.35	10.0	5.48
19173806	histidine-rich glycoprotein [Rattus norvegicus]	Hrg	171016	18.48	3	22	1.64	3.05	3.72	59.0	7.84
6978501	annexin A1 [Rattus norvegicus]	Anxa1	25380	52.02	17	338	1.55	2.88	4.07	38.8	7.34
293342244	PREDICTED: potassium channel tetramerisation domain containing 12 [Rattus norvegicus]	Kctd12	364458	6.42	1	2	1.46	2.88	3.57	35.9	5.81
157823033	beta-actin-like protein 2 [Rattus norvegicus]	Actbl2	294732	15.96	1	136	0.95	2.86	3.02	41.9	5.49
157823757	**periostin [Rattus norvegicus]**	**Postn**	**361945**	**22.22**	**14**	**92**	**1.23**	**2.82**	**3.69**	**90.0**	**7.53**
40254796	lysozyme C-1 precursor [Rattus norvegicus]	Lyz2	25211	22.30	3	9	0.90	2.75	5.99	16.7	8.94
16924006	complement component C9 [Rattus norvegicus]	C9	117512	10.58	5	12	1.41	2.69	3.31	63.7	6.10
13928744	transgelin [Rattus norvegicus]	Tagln	25123	28.36	5	26	0.95	2.67	3.02	22.6	8.84
293349337	PREDICTED: collagen, type XII, alpha 1 [Rattus norvegicus]	Col12a1	25683	17.69	41	137	0.87	2.64	4.85	332.8	5.72
8393197	C-reactive protein precursor [Rattus norvegicus]	Crp	25419	4.35	1	2	1.69	2.63	2.98	25.5	5.00
51036655	alpha-1-antiproteinase precursor [Rattus norvegicus]	Serpina1	24648	27.98	10	38	1.29	2.60	3.08	46.1	6.07
62078737	aminomethyltransferase, mitochondrial [Rattus norvegicus]	Amt	306586	1.99	1	1	1.76	2.59	7.12	44.0	8.95
11177880	vesicle-associated membrane protein-associated protein B [Rattus norvegicus]	Vapb	60431	12.76	1	3	0.96	2.56	2.96	26.9	7.78
18543345	neutrophil gelatinase-associated lipocalin precursor [Rattus norvegicus]	Lcn2	170496	3.03	1	1	1.27	2.54	8.53	22.5	8.25
293360225	PREDICTED: mCG140411-like [Rattus norvegicus]	LOC691886	691886	10.32	1	2	1.42	2.53	3.13	14.4	7.85
8393057	serpin H1 precursor [Rattus norvegicus]	Serpinh1	29345	23.98	8	44	1.05	2.51	2.87	46.5	8.82
19705543	MOSC domain-containing protein 2, mitochondrial precursor [Rattus norvegicus]	Marc2	171451	4.44	1	1	1.78	2.50	2.83	38.2	8.68
6978477	alpha-2-HS-glycoprotein precursor [Rattus norvegicus]	Ahsg	25373	23.30	6	36	1.37	2.49	2.64	38.0	6.77
157821823	neutrophilic granule protein [Rattus norvegicus]	Ngp	301026	30.95	5	14	1.14	2.49	5.38	19.4	8.02
293340913	PREDICTED: insulin-like growth factor 2 mRNA binding protein 1-like isoform 2 [Rattus norvegicus]	Igf2bp2	303824	2.19	1	1	0.95	2.47	3.07	61.1	8.02
60097941	haptoglobin precursor [Rattus norvegicus]	Hp	24464	26.51	8	60	1.60	2.46	2.70	38.5	6.54
186972114	fibronectin precursor [Rattus norvegicus]	Fn1	25661	14.17	26	80	1.18	2.46	2.80	272.3	5.67
158138496	receptor-type tyrosine-protein phosphatase C isoform 1 [Rattus norvegicus]	Ptprc	24699	1.84	2	2	0.75	2.46	3.45	129.6	6.34
58865656	plastin-2 [Rattus norvegicus]	Lcp1	306071	44.98	20	93	1.15	2.45	4.44	70.1	5.29

6978695	ceruloplasmin precursor [Rattus norvegicus]	Cp	24268	17.37	14	63	1.43	2.42	3.90	120.8	5.58
13592079	protein S100-A10 [Rattus norvegicus]	S100a10	81778	17.89	1	15	1.57	2.40	2.91	11.1	6.77
157820285	myeloperoxidase [Rattus norvegicus]	Mpo	303413	31.22	11	79	1.21	2.37	6.00	51.9	9.94
16758014	hemopexin precursor [Rattus norvegicus]	Hpx	58917	35.87	19	207	1.55	2.36	2.88	51.3	7.65
18266706	elongator complex protein 1 [Rattus norvegicus]	Ikbkap	140934	1.73	1	2	1.02	2.34	4.98	149.1	6.39
57528407	leucine-rich alpha-2-glycoprotein [Rattus norvegicus]	Lrg1	367455	7.53	2	4	2.37	2.32	5.06	36.3	7.23
189491879	sorcin [Rattus norvegicus]	Sri	683667	10.10	2	8	0.94	2.30	4.40	21.6	5.90
13591993	matrix metalloproteinase-9 precursor [Rattus norvegicus]	Mmp9	81687	7.20	3	5	1.03	2.30	5.07	78.5	6.33
6981574	SPARC precursor [Rattus norvegicus]	Sparc	24791	8.31	2	4	1.32	2.28	2.53	34.3	4.89
8393218	dipeptidyl peptidase 1 [Rattus norvegicus]	Ctsc	25423	8.87	4	27	1.40	2.22	3.00	52.2	6.89
162287337	apolipoprotein E precursor [Rattus norvegicus]	Apoe	25728	16.35	4	18	1.31	2.18	2.66	35.7	5.27
163937849	integrin beta 2 [Rattus norvegicus]	Itgb2	309684	10.94	7	15	1.13	2.15	4.20	84.8	7.23
156231040	kininogen-1 isoform 2 [Rattus norvegicus]	Kng2	25087	7.39	2	16	1.41	2.15	2.52	47.9	6.39
109470046	PREDICTED: integrin, alpha 6 isoform 2 [Rattus norvegicus]	Itga6	114517	6.71	5	12	1.11	2.11	2.22	119.4	7.06
189011669	fermitin family homolog 3 [Rattus norvegicus]	Fermt3	309186	2.41	1	4	1.37	2.09	3.14	75.6	7.08
293350447	PREDICTED: MHC class I RT1.Aw3 protein-like, partial [Rattus norvegicus]	LOC683761	683761	10.70	2	11	1.27	2.08	2.77	27.1	5.52
6978503	annexin A3 [Rattus norvegicus]	Anxa3	25291	26.23	8	18	1.26	2.07	3.24	36.3	6.47
14010873	clathrin light chain A [Rattus norvegicus]	Clta	83800	2.82	1	3	1.03	2.06	2.85	27.0	4.50
109494457	PREDICTED: stefin A3-like isoform 1 [Rattus norvegicus]	LOC684525	684525	30.10	2	7	2.78	2.04	3.23	11.8	5.54
142349612	glutamine synthetase [Rattus norvegicus]	Glul	24957	5.36	1	1	1.77	2.03	2.55	42.2	7.08
13027416	cytochrome b-245, beta polypeptide [Rattus norvegicus]	Cybb	66021	5.96	3	7	1.03	2.02	3.24	65.3	8.65
124249068	carbonic anhydrase 12 [Rattus norvegicus]	Car12	363085	3.11	1	4	1.42	2.01	2.51	39.5	7.11

Sequentially Down regulated proteins from Papilloma (51)

158341658	serpin A12 [Rattus norvegicus]	Serpina12	191570	4.59	1	4	0.52	0.06	0.04	47.9	9.44
57012430	keratin, type I cuticular Ha5 [Rattus norvegicus]	Krt35	287697	12.53	1	54	0.26	0.06	0.04	50.6	5.01
6981610	seminal vesicle secretory protein 4 [Rattus norvegicus]	Svs4	100909594	15.32	1	1	0.30	0.06	0.05	11.9	9.04
157821865	hypothetical protein LOC499657 [Rattus norvegicus]	RGD1562234	499657	45.05	5	70	0.22	0.07	0.05	10.7	7.25
71043742	chromosome 20 open reading frame 165 [Rattus norvegicus]	Spata25	499943	5.78	1	1	0.49	0.08	0.04	23.6	7.74
57012372	keratin, type II cytoskeletal 2 oral [Rattus norvegicus]	Krt76	407757	49.74	24	684	0.54	0.08	0.05	61.7	8.38
57012366	keratin, type II cuticular Hb4 [Rattus norvegicus]	Krt84	315320	29.98	18	269	0.38	0.09	0.07	61.2	7.84
109467091	**PREDICTED: cornulin [Rattus norvegicus]**	**Crnn**	**295186**	**19.05**	**6**	**17**	**0.26**	**0.10**	**0.08**	**58.2**	**6.02**
293345493	**PREDICTED: trichohyalin [Rattus norvegicus]**	**Tchh**	**310588**	**21.55**	**34**	**368**	**0.45**	**0.10**	**0.07**	**205.1**	**5.78**

149944672	lysozyme g-like protein 1 precursor [Rattus norvegicus]	Lyg1	100910070	19.29	4	30	0.28	0.11	0.11	21.9	9.14
18426812	adenosine deaminase [Rattus norvegicus]	Ada	24165	46.59	16	325	0.38	0.11	0.11	39.9	5.50
51889726	keratin, type I cytoskeletal 24 [Rattus norvegicus]	Krt24	287675	28.11	14	143	0.54	0.13	0.09	52.3	5.01
293342784	PREDICTED: enhancer of polycomb homolog 1-like [Rattus norvegicus]	LOC100362678	100362678	1.57	1	1	0.49	0.13	0.10	84.6	8.57
109493951	PREDICTED: hypothetical protein [Rattus norvegicus]	LOC684640	684640	9.77	1	1	1.42	0.14	0.12	18.5	7.99
57012378	keratin, type II cuticular Hb6 [Rattus norvegicus]	Krt86	407760	17.02	2	30	1.28	0.15	0.11	56.6	6.29
57012436	keratin, type I cytoskeletal 10 [Rattus norvegicus]	Krt10	450225	28.90	6	111	0.60	0.17	0.11	56.5	5.15
56912229	keratin, type I cuticular Ha6 [Rattus norvegicus]	Krt36	287698	48.72	16	336	0.52	0.17	0.12	52.2	5.05
293340723	PREDICTED: keratin associated protein 11-1-like isoform 2 [Rattus norvegicus]	LOC100359886	100359886	8.59	1	2	2.30	0.18	0.07	17.0	7.94
199560677	coiled-coil domain-containing protein 8 [Rattus norvegicus]	Ccdc8	494320	2.46	1	1	0.68	0.18	0.14	70.0	9.76
57012446	keratin, type I cytoskeletal 42 [Rattus norvegicus]	Krt42	450231	47.12	7	550	0.63	0.20	0.11	50.2	5.16
293340674	PREDICTED: hypothetical protein [Rattus norvegicus]	LOC100364664	100363136	12.57	1	2	0.72	0.22	0.10	19.1	8.97
54234046	cystatin-C precursor [Rattus norvegicus]	Cst3	25307	25.00	2	7	0.75	0.25	0.23	15.4	9.22
71043890	acid sphingomyelinase-like phosphodiesterase 3b [Rattus norvegicus]	Smpdl3b	362619	6.80	2	4	0.80	0.25	0.18	51.6	5.88
31377484	carbonic anhydrase 3 [Rattus norvegicus]	Car3	54232	6.92	1	2	0.49	0.26	0.20	29.4	7.37
57012440	keratin 33B [Rattus norvegicus]	Krt33b	450227	16.58	3	105	1.66	0.27	0.20	45.8	4.82
156119593	keratin, type II cuticular Hb3 [Rattus norvegicus]	Krt83	681126	19.80	3	31	1.03	0.29	0.23	54.7	6.37
48040390	LIM domain only protein 7 [Rattus norvegicus]	Lmo7	361084	1.97	2	3	0.38	0.30	0.17	195.5	6.60
197927125	galectin-related protein [Rattus norvegicus]	Lgalsl	360983	22.67	3	6	0.63	0.31	0.25	18.9	5.35
57012388	keratin, type II cytoskeletal 80 [Rattus norvegicus]	Krt80	315318	6.42	2	9	0.86	0.31	0.20	50.5	6.20
157822763	serpin A9 [Rattus norvegicus]	Serpina9	299274	17.03	6	10	1.00	0.33	0.29	46.8	9.60
164663841	chloride intracellular channel 3 [Rattus norvegicus]	Clic3	296566	20.25	3	7	0.55	0.33	0.24	26.8	5.97
157823103	cytosolic 5'-nucleotidase 3 [Rattus norvegicus]	Nt5c3	312373	8.42	2	4	0.63	0.33	0.28	33.8	5.47
157819539	kallikrein-8 precursor [Rattus norvegicus]	Klk8	308565	4.23	1	2	0.64	0.33	0.28	28.5	8.25
290563809	dermokine [Rattus norvegicus]	Dmkn	361548	18.22	8	61	0.74	0.36	0.30	52.2	6.84
6981182	microtubule-associated protein 2 [Rattus norvegicus]	Map2	25595	2.58	3	5	0.83	0.37	0.22	198.4	4.84
162287127	myosin-14 [Rattus norvegicus]	Myh14	308572	8.45	7	62	0.83	0.37	0.27	228.8	5.55
31542401	creatine kinase B-type [Rattus norvegicus]	Ckb	24264	30.18	7	41	0.61	0.41	0.31	42.7	5.58
16758346	von Ebner gland protein 2 precursor [Rattus norvegicus]	Vegp2	94106	11.86	2	6	1.99	0.41	0.21	19.7	6.54
61557414	tubulin-specific chaperone A [Rattus norvegicus]	Tbca	366995	8.33	1	1	0.62	0.41	0.31	12.7	5.47
293342967	PREDICTED: desmoglein 1 beta [Rattus norvegicus]	Dsg1b	291755	21.60	17	138	0.85	0.41	0.37	112.3	4.91
290563194	catenin, beta-interacting protein 1 [Rattus norvegicus]	Ctnnbip1	503000	17.28	1	2	0.73	0.42	0.35	9.2	5.41

12018322	transmembrane protease serine 11D isoform b precursor [Rattus norvegicus]	Tmprss11d	64565	12.19	2	4	1.00	0.42	0.32	30.5	6.38
62543549	ubiquitin-like protein 3 precursor [Rattus norvegicus]	Ubl3	363869	18.80	3	3	0.80	0.43	0.39	13.2	6.92
82617596	tumor-associated calcium signal transducer 2 precursor [Rattus norvegicus]	Tacstd2	494343	7.26	1	1	0.50	0.43	0.33	35.5	8.60
57012360	keratin, type II cytoskeletal 4 [Rattus norvegicus]	Krt4	315323	50.56	24	714	0.66	0.46	0.27	57.6	7.64
6978469	afadin [Rattus norvegicus]	Mllt4	26955	2.68	3	4	0.77	0.47	0.38	207.5	6.16
56847618	keratin, type I cytoskeletal 16 [Rattus norvegicus]	Krt16	303530	62.20	19	954	1.06	0.48	0.37	50.7	5.12
157821563	magnesium transporter NIPA4 [Rattus norvegicus]	Nipal4	303070	3.45	1	3	0.90	0.48	0.38	44.1	8.47
56605806	target of Myb protein 1 [Rattus norvegicus]	Tom1	361370	3.46	1	4	0.82	0.48	0.36	54.1	4.96
19705477	RING finger protein 39 [Rattus norvegicus]	Rnf39	171387	5.11	1	1	0.86	0.49	0.33	38.3	6.55
157819247	carboxypeptidase A4 [Rattus norvegicus]	Cpa4	502736	10.45	4	8	0.72	0.49	0.38	47.4	6.61

Sequentially up regulated proteins from SCC (154)

28212254	insulin-like growth factor 2 mRNA-binding protein 1 [Rattus norvegicus]	Igf2bp1	303477	2.25	1	2	1.29	12.81	16.26	63.4	9.20
18543345	neutrophil gelatinase-associated lipocalin precursor [Rattus norvegicus]	Lcn2	170496	3.03	1	1	1.27	2.54	8.53	22.5	8.25
187937026	neutrophil cytosol factor 4 [Rattus norvegicus]	Ncf4	500904	4.72	1	2	1.67	5.16	8.29	38.7	5.91
158262001	cathelicidin antimicrobial peptide [Rattus norvegicus]	Camp	316010	34.29	5	22	1.33	3.70	7.26	19.7	8.13
62078737	aminomethyltransferase, mitochondrial [Rattus norvegicus]	Amt	306586	1.99	1	1	1.76	2.59	7.12	44.0	8.95
157823473	eosinophil peroxidase [Rattus norvegicus]	Epx	303414	2.52	1	8	1.25	3.12	6.94	81.2	10.14
281332082	**thrombospondin 2 precursor [Rattus norvegicus]**	**Thbs2**	**292406**	**2.47**	**2**	**3**	**0.65**	**4.71**	**6.88**	**129.6**	**4.82**
18426834	**coronin-1A [Rattus norvegicus]**	**Coro1a**	**155151**	**12.80**	**5**	**21**	**1.50**	**3.42**	**6.50**	**51.0**	**6.48**
198278475	apolipoprotein L3 [Rattus norvegicus]	RGD1309808	100911562	3.84	1	1	1.67	1.86	6.46	39.7	5.17
40254742	neutrophil cytosol factor 1 [Rattus norvegicus]	Ncf1	114553	2.31	1	2	1.51	3.35	6.44	44.7	9.17
71043724	proteasome subunit beta type-10 [Rattus norvegicus]	Psmb10	291983	8.79	2	7	1.94	3.61	6.17	29.0	6.64
51854235	rano class II histocompatibility antigen, B-1 beta chain precursor [Rattus norvegicus]	RT1-Bb	309622	6.08	1	2	1.86	3.39	6.15	30.0	7.85
157817107	grancalcin [Rattus norvegicus]	Gca	295647	3.18	1	2	0.81	4.14	6.03	24.6	5.07
157820285	myeloperoxidase [Rattus norvegicus]	Mpo	303413	31.22	11	79	1.21	2.37	6.00	51.9	9.94
40254796	lysozyme C-1 precursor [Rattus norvegicus]	Lyz2	25211	22.30	3	9	0.90	2.75	5.99	16.7	8.94
109494445	PREDICTED: stefin A1 like 1-like [Rattus norvegicus]	LOC684499	689230	37.11	2	14	2.90	4.47	5.76	11.1	6.30
55742723	dimethylglycine dehydrogenase, mitochondrial precursor [Rattus	Dmgdh	245961	0.82	1	1	0.85	1.47	5.62	95.9	7.24

	norvegicus]										
57526868	T-kininogen 2 precursor [Rattus norvegicus]	Kng1l1	288001	28.84	3	67	1.02	1.68	5.58	47.7	6.35
20301952	solute carrier family 2, facilitated glucose transporter member 1 [Rattus norvegicus]	Slc2a1	24778	8.54	4	11	1.51	4.15	5.38	53.9	8.72
157821823	neutrophilic granule protein [Rattus norvegicus]	Ngp	301026	30.95	5	14	1.14	2.49	5.38	19.4	8.02
16758364	protein S100-A9 [Rattus norvegicus]	S100a9	94195	34.51	4	90	1.55	4.47	5.26	13.2	7.94
13928980	aquaporin-3 [Rattus norvegicus]	Aqp3	65133	2.74	1	1	1.72	5.32	5.22	31.4	7.12
56090431	serpin B9 [Rattus norvegicus]	Serpinb9	361241	12.03	3	7	2.15	5.16	5.17	42.3	5.86
13591993	matrix metalloproteinase-9 precursor [Rattus norvegicus]	Mmp9	81687	7.20	3	5	1.03	2.30	5.07	78.5	6.33
57528407	leucine-rich alpha-2-glycoprotein [Rattus norvegicus]	Lrg1	367455	7.53	2	4	2.37	2.32	5.06	36.3	7.23
18266706	elongator complex protein 1 [Rattus norvegicus]	Ikbkap	140934	1.73	1	2	1.02	2.34	4.98	149.1	6.39
31543514	legumain precursor [Rattus norvegicus]	Lgmn	63865	3.91	1	4	1.47	3.44	4.88	49.4	6.58
293349337	PREDICTED: collagen, type XII, alpha 1 [Rattus norvegicus]	Col12a1	25683	17.69	41	137	0.87	2.64	4.85	332.8	5.72
21245096	multiple coagulation factor deficiency protein 2 homolog precursor [Rattus norvegicus]	Mcfd2	246117	11.72	1	1	2.42	4.98	4.49	16.1	4.74
11560135	brain acid soluble protein 1 [Rattus norvegicus]	Basp1	64160	10.45	1	1	1.26	1.74	4.47	21.8	4.51
58865656	plastin-2 [Rattus norvegicus]	Lcp1	306071	44.98	20	93	1.15	2.45	4.44	70.1	5.29
189491879	sorcin [Rattus norvegicus]	Sri	683667	10.10	2	8	0.94	2.30	4.40	21.6	5.90
80861401	T-kininogen 1 [Rattus norvegicus]	Kng1	24903	25.35	3	69	1.33	1.51	4.33	47.7	6.74
163937849	integrin beta 2 [Rattus norvegicus]	Itgb2	309684	10.94	7	15	1.13	2.15	4.20	84.8	7.23
139948891	lipopolysaccharide-binding protein precursor [Rattus norvegicus]	Lbp	29469	2.08	1	2	0.67	1.82	4.14	53.5	9.01
6978501	annexin A1 [Rattus norvegicus]	Anxa1	25380	52.02	17	338	1.55	2.88	4.07	38.8	7.34
48040428	hyaluronan-binding protein 2 [Rattus norvegicus]	Habp2	292126	2.33	1	1	1.13	3.33	3.92	62.1	6.27
6978695	ceruloplasmin precursor [Rattus norvegicus]	Cp	24268	17.37	14	63	1.43	2.42	3.90	120.8	5.58
281485600	protein S100-A8 [Rattus norvegicus]	S100a8	116547	33.71	3	120	1.40	3.27	3.81	10.2	6.05
19173806	histidine-rich glycoprotein [Rattus norvegicus]	Hrg	171016	18.48	3	22	1.64	3.05	3.72	59.0	7.84
157823757	**periostin [Rattus norvegicus]**	**Postn**	**361945**	**22.22**	**14**	**92**	**1.23**	**2.82**	**3.69**	**90.0**	**7.53**
293342244	PREDICTED: potassium channel tetramerisation domain containing 12 [Rattus norvegicus]	Kctd12	364458	6.42	1	2	1.46	2.88	3.57	35.9	5.81
6978565	zinc-alpha-2-glycoprotein precursor [Rattus norvegicus]	Azgp1	25294	3.38	1	1	1.76	3.83	3.48	34.0	6.24
158138496	receptor-type tyrosine-protein phosphatase C isoform 1 [Rattus	Ptprc	24699	1.84	2	2	0.75	2.46	3.45	129.6	6.34

	norvegicus]										
16758986	protein S100-A6 [Rattus norvegicus]	S100a6	85247	24.72	3	8	1.69	3.11	3.35	10.0	5.48
16924006	complement component C9 [Rattus norvegicus]	C9	117512	10.58	5	12	1.41	2.69	3.31	63.7	6.10
306922366	splicing factor, arginine/serine-rich 6 [Rattus norvegicus]	Srsf6	362264	4.72	1	7	0.84	3.12	3.30	39.0	11.46
201066380	**fascin [Rattus norvegicus]**	**LOC683788**	**683788**	**14.00**	**5**	**11**	**1.76**	**3.29**	**3.29**	**54.5**	**6.74**
29789036	integrin alpha-M [Rattus norvegicus]	Itgam	25021	7.65	7	11	1.30	1.63	3.26	126.9	6.65
6978503	annexin A3 [Rattus norvegicus]	Anxa3	25291	26.23	8	18	1.26	2.07	3.24	36.3	6.47
13027416	cytochrome b-245, beta polypeptide [Rattus norvegicus]	Cybb	66021	5.96	3	7	1.03	2.02	3.24	65.3	8.65
61556986	serotransferrin precursor [Rattus norvegicus]	Tf	24825	43.41	28	473	1.90	2.61	3.17	76.3	7.28
189011669	fermitin family homolog 3 [Rattus norvegicus]	Fermt3	309186	2.41	1	4	1.37	2.09	3.14	75.6	7.08
293360225	PREDICTED: mCG140411-like [Rattus norvegicus]	LOC691886	691886	10.32	1	2	1.42	2.53	3.13	14.4	7.85
51036655	alpha-1-antiproteinase precursor [Rattus norvegicus]	Serpina1	24648	27.98	10	38	1.29	2.60	3.08	46.1	6.07
293340913	PREDICTED: insulin-like growth factor 2 mRNA binding protein 1-like isoform 2 [Rattus norvegicus]	Igf2bp2	303824	2.19	1	1	0.95	2.47	3.07	61.1	8.02
13928744	transgelin [Rattus norvegicus]	Tagln	25123	28.36	5	26	0.95	2.67	3.02	22.6	8.84
157823033	beta-actin-like protein 2 [Rattus norvegicus]	Actbl2	294732	15.96	1	136	0.95	2.86	3.02	41.9	5.49
8393218	dipeptidyl peptidase 1 [Rattus norvegicus]	Ctsc	25423	8.87	4	27	1.40	2.22	3.00	52.2	6.89
57527565	rho GDP-dissociation inhibitor 2 [Rattus norvegicus]	Arhgdib	362456	7.00	1	3	1.42	1.61	2.99	22.9	5.11
8393197	C-reactive protein precursor [Rattus norvegicus]	Crp	25419	4.35	1	2	1.69	2.63	2.98	25.5	5.00
11177880	vesicle-associated membrane protein-associated protein B [Rattus norvegicus]	Vapb	60431	12.76	1	3	0.96	2.56	2.96	26.9	7.78
13592079	protein S100-A10 [Rattus norvegicus]	S100a10	81778	17.89	1	15	1.57	2.40	2.91	11.1	6.77
16758014	hemopexin precursor [Rattus norvegicus]	Hpx	58917	35.87	19	207	1.55	2.36	2.88	51.3	7.65
8393057	serpin H1 precursor [Rattus norvegicus]	Serpinh1	29345	23.98	8	44	1.05	2.51	2.87	46.5	8.82
16758004	mitochondrial peptide methionine sulfoxide reductase [Rattus norvegicus]	Msra	29447	2.58	1	1	1.30	1.71	2.86	25.8	8.07
14010873	clathrin light chain A [Rattus norvegicus]	Clta	83800	2.82	1	3	1.03	2.06	2.85	27.0	4.50
118142811	vesicle-associated membrane protein-associated protein A [Rattus norvegicus]	Vapa	58857	13.25	2	7	1.36	3.84	2.85	27.8	8.40
19705543	MOSC domain-containing protein 2, mitochondrial precursor [Rattus norvegicus]	Marc2	171451	4.44	1	1	1.78	2.50	2.83	38.2	8.68
13591902	alpha-actinin-1 [Rattus norvegicus]	Actn1	81634	32.40	11	125	1.49	2.81	2.82	102.9	5.38

77861917	complement factor H [Rattus norvegicus]	Cfh	155012	6.40	6	16	1.57	2.27	2.82	140.1	6.77
58865500	erythrocyte band 7 integral membrane protein [Rattus norvegicus]	Stom	296655	16.55	3	17	1.00	1.69	2.80	31.4	7.03
186972114	fibronectin precursor [Rattus norvegicus]	Fn1	25661	14.17	26	80	1.18	2.46	2.80	272.3	5.67
293350447	PREDICTED: MHC class I RT1.Aw3 protein-like, partial [Rattus norvegicus]	LOC683761	683761	10.70	2	11	1.27	2.08	2.77	27.1	5.52
298231229	sulfated glycoprotein 1 isoform D preproprotein [Rattus norvegicus]	Psap	25524	3.62	2	10	0.81	1.67	2.75	61.0	5.25
158138568	serum albumin precursor [Rattus norvegicus]	Alb	24186	54.61	32	1051	1.37	2.26	2.72	68.7	6.48
218156285	complement factor B [Rattus norvegicus]	Cfb	294257	4.85	3	4	2.08	2.30	2.71	85.3	6.96
60097941	haptoglobin precursor [Rattus norvegicus]	Hp	24464	26.51	8	60	1.60	2.46	2.70	38.5	6.54
157820929	neutrophil elastase [Rattus norvegicus]	Elane	299606	16.97	4	12	0.96	1.31	2.67	29.5	9.28
162287337	apolipoprotein E precursor [Rattus norvegicus]	Apoe	25728	16.35	4	18	1.31	2.18	2.66	35.7	5.27
11560008	neutrophil collagenase precursor [Rattus norvegicus]	Mmp8	63849	1.50	1	1	0.82	1.10	2.65	53.2	6.76
293340942	PREDICTED: rCG36783-like [Rattus norvegicus]	LOC100363836	100363836	12.93	1	2	2.02	2.35	2.65	12.6	4.42
6978477	alpha-2-HS-glycoprotein precursor [Rattus norvegicus]	Ahsg	25373	23.30	6	36	1.37	2.49	2.64	38.0	6.77
293345034	PREDICTED: hypothetical protein isoform 2 [Rattus norvegicus]	LOC294446	294446	21.80	4	12	1.40	2.55	2.58	27.9	4.31
293346859	PREDICTED: Igk protein-like isoform 1 [Rattus norvegicus]	LOC683399	683399	18.57	4	235	1.89	2.44	2.58	26.1	7.72
18426838	src kinase-associated phosphoprotein 2 [Rattus norvegicus]	Skap2	155183	4.75	1	1	0.83	1.09	2.57	40.7	4.65
142349612	glutamine synthetase [Rattus norvegicus]	Glul	24957	5.36	1	1	1.77	2.03	2.55	42.2	7.08
13592119	thymosin beta-4 [Rattus norvegicus]	Tmsb4x	81814	15.91	1	5	0.98	1.79	2.54	5.0	5.06
6981574	SPARC precursor [Rattus norvegicus]	Sparc	24791	8.31	2	4	1.32	2.28	2.53	34.3	4.89
156231040	kininogen-1 isoform 2 [Rattus norvegicus]	Kng2	25087	7.39	2	16	1.41	2.15	2.52	47.9	6.39
6978879	vitamin D-binding protein precursor [Rattus norvegicus]	Gc	24384	21.22	10	41	1.57	2.42	2.51	53.5	5.86
124249068	carbonic anhydrase 12 [Rattus norvegicus]	Car12	363085	3.11	1	4	1.42	2.01	2.51	39.5	7.11
161333847	prothrombin [Rattus norvegicus]	F2	29251	19.29	10	28	1.31	1.84	2.48	70.3	6.71
162287322	lymphocyte specific 1 [Rattus norvegicus]	Lsp1	361680	9.97	2	3	0.96	1.78	2.48	36.5	4.67
158138561	complement C3 [Rattus norvegicus]	C3	24232	34.64	44	272	1.66	2.31	2.48	186.2	6.47
312922379	**tenascin-N [Rattus norvegicus]**	**Tnn**	**304913**	**3.52**	**4**	**6**	**0.79**	**0.75**	**2.45**	**173.1**	**5.63**
16975494	TAP-binding protein [Rattus norvegicus]	Tapbp	25217	2.80	1	1	1.03	1.76	2.44	50.0	7.88

145386553	vitamin K-dependent protein S precursor [Rattus norvegicus]	Pros1	81750	0.89	1	1	1.05	2.45	2.43	74.6	5.48
27465603	aldose reductase-related protein 2 [Rattus norvegicus]	Akr1b8	286921	37.66	10	73	2.08	2.21	2.42	36.2	7.46
66730380	myeloblastin [Rattus norvegicus]	Prtn3	314615	9.84	2	6	1.15	1.30	2.37	27.7	7.97
57528174	beta-2-glycoprotein 1 [Rattus norvegicus]	Apoh	287774	13.62	4	14	1.41	1.84	2.36	38.4	8.21
67514516	calpain small subunit 1 [Rattus norvegicus]	Capns1	29156	7.41	2	8	1.53	2.21	2.36	28.6	5.47
34328542	myeloid-associated differentiation marker [Rattus norvegicus]	Myadm	369016	9.43	2	10	1.41	2.03	2.36	35.1	8.18
13929060	carcinoembryonic antigen-related cell adhesion molecule 1 isoform 4 [Rattus norvegicus]	Ceacam1	81613	2.68	1	9	0.94	1.46	2.35	50.4	5.59
9506467	carbonyl reductase [NADPH] 1 [Rattus norvegicus]	Cbr1	29224	25.99	6	43	1.75	1.79	2.33	30.6	8.06
109494630	PREDICTED: histidine-rich glycoprotein-like [Rattus norvegicus]	LOC681544	681544	22.62	4	22	1.23	1.94	2.29	59.3	7.74
293350046	PREDICTED: immunoglobulin light chain-like [Rattus norvegicus]	LOC502795	502795	8.87	2	6	1.56	1.81	2.29	31.8	9.07
157823295	olfactomedin-4 [Rattus norvegicus]	Olfm4	290409	14.09	6	17	0.89	2.11	2.29	57.9	5.87
189011598	calcium-binding mitochondrial carrier protein SCaMC-1 [Rattus norvegicus]	Slc25a24	310791	3.58	2	5	1.01	1.76	2.29	52.9	8.05
9506405	actin-related protein 2/3 complex subunit 1B [Rattus norvegicus]	Arpc1b	54227	22.58	6	18	1.49	1.79	2.29	41.0	8.35
14389303	protein RoBo-1 [Rattus norvegicus]	LOC24906	24906	9.17	2	7	0.90	1.05	2.26	26.2	7.33
72255515	leukocyte elastase inhibitor A [Rattus norvegicus]	Serpinb1a	291091	36.94	16	122	1.44	1.40	2.26	42.7	6.32
34328540	cathepsin Z precursor [Rattus norvegicus]	Ctsz	252929	4.58	1	4	1.12	2.22	2.25	34.2	7.15
293349296	PREDICTED: rCG57864-like [Rattus norvegicus]	Plekho2	315764	3.05	1	1	0.56	1.22	2.24	50.1	5.20
109470046	PREDICTED: integrin, alpha 6 isoform 2 [Rattus norvegicus]	Itga6	114517	6.71	5	12	1.11	2.11	2.22	119.4	7.06
157823539	copine-7 [Rattus norvegicus]	Cpne7	361433	1.85	1	5	1.12	1.59	2.21	54.1	5.03
110625958	actin, aortic smooth muscle [Rattus norvegicus]	Acta2	81633	39.52	1	421	0.62	1.87	2.20	42.0	5.39
126722991	inter-alpha-inhibitor H4 heavy chain [Rattus norvegicus]	Itih4	54404	12.54	10	29	1.23	1.80	2.20	103.7	6.20
148747414	guanine deaminase [Rattus norvegicus]	Gda	83585	23.35	9	22	0.74	1.16	2.19	50.9	5.72
8393901	serine/threonine-protein kinase PAK 1 [Rattus norvegicus]	Pak1	29431	5.33	1	6	1.53	2.17	2.17	60.5	5.86
14389299	**vimentin [Rattus norvegicus]**	**Vim**	**81818**	**48.50**	**17**	**175**	**0.79**	**1.91**	**2.16**	**53.7**	**5.12**
58865630	antithrombin-III [Rattus norvegicus]	Serpinc1	304917	11.61	6	16	1.42	1.77	2.16	52.2	6.57
157823499	procollagen galactosyltransferase 1 [Rattus norvegicus]	Glt25d1	290637	1.62	1	1	1.16	1.80	2.15	71.1	7.12
157823071	tumor necrosis factor alpha-induced protein 8 [Rattus norvegicus]	Tnfaip8	307428	4.22	1	2	1.13	1.72	2.15	19.4	7.94

214010196	DNA (cytosine-5)-methyltransferase 1 [Rattus norvegicus]	Dnmt1	84350	0.56	1	1	1.07	1.82	2.14	182.9	8.07
293340790	PREDICTED: high mobility group protein B2-like [Rattus norvegicus]	RGD1564519	498072	25.24	5	27	1.00	1.41	2.13	24.1	7.39
51948402	peptidyl-prolyl cis-trans isomerase C [Rattus norvegicus]	Ppic	291463	5.66	1	2	1.23	1.92	2.13	23.0	8.13
293360190	PREDICTED: hCG2042717-like [Rattus norvegicus]	LOC691801	691801	7.10	1	2	1.33	1.60	2.13	17.8	8.27
13242285	heparin cofactor 2 precursor [Rattus norvegicus]	Serpind1	79224	5.01	2	5	1.32	1.51	2.11	54.5	6.96
9506475	cyclin-dependent kinase 1 [Rattus norvegicus]	Cdk1	54237	3.70	1	1	1.64	1.99	2.11	34.1	8.41
164698508	septin-9 isoform 2 [Rattus norvegicus]	Sept9	83788	14.16	6	11	1.11	1.99	2.11	63.8	8.32
16758438	barrier-to-autointegration factor [Rattus norvegicus]	Banf1	114087	26.97	1	3	1.45	1.54	2.11	10.0	6.09
25742568	dihydropyrimidinase-related protein 3 [Rattus norvegicus]	Dpysl3	25418	20.18	6	28	1.29	2.10	2.10	61.9	6.49
14010871	dipeptidyl peptidase 2 precursor [Rattus norvegicus]	Dpp7	83799	4.80	2	7	1.24	1.22	2.10	55.1	5.05
70778983	splicing factor, proline- and glutamine-rich [Rattus norvegicus]	Sfpq	252855	10.01	4	11	1.01	1.31	2.09	75.4	9.44
13928704	myosin-10 [Rattus norvegicus]	Myh10	79433	5.31	3	119	1.25	1.71	2.09	228.8	5.60
197386807	filamin-A [Rattus norvegicus]	Flna	293860	33.72	59	267	1.21	1.95	2.08	280.3	6.04
7549746	beta-2-microglobulin precursor [Rattus norvegicus]	B2m	24223	11.76	2	5	1.31	1.60	2.08	13.7	8.00
6978553	sodium/potassium-transporting ATPase subunit beta-3 [Rattus norvegicus]	Atp1b3	25390	13.62	5	14	1.27	1.88	2.07	31.8	7.96
158262052	antigen peptide transporter 1 [Rattus norvegicus]	Tap1	24811	1.10	1	1	1.57	1.42	2.07	79.1	8.62
16758534	embigin precursor [Rattus norvegicus]	Emb	114511	3.96	1	2	0.58	1.92	2.07	37.0	5.36
157822653	CD2 antigen cytoplasmic tail-binding protein 2 [Rattus norvegicus]	Cd2bp2	293505	4.12	1	2	1.13	1.71	2.07	37.5	4.61
6978721	pro-cathepsin H precursor [Rattus norvegicus]	Ctsh	25425	3.90	1	4	1.13	1.60	2.06	37.1	8.41
162138932	AP-2 complex subunit alpha-2 [Rattus norvegicus]	Ap2a2	81637	3.62	3	3	1.34	1.64	2.04	104.1	6.83
34328538	L-lactate dehydrogenase A-like 6B [Rattus norvegicus]	Ldhal6b	369018	2.62	1	2	0.89	0.87	2.04	42.0	9.41
11559937	hexokinase-3 [Rattus norvegicus]	Hk3	25060	4.00	2	8	0.97	1.26	2.04	100.2	5.48
158303310	beta-glucuronidase precursor [Rattus norvegicus]	Gusb	24434	2.31	1	1	0.90	1.22	2.04	74.8	6.74
6981464	retinol-binding protein 1 [Rattus norvegicus]	Rbp1	25056	26.67	3	8	1.23	1.98	2.03	15.8	5.25
8392983	biglycan precursor [Rattus norvegicus]	Bgn	25181	15.18	4	28	0.70	1.64	2.02	41.7	7.52
56605840	ras-related C3 botulinum toxin substrate 2 [Rattus norvegicus]	Rac2	366957	21.35	2	6	0.96	1.32	2.01	21.4	7.61
109469622	PREDICTED: complement component 5 [Rattus norvegicus]	C5	362119	4.28	6	8	1.27	2.04	2.01	188.9	6.62
307746876	alpha-1-macroglobulin precursor [Rattus norvegicus]	Pzp	252922	22.13	26	103	1.29	2.00	2.00	167.0	6.90

109476830	PREDICTED: complement component 8, beta polypeptide [Rattus norvegicus]	C8b	313421	2.04	1	2	1.35	1.94	2.00	66.6	8.05
166091476	transcription elongation regulator 1 [Rattus norvegicus]	Tcerg1	307474	1.20	1	2	0.88	1.94	2.00	121.8	8.65

Sequentially down regulated proteins from SCC (170)

57012430	keratin, type I cuticular Ha5 [Rattus norvegicus]	Krt35	287697	12.53	1	54	0.26	0.06	0.04	50.6	5.01
71043742	chromosome 20 open reading frame 165 [Rattus norvegicus]	Spata25	499943	5.78	1	1	0.49	0.08	0.04	23.6	7.74
158341658	serpin A12 [Rattus norvegicus]	Serpina12	191570	4.59	1	4	0.52	0.06	0.04	47.9	9.44
6981610	seminal vesicle secretory protein 4 [Rattus norvegicus]	Svs4	100909594	15.32	1	1	0.30	0.06	0.05	11.9	9.04
157821865	hypothetical protein LOC499657 [Rattus norvegicus]	RGD15622 34	499657	45.05	5	70	0.22	0.07	0.05	10.7	7.25
57012372	keratin, type II cytoskeletal 2 oral [Rattus norvegicus]	Krt76	407757	49.74	24	684	0.54	0.08	0.05	61.7	8.38
57012366	keratin, type II cuticular Hb4 [Rattus norvegicus]	Krt84	315320	29.98	18	269	0.38	0.09	0.07	61.2	7.84
293340723	PREDICTED: keratin associated protein 11-1-like isoform 2 [Rattus norvegicus]	LOC10035 9886	100359886	8.59	1	2	2.30	0.18	0.07	17.0	7.94
8394193	gastric triacylglycerol lipase precursor [Rattus norvegicus]	Lipf	50682	4.81	1	1	2.12	0.50	0.07	44.6	6.60
293345493	**PREDICTED: trichohyalin [Rattus norvegicus]**	**Tchh**	**310588**	**21.55**	**34**	**368**	**0.45**	**0.10**	**0.07**	**205.1**	**5.78**
109467091	**PREDICTED: cornulin [Rattus norvegicus]**	**Crnn**	**295186**	**19.05**	**6**	**17**	**0.26**	**0.10**	**0.08**	**58.2**	**6.02**
51889726	keratin, type I cytoskeletal 24 [Rattus norvegicus]	Krt24	287675	28.11	14	143	0.54	0.13	0.09	52.3	5.01
293340674	PREDICTED: hypothetical protein [Rattus norvegicus]	LOC10036 4664	100363136	12.57	1	2	0.72	0.22	0.10	19.1	8.97
16758840	mu-crystallin homolog [Rattus norvegicus]	Crym	117024	4.79	1	2	0.16	0.13	0.10	33.5	5.53
293342784	PREDICTED: enhancer of polycomb homolog 1-like [Rattus norvegicus]	LOC10036 2678	100362678	1.57	1	1	0.49	0.13	0.10	84.6	8.57
57012436	keratin, type I cytoskeletal 10 [Rattus norvegicus]	Krt10	450225	28.90	6	111	0.60	0.17	0.11	56.5	5.15
57012378	keratin, type II cuticular Hb6 [Rattus norvegicus]	Krt86	407760	17.02	2	30	1.28	0.15	0.11	56.6	6.29
18426812	adenosine deaminase [Rattus norvegicus]	Ada	24165	46.59	16	325	0.38	0.11	0.11	39.9	5.50
149944672	lysozyme g-like protein 1 precursor [Rattus norvegicus]	Lyg1	100910070	19.29	4	30	0.28	0.11	0.11	21.9	9.14
57012446	keratin, type I cytoskeletal 42 [Rattus norvegicus]	Krt42	450231	47.12	7	550	0.63	0.20	0.11	50.2	5.16
56912229	keratin, type I cuticular Ha6 [Rattus norvegicus]	Krt36	287698	48.72	16	336	0.52	0.17	0.12	52.2	5.05
109493951	PREDICTED: hypothetical protein [Rattus norvegicus]	LOC68464 0	684640	9.77	1	1	1.42	0.14	0.12	18.5	7.99
56847624	keratin, type I cytoskeletal 23 [Rattus norvegicus]	Krt23	287678	7.58	1	39	0.36	0.13	0.13	48.1	6.05
117940041	long palate, lung and nasal epithelium carcinoma-associated protein 1 precursor [Rattus norvegicus]	Bpifb1	499926	5.31	3	4	1.32	0.58	0.13	52.2	6.13
199560677	coiled-coil domain-containing protein 8 [Rattus norvegicus]	Ccdc8	494320	2.46	1	1	0.68	0.18	0.14	70.0	9.76
6978661	creatine kinase M-type [Rattus norvegicus]	Ckm	24265	26.51	8	36	0.25	0.13	0.14	43.0	7.06
6981146	L-lactate dehydrogenase B chain [Rattus norvegicus]	Ldhb	24534	21.26	4	24	0.32	0.16	0.16	36.6	6.05

48040390	LIM domain only protein 7 [Rattus norvegicus]	Lmo7	361084	1.97	2	3	0.38	0.30	0.17	195.5	6.60
157822549	protein-glutamine gamma-glutamyltransferase E [Rattus norvegicus]	Tgm3	366189	32.90	19	164	0.84	0.18	0.18	77.2	6.89
293339922	PREDICTED: rCG35247-like isoform 1 [Rattus norvegicus]	LOC100364462	681124	3.92	1	2	0.68	0.20	0.18	36.8	4.89
71043890	acid sphingomyelinase-like phosphodiesterase 3b [Rattus norvegicus]	Smpdl3b	362619	6.80	2	4	0.80	0.25	0.18	51.6	5.88
145966774	tubulin beta-3 chain [Rattus norvegicus]	Tubb3	246118	35.11	1	154	0.82	0.31	0.18	50.4	4.93
158138498	glycogen phosphorylase, muscle form [Rattus norvegicus]	Pygm	24701	4.87	2	10	0.44	0.29	0.20	97.2	7.11
57012440	keratin 33B [Rattus norvegicus]	Krt33b	450227	16.58	3	105	1.66	0.27	0.20	45.8	4.82
57012388	keratin, type II cytoskeletal 80 [Rattus norvegicus]	Krt80	315318	6.42	2	9	0.86	0.31	0.20	50.5	6.20
31377484	carbonic anhydrase 3 [Rattus norvegicus]	Car3	54232	6.92	1	2	0.49	0.26	0.20	29.4	7.37
39930606	oncomodulin [Rattus norvegicus]	Ocm	25503	44.95	4	9	1.02	0.95	0.21	12.2	4.27
109491332	PREDICTED: family with sequence similarity 57, member A-like [Rattus norvegicus]	RGD1307493	100360533	6.23	1	2	0.59	0.21	0.21	29.3	9.17
16758346	von Ebner gland protein 2 precursor [Rattus norvegicus]	Vegp2	94106	11.86	2	6	1.99	0.41	0.21	19.7	6.54
6981598	steryl-sulfatase precursor [Rattus norvegicus]	Sts	24800	1.73	1	1	0.64	0.24	0.21	62.6	7.31
310703584	suprabasin isoform 1 [Rattus norvegicus]	Sbsn	292793	34.81	9	67	0.64	0.26	0.21	69.4	7.15
11968064	parvalbumin alpha [Rattus norvegicus]	Pvalb	25269	20.91	2	3	0.71	0.15	0.21	11.9	5.19
157818163	protein POF1B [Rattus norvegicus]	Pof1b	302328	19.59	10	38	0.57	0.29	0.21	67.6	6.28
293348974	PREDICTED: type II keratin Kb40 [Rattus norvegicus]	Krt78	315324	35.93	14	54	0.75	0.32	0.21	107.8	7.14
19424346	common salivary protein 1 [Rattus norvegicus]	LOC171161	171161	32.08	4	156	0.84	0.26	0.21	17.6	7.90
6981182	microtubule-associated protein 2 [Rattus norvegicus]	Map2	25595	2.58	3	5	0.83	0.37	0.22	198.4	4.84
54234046	cystatin-C precursor [Rattus norvegicus]	Cst3	25307	25.00	2	7	0.75	0.25	0.23	15.4	9.22
157817051	hypothetical protein LOC307124 [Rattus norvegicus]	Camk1d	307124	2.86	1	1	0.61	0.27	0.23	42.9	7.17
156119593	keratin, type II cuticular Hb3 [Rattus norvegicus]	Krt83	681126	19.80	3	31	1.03	0.29	0.23	54.7	6.37
164663841	chloride intracellular channel 3 [Rattus norvegicus]	Clic3	296566	20.25	3	7	0.55	0.33	0.24	26.8	5.97
197927125	galectin-related protein [Rattus norvegicus]	Lgalsl	360983	22.67	3	6	0.63	0.31	0.25	18.9	5.35
9506531	cytochrome P450 2F2 [Rattus norvegicus]	Cyp2f4	54246	11.81	4	11	0.57	0.28	0.25	55.9	7.84
109509676	PREDICTED: hypothetical protein isoform 1 [Rattus norvegicus]	LOC682408	682408	14.26	5	21	0.84	0.32	0.25	53.0	9.04
19705467	cytochrome P450, family 2, subfamily t, polypeptide 1 [Rattus norvegicus]	Cyp2t1	171380	13.74	6	17	0.68	0.36	0.26	55.8	6.67
300360521	smoothelin-like protein 2 [Rattus norvegicus]	Smtnl2	679629	3.07	1	1	0.81	0.31	0.26	49.5	8.54
109467082	PREDICTED: repetin [Rattus norvegicus]	Rptn	295190	11.20	7	130	0.87	0.32	0.26	128.0	7.53
71361623	protein MEMO1 [Rattus norvegicus]	Memo1	298787	4.38	1	2	0.72	0.37	0.26	33.7	7.14
109486870	PREDICTED: similar to class-alpha glutathione S-transferase [Rattus norvegicus]	RGD1562107	363205	7.14	2	3	0.67	0.35	0.26	27.6	4.94

57012360	keratin, type II cytoskeletal 4 [Rattus norvegicus]	Krt4	315323	50.56	24	714	0.66	0.46	0.27	57.6	7.64
162287127	myosin-14 [Rattus norvegicus]	Myh14	308572	8.45	7	62	0.83	0.37	0.27	228.8	5.55
157819539	kallikrein-8 precursor [Rattus norvegicus]	Klk8	308565	4.23	1	2	0.64	0.33	0.28	28.5	8.25
57526937	tubulin polymerization-promoting protein family member 3 [Rattus norvegicus]	Tppp3	291966	12.50	2	6	0.58	0.39	0.28	19.0	9.11
157823103	cytosolic 5'-nucleotidase 3 [Rattus norvegicus]	Nt5c3	312373	8.42	2	4	0.63	0.33	0.28	33.8	5.47
157822763	serpin A9 [Rattus norvegicus]	Serpina9	299274	17.03	6	10	1.00	0.33	0.29	46.8	9.60
109482461	PREDICTED: epiplakin 1-like [Rattus norvegicus]	LOC686567	680860	29.76	41	189	0.71	0.37	0.30	381.0	5.87
290563809	dermokine [Rattus norvegicus]	Dmkn	361548	18.22	8	61	0.74	0.36	0.30	52.2	6.84
293344027	PREDICTED: kallikrein related-peptidase 14 [Rattus norvegicus]	Klk14	308562	13.20	2	7	0.87	0.40	0.30	27.2	9.11
78214356	general transcription factor II-I [Rattus norvegicus]	Gtf2i	353256	1.75	1	1	1.00	0.60	0.30	103.0	8.76
31542401	creatine kinase B-type [Rattus norvegicus]	Ckb	24264	30.18	7	41	0.61	0.41	0.31	42.7	5.58
109494239	PREDICTED: similar to Dermal papilla derived protein 7 [Rattus norvegicus]	RGD1310935	360707	3.70	1	2	0.87	0.31	0.31	33.6	8.28
61557414	tubulin-specific chaperone A [Rattus norvegicus]	Tbca	366995	8.33	1	1	0.62	0.41	0.31	12.7	5.47
14192935	retinal dehydrogenase 1 [Rattus norvegicus]	Aldh1a1	24188	17.76	2	20	0.80	0.39	0.31	54.4	7.83
52486810	transforming acidic coiled coil 2 isoform 1 [Rattus norvegicus]	Tacc2	309025	0.88	2	2	1.19	0.58	0.32	300.0	4.78
19424152	proline rich, lacrimal 1 [Rattus norvegicus]	Prol1	65182	9.94	3	7	0.63	0.51	0.32	35.0	9.73
12018322	transmembrane protease serine 11D isoform b precursor [Rattus norvegicus]	Tmprss11d	64565	12.19	2	4	1.00	0.42	0.32	30.5	6.38
19705477	RING finger protein 39 [Rattus norvegicus]	Rnf39	171387	5.11	1	1	0.86	0.49	0.33	38.3	6.55
82617596	tumor-associated calcium signal transducer 2 precursor [Rattus norvegicus]	Tacstd2	494343	7.26	1	1	0.50	0.43	0.33	35.5	8.60
293354064	PREDICTED: DIP13 alpha [Rattus norvegicus]	RGD1309388	290537	1.41	1	1	0.64	0.60	0.33	79.3	5.38
157819905	kallikrein-12 [Rattus norvegicus]	Klk12	308564	4.44	1	2	0.89	0.34	0.33	19.5	8.25
92373398	nuclease-sensitive element-binding protein 1 [Rattus norvegicus]	Ybx1	500538	16.46	1	5	1.90	1.39	0.33	35.7	9.88
14010869	aldehyde dehydrogenase, dimeric NADP-preferring [Rattus norvegicus]	Aldh3a1	25375	22.30	9	37	0.80	0.40	0.34	50.3	6.80
61098212	ubiquitin carboxyl-terminal hydrolase isozyme L1 [Rattus norvegicus]	Uchl1	29545	8.07	1	1	0.52	0.41	0.34	24.8	5.24
16758388	ATP synthase-coupling factor 6, mitochondrial precursor [Rattus norvegicus]	Atp5j	94271	35.19	3	5	0.68	0.53	0.34	12.5	9.44
13242273	polypeptide N-acetylgalactosaminyltransferase 1 [Rattus norvegicus]	Galnt1	79214	2.15	1	2	0.56	0.36	0.35	64.2	7.72
120586975	dual specificity phosphatase 14 [Rattus norvegicus]	Dusp14	360580	10.61	2	15	0.88	0.37	0.35	22.3	9.54
290563194	catenin, beta-interacting protein 1 [Rattus norvegicus]	Ctnnbip1	503000	17.28	1	2	0.73	0.42	0.35	9.2	5.41
12083661	ADP-ribosylation factor-like protein 3 [Rattus norvegicus]	Arl3	64664	10.44	1	2	0.70	0.40	0.35	20.4	7.24
293347512	PREDICTED: ribosomal protein L32-like [Rattus norvegicus]	LOC10036	688684	12.60	2	5	0.90	0.55	0.36	15.0	11.2

		3713									1
140969642	carboxylesterase 3 precursor [Rattus norvegicus]	Ces1d	113902	11.33	5	12	0.92	0.39	0.36	62.1	6.54
157786978	serpin B8 [Rattus norvegicus]	Serpinb8	288937	8.80	3	10	0.95	0.41	0.36	42.2	6.21
56605806	target of Myb protein 1 [Rattus norvegicus]	Tom1	361370	3.46	1	4	0.82	0.48	0.36	54.1	4.96
000000000037	Tax_Id=9606 Gene_Symbol=KRT1 Keratin, type II cytoskeletal 1	Pter	Protein not found	15.68	3	228	0.72	0.52	0.36	66.0	8.12
40445397	hemoglobin, beta adult major chain [Rattus norvegicus]	Hbb-b1	361619	61.22	5	189	0.49	0.45	0.36	16.0	7.30
16923936	vesicle-associated membrane protein 3 [Rattus norvegicus]	Vamp3	29528	32.04	2	3	1.03	0.63	0.36	11.5	8.50
198442840	hypothetical protein LOC684972 [Rattus norvegicus]	Fam25a	684972	38.20	3	39	0.75	0.45	0.36	9.2	6.05
293342967	PREDICTED: desmoglein 1 beta [Rattus norvegicus]	Dsg1b	291755	21.60	17	138	0.85	0.41	0.37	112.3	4.91
55742713	extracellular matrix protein 1 precursor [Rattus norvegicus]	Ecm1	116662	23.49	9	31	0.69	0.45	0.37	63.2	6.87
56847618	keratin, type I cytoskeletal 16 [Rattus norvegicus]	Krt16	303530	62.20	19	954	1.06	0.48	0.37	50.7	5.12
6978469	afadin [Rattus norvegicus]	Mllt4	26955	2.68	3	4	0.77	0.47	0.38	207.5	6.16
158517925	galectin-7 [Rattus norvegicus]	Lgals7	29518	24.26	3	34	0.66	0.49	0.38	15.3	6.96
157818431	periplakin [Rattus norvegicus]	Ppl	302934	23.66	38	145	0.84	0.50	0.38	204.0	5.45
109506062	PREDICTED: calmodulin 4 isoform 1 [Rattus norvegicus]	Calm4	364774	61.22	10	357	1.00	0.40	0.38	16.8	4.74
157823017	ferrochelatase, mitochondrial [Rattus norvegicus]	Fech	361338	3.32	1	4	0.73	0.46	0.38	47.5	8.72
157819247	carboxypeptidase A4 [Rattus norvegicus]	Cpa4	502736	10.45	4	8	0.72	0.49	0.38	47.4	6.61
6981076	insulin-degrading enzyme [Rattus norvegicus]	Ide	25700	21.49	20	101	0.92	0.42	0.38	117.6	6.61
189011675	lethal(2) giant larvae protein homolog 2 [Rattus norvegicus]	Llgl2	360661	1.27	1	2	0.88	0.58	0.38	114.0	7.53
157821563	magnesium transporter NIPA4 [Rattus norvegicus]	Nipal4	303070	3.45	1	3	0.90	0.48	0.38	44.1	8.47
62543549	ubiquitin-like protein 3 precursor [Rattus norvegicus]	Ubl3	363869	18.80	3	3	0.80	0.43	0.39	13.2	6.92
157818855	envoplakin [Rattus norvegicus]	Evpl	303687	9.91	16	36	0.99	0.55	0.39	231.0	6.58
57164095	pirin [Rattus norvegicus]	Pir	363465	10.65	2	4	0.79	0.44	0.39	32.2	6.70
11693174	branched-chain-amino-acid aminotransferase, mitochondrial precursor [Rattus norvegicus]	Bcat2	64203	6.87	2	4	0.59	0.50	0.39	44.2	8.16
56090383	transmembrane protein 43 [Rattus norvegicus]	Tmem43	362401	14.75	5	19	0.60	0.56	0.39	44.7	7.36
77157795	MAL2 proteolipid protein [Rattus norvegicus]	Mal2	362911	6.25	1	6	1.33	0.55	0.40	19.2	6.48
19705557	pancreatic secretory granule membrane major glycoprotein GP2 precursor [Rattus norvegicus]	Gp2	171459	3.02	1	1	1.11	0.65	0.40	58.7	5.11
219277681	budding uninhibited by benzimidazoles 3 homolog [Rattus norvegicus]	Bub3	361662	6.13	1	1	0.70	0.60	0.40	36.9	6.84
157819321	gasdermin-A [Rattus norvegicus]	Gsdma	360619	5.16	2	3	1.02	0.53	0.40	49.8	5.41
57114298	ubiquitin thioesterase OTU1 [Rattus norvegicus]	Yod1	363982	2.64	1	1	1.06	0.50	0.41	33.8	5.45
9507135	spectrin beta chain, brain 2 [Rattus norvegicus]	Sptbn2	29211	10.85	15	46	0.77	0.54	0.41	270.8	5.85
51591909	keratin, type I cytoskeletal 13 [Rattus norvegicus]	Krt13	287699	64.38	18	849	0.71	0.51	0.41	47.7	4.91

158341689	NADP-dependent malic enzyme [Rattus norvegicus]	Me1	24552	16.08	7	17	0.87	0.38	0.41	63.9	6.80
000000000039	Keratin, type II cytoskeletal 2 epidermal	Psmd11	Protein not found	11.16	2	179	0.96	0.48	0.41	65.8	8.00
157817091	myelin protein zero-like protein 3 [Rattus norvegicus]	Mpzl3	363054	3.81	1	2	0.94	0.47	0.41	25.9	7.93
114145540	arylsulfatase E [Rattus norvegicus]	Arse	310326	1.15	1	1	0.78	0.42	0.41	65.6	7.43
157823875	epidermal growth factor receptor kinase substrate 8-like protein 1 [Rattus norvegicus]	Eps8l1	361503	9.62	5	21	0.66	0.49	0.42	80.0	6.43
157787014	V-set and immunoglobulin domain-containing protein 8 [Rattus norvegicus]	Vsig8	289236	7.43	2	5	0.69	0.57	0.42	44.2	7.08
157820297	hypothetical protein LOC498967 [Rattus norvegicus]	RGD1559896	498967	4.94	1	2	0.75	0.42	0.42	35.6	5.07
157823027	ATP-dependent RNA helicase DDX3X [Rattus norvegicus]	Ddx3x	317335	18.65	1	18	0.90	0.47	0.42	34.3	7.02
157823873	myeloid leukemia factor 2 [Rattus norvegicus]	Mlf2	312709	4.86	1	3	0.77	0.48	0.42	28.0	6.98
109481640	PREDICTED: caspase 14 [Rattus norvegicus]	Casp14	299587	12.60	2	8	0.76	0.56	0.43	28.2	5.33
315630402	hydroxyacylglutathione hydrolase, mitochondrial precursor [Rattus norvegicus]	Hagh	24439	6.15	2	9	0.92	0.49	0.43	34.1	7.94
000000000040	Tax_Id=9606 Gene_Symbol=KRT10 Keratin, type I cytoskeletal 10	LOC100360710	Protein not found	18.72	2	133	0.95	0.41	0.43	59.5	5.21
6981110	inositol 1,4,5-trisphosphate receptor type 3 [Rattus norvegicus]	Itpr3	25679	0.79	2	3	0.81	0.52	0.43	304.1	6.51
157821605	plakophilin-1 [Rattus norvegicus]	Pkp1	304822	23.80	12	75	0.79	0.70	0.43	67.2	8.70
8394405	large neutral amino acids transporter small subunit 1 [Rattus norvegicus]	Slc7a5	50719	2.73	1	3	0.66	0.55	0.43	55.9	7.90
293349573	PREDICTED: RGD1563398 [Rattus norvegicus]	RGD1563398	301075	0.64	1	1	1.63	0.77	0.43	176.0	8.13
56090295	PDZ and LIM domain protein 2 [Rattus norvegicus]	Pdlim2	290354	8.88	2	3	0.72	0.62	0.43	37.6	8.65
197386455	non-specific cytotoxic cell receptor protein 1 homolog [Rattus norvegicus]	Nccrp1	292755	15.12	3	9	1.01	0.58	0.43	33.0	6.54
50054216	cyclin-dependent kinase inhibitor 1B [Rattus norvegicus]	Cdkn1b	83571	14.21	2	2	0.84	0.47	0.44	22.1	7.02
142976607	selenoprotein T precursor [Rattus norvegicus]	Selt	365802	6.67	1	1	0.74	0.66	0.44	22.3	8.60
157817610	tuftelin [Rattus norvegicus]	Tuft1	365864	3.50	1	4	0.81	0.42	0.44	29.2	6.07
157821835	sciellin [Rattus norvegicus]	Scel	361086	12.88	7	16	0.90	0.66	0.45	72.8	9.42
261337175	Stg protein [Rattus norvegicus]	RGD1562885	502412	6.25	2	6	0.93	0.54	0.45	39.9	7.87
50845396	lymphocyte antigen 6 complex G6C [Rattus norvegicus]	Ly6g6c	294241	7.14	1	1	0.74	0.47	0.45	14.0	7.94
157821833	exportin-T [Rattus norvegicus]	Xpot	314879	1.79	1	2	0.73	0.50	0.45	83.4	5.47
109511452	PREDICTED: GABA(A) receptor-associated protein-like 2-like [Rattus norvegicus]	LOC501441	501441	12.82	1	2	0.98	0.60	0.45	13.7	7.33
000000000041	Tax_Id=9606 Gene_Symbol=KRT9 Keratin, type I cytoskeletal 9	Palm	Protein not found	11.24	5	58	0.66	0.56	0.46	62.1	5.30
6980970	aspartate aminotransferase, cytoplasmic [Rattus norvegicus]	Got1	24401	3.87	1	4	0.68	0.56	0.46	46.3	6.74

112984140	atlastin-3 [Rattus norvegicus]	Atl3	309187	2.24	1	1	0.60	0.40	0.46	60.2	5.55
56605724	phosphomannomutase 1 [Rattus norvegicus]	Pmm1	300089	5.73	1	1	0.58	0.46	0.46	29.7	5.59
27501444	NAD(P)H dehydrogenase [quinone] 1 [Rattus norvegicus]	Nqo1	24314	20.07	5	62	1.13	0.48	0.46	30.9	8.43
157820561	hypothetical protein LOC311257 [Rattus norvegicus]	Nat10	311257	3.08	2	3	0.93	0.50	0.46	102.1	8.28
109482100	PREDICTED: orphan short-chain dehydrogenase / reductase [Rattus norvegicus]	Sdr-o	259235	4.79	1	2	0.84	0.58	0.46	35.1	8.91
300390197	dedicator of cytokinesis protein 9 [Rattus norvegicus]	Dock9	259237	0.58	1	2	0.79	0.44	0.47	234.7	7.52
11560105	glutaredoxin-1 [Rattus norvegicus]	Glrx	64045	25.23	4	27	0.97	0.54	0.47	11.9	8.62
157819655	heme-binding protein 2 [Rattus norvegicus]	Hebp2	308632	7.39	1	2	0.91	0.54	0.47	22.9	4.58
82524639	DNA-binding protein A [Rattus norvegicus]	Csda	83807	19.94	2	15	0.83	0.71	0.47	38.8	9.69
198041631	huntingtin interacting protein 1 related isoform 2 [Rattus norvegicus]	Hip1r	81917	2.53	2	9	1.31	0.67	0.47	119.4	6.55
293346079	PREDICTED: titin [Rattus norvegicus]	Ttn	84015	0.07	2	3	0.62	0.48	0.48	3702.7	6.44
68163503	hypothetical protein LOC498796 [Rattus norvegicus]	Fam107b	498796	9.92	1	2	0.89	0.82	0.48	15.6	8.31
293345557	PREDICTED: annexin A2-like [Rattus norvegicus]	Anxa9	689830	3.78	1	4	0.86	0.67	0.48	38.1	6.02
62945278	2-oxoglutarate dehydrogenase, mitochondrial precursor [Rattus norvegicus]	Ogdh	360975	6.35	4	6	0.80	0.57	0.48	116.2	6.77
58865906	phospholipase D3 [Rattus norvegicus]	Pld3	361527	4.51	2	3	0.83	0.49	0.48	54.4	6.52
77681960	bleomycin hydrolase [Rattus norvegicus]	Blmh	287552	22.64	8	114	0.98	0.57	0.48	52.4	6.38
293344068	PREDICTED: hydroxysteroid (17-beta) dehydrogenase 14 [Rattus norvegicus]	Hsd17b14	691018	5.19	1	2	0.76	0.55	0.48	28.1	5.41
28461161	low-density lipoprotein receptor precursor [Rattus norvegicus]	Ldlr	300438	1.48	1	1	0.80	0.59	0.49	96.6	4.98
109505631	PREDICTED: desmoplakin isoform 2 [Rattus norvegicus]	Dsp	306871	29.72	79	532	0.92	0.62	0.49	332.2	6.83
000000000001	Trypsin - Sus scrofa [109]	Cops4	Protein not found	31.17	5	137	1.38	0.62	0.49	24.4	7.18
47059179	heat shock 70kD protein 1B [Rattus norvegicus]	Hspa1b	294254	31.36	11	130	0.94	0.64	0.49	70.1	5.82
14485281	aldehyde dehydrogenase, cytosolic 1 [Rattus norvegicus]	Aldh1a7	29651	31.14	6	43	0.91	0.52	0.49	54.5	7.42
126723393	beta-enolase [Rattus norvegicus]	Eno3	25438	17.74	3	79	0.22	0.12	0.13	47.0	7.44

The highlighted proteins have been validated either by IHC or qRT-PCR

PUBLICATION

Clinical Communications-Oncology

Original Article

Quantitative proteomic analysis of different stages of rat lingual carcinogenesis

Bihari Lal Soni[1], Arivusudar Marimuthu[2], Harsh Pawar[2], Sharada S. Sawant[1], Anita Borges[3], Ranganathan Kannan[4], Akhilesh Pandey[5,6,7,8], Arvind D. Ingle[9], Hindahally Chandregowda Harsha[2], Milind Murlidhar Vaidya[1]*

[1]Vaidya Laboratory, TMC-Advanced Centre for Treatment, Research and Education in Cancer, Kharghar Navi-Mumbai, [2]Institute of Bioinformatics, International Technology Park Limited, Whitefield, Bengaluru, Karnataka, [3]Asian Institute of Oncology, Department of Pathology, S L Raheja Hospital, Mahim, Mumbai, Maharashtra, [4]Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai, Tamil Nadu, India, [5]McKusick-Nathans Institute of Genetic Medicine, [6]Departments of Biological Chemistry, [7]Oncology, and [8]Pathology, Johns Hopkins University School of Medicine, Baltimore, MD, USA, [9]Animal House facility, TMC-Advanced Centre for Treatment, Research and Education in Cancer, Kharghar Navi-Mumbai, India

E-mail: mvaidya@actrec.gov.in
*Corresponding author

Published: 09 May, 2014
Journal of Carcinogenesis 2014, 12:0*
This article is available from: http://www.carcinogenesis.com/content/12/1/0*

Received: 22 January, 2014
Accepted: 07 March, 2014

Abstract

Background: In India, oral squamous cell carcinoma (OSCC) is the single largest group of malignancies in males. Early diagnosis of cancer is difficult because of the lack of specific symptoms and/or biomarkers for early disease. Animal models provide an opportunity to study development and progression of cancers. **Materials and Methods:** In this study, we have explored the 4-nitroquinoline 1-oxide (4NQO)-induced tongue cancer model in Sprague Dawley rats. We compared the protein expression profiles of normal tissues with different stages of rat tongue cancer using isobaric tags for relative and absolute quantitation (iTRAQ)-liquid chromatography-tandem mass spectrometry (LC-MS/MS) based proteomics strategy. We validated some known and novel proteins by immunohistochemistry (IHC) and real-time polymerase chain reaction (PCR). **Results:** We observed hyperplasia, papillomas, and carcinomas after 120, 160, and 200 days treatment of 4NQO, respectively. LC-MS/MS analysis resulted in identification of 2223 proteins. Of these, 415 proteins were found to be differentially expressed in tumors, 333 proteins in papilloma and 109 proteins in hyperplasia. We have found alterations in several previously reported as well as novel proteins during rat tongue carcinogenesis. We validated known molecules such as vimentin, fascin, periostin, transglutaminase 3 by IHC and cornulin by real-time PCR on rat tissues. We also validated tenascin N, a novel protein by IHC on rat as well as in human tongue tissues. **Conclusion:** To the best of our knowledge, this is the first in-depth differential proteomics study carried out using an experimental rat model of OSCC. Proteomic alterations observed in this study provide insights into carcinogenesis process and may serve as a valuable resource for oral cancer biomarker discovery.

Keywords: Chemical carcinogenesis, isobaric tags for relative and absolute quantitation, mass spectrometry, oral cancer, rat model

Access this article online

Quick Response Code:

Website: www.carcinogenesis.com

DOI: 10.4103/WKMP-0062.132172

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the sixth largest group of malignancies globally and represents one of the leading causes of mortality.[1] It remains a major cancer in the Indian subcontinent, comprising >40% of all cancer cases. The most commonly involved sites of tumor development in

the Indian population are buccal mucosa and tongue.[2] The major risk factors for oral cancer include chewing tobacco either alone or with substances such as betel nut and alcohol. Precancerous lesions like leukoplakia and submucous fibrosis are also quite prevalent in India due to these habits.[3] The malignant transformation of oral leukoplakia has been proposed to range from 15%-18%, respectively.[3,4] Despite advances in treatment and therapeutic modalities, the 5 year survival rate of OSCC has not changed much in the last few decades. The possible reasons for poor survival rates are late detection and local recurrence/regional lymph node metastasis.

In patients, the molecular analysis of multiple stages of carcinogenesis is hampered by the unavailability of biopsies of all the stages of oral carcinogenesis (e.g., normal, premalignant, dysplastic, and malignant lesions). However, animal models of carcinogenesis allow the reproducible isolation of all stages, including normal tissues, which are then amenable to pathological, genetic, and biochemical analyses.[5] We chose the 4-nitroquinoline 1-oxide (4NQO)-induced rat model of carcinogenesis as our model for studies related to oral carcinogenesis because it mimics molecular and pathological changes observed in patients.[5,6]

Proteomics has grown as powerful tool for biomarker discovery in various cancers.[7,8] A few proteomics studies on human samples have been conducted to dissect the molecular events, which lead to development of OSCC from leukoplakia.[9,10] Isobaric tags for relative and absolute quantitation (iTRAQ)-based liquid chromatography-tandem mass spectrometry (LC-MS/MS) is one of the useful quantitative approaches in proteomics to identify the differences between protein expression profiles of normal and diseased samples.[8]

In this study, we utilized 4NQO-induced rat model for tongue cancer because it recapitulates all the histological grades of human lingual carcinogenesis.[6,11] We obtained hyperplasia, papilloma, and carcinoma stages after 120, 160, and 200 days treatment of 4NQO, respectively. An iTRAQ-based differential proteomic analysis was carried out by labeling tryptic peptides derived from protein samples isolated from different stages of rat lingual carcinogenesis followed by LC-MS/MS analysis. This resulted in identification of 2223 total proteins. Of these, 415 proteins were found to be differentially expressed in SCC as compared with normal tissues. Among these, 109 proteins were differentially expressed in hyperplasia, while 333 proteins were differentially expressed in papillomas as compared with normal tissues. We validated some known molecules, including vimentin (*Vim*), fascin (*Fscn1*), and periostin (*Postn*) by immunohistochemistry (IHC). We also validated a novel protein, tenascin N (*Tnn*) in both rat and human tissues by IHC.

Thus, to the best of our knowledge, this is the first in-depth differential proteomics study on rat model of tongue carcinogenesis, which led to the identification of several known as well as novel molecules as candidate biomarkers for lingual carcinogenesis. Our studies demonstrate the utility of this model in the study of oral carcinogenesis and as tool for early biomarker discovery of tongue cancer.

MATERIALS AND METHODS

Animal model for tongue cancer

All animal experiments were approved by the Institutional Animal Ethics Committee. 5-6 weeks old male Sprague Dawley rats were used to induce oral tongue cancer. Animals were randomized and grouped in three groups: Untreated group (n = 36), acetone (vehicle) treated (n = 36), and 4NQO treated (n = 48). Each group was further sub divided into three sub-groups and treated for 120, 160, and 200 days, respectively. For 4NQO treatment animals were distributed into three groups (12 animals for 120 and 160 days while 24 animals for 200 days). 4NQO was dissolved in acetone and finally given to the animals at 30 ppm concentration in normal drinking water[Figure 1a]. After each time point of treatment, animals were fed with normal drinking water for another 15 days to get the stable changes. Animals were sacrificed by CO_2 inhalation followed by cervical dislocation. Detail distribution of 4NQO treated animals with their corresponding lesions on the tongue is given in Table 1.

Histopathology

Gross lesions were seen on the base of the dorsal tongue of rats [Figure 1b]. Histopathological observations were made by an experienced pathologist using hematoxylin and eosin stained slides [Figure 1c].

Table 1: Incidence of histopathological lesions in tongue of 4NQO treated rats for the development of oral carcinogenesis model

Group	Histopathological analysis of lingual tissues treated with 4NQO			
	Normal (no change)	Hyperplasia/ atypical hyperplasia	papilloma/ atypical papilloma	SCC
120 days (n=12)	-	7	5	0
160 days (n=12)	3*	2	5	2
200 days (n=24)	6*	0	8	10

*Animals died during experiment. 4NQO: 4-nitroquinoline 1-oxide; SCC: Squamous cell carcinoma

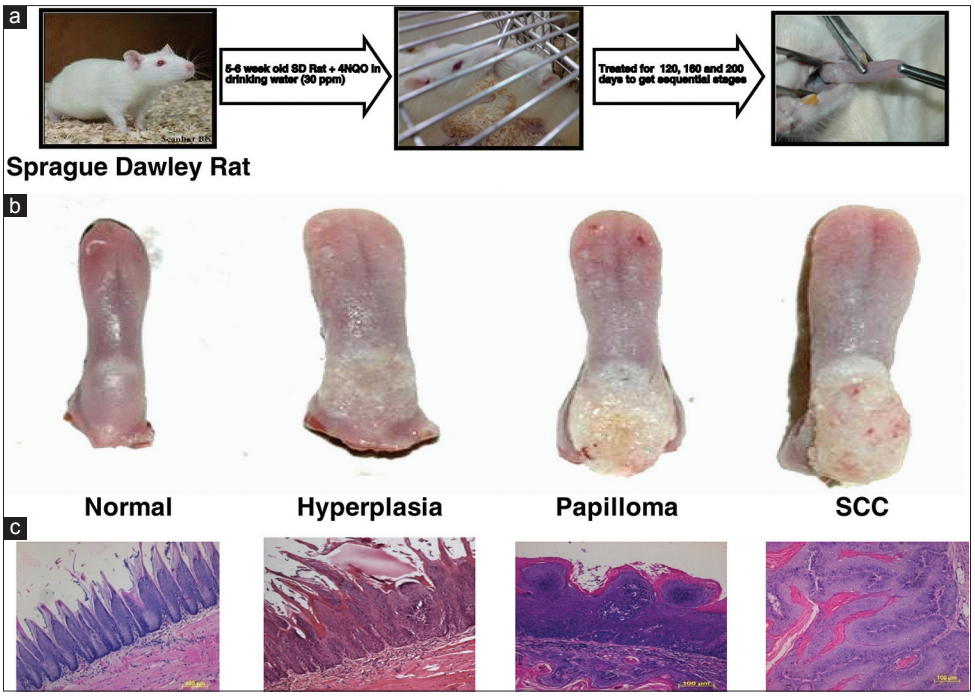


Figure 1: Rat lingual carcinogenesis model. (a) Protocol for lingual carcinogenesis, 5-6 weeks old Sprague Dawley male rats were taken and treated with 30 ppm of 4NQO in drinking water for 120, 160, and 200 days, respectively (b) Morphological alterations after 4-nitroquinoline 1-oxide (4NQO) treatment. (c) Photomicrograph of Hematoxylin and Eosin staining of different stages of rat lingual carcinogenesis (×100)

Protein extraction and pooling of samples

Approximately 30 mg of epithelial tissue from the rat tongue was pulverized in liquid nitrogen by mortar and pestle. The powdered tissue was reconstituted in 0.5% sodium dodecyl sulfate and sonicated using ultrsonicator on ice. Each sonication cycle was of 20s of pulsing at 50% output with intermittent gap of 45s; this cycle was repeated 3 times. Subsequently, the cell lysate was centrifuged at 14,000 rpm for 10 min at 40°C. Supernatant was transferred into fresh eppendorf tube and total protein content was measured using Lowry's method.[12] 100 µg of protein was pooled from each group normal (n = 10), hyperplasia (n = 5), papilloma (n = 5), and tumor (n = 5). Cell lysates were stored at −80°C until further use.

Protein digestion and isobaric tags for relative and absolute quantitation labeling

100 µg of total protein from each pool representing control, hyperplasia, papilloma, and carcinoma was used for iTRAQ labeling. Labeling was carried out as per manufacturer's instructions. Briefly, proteins were subjected to reduction using 2 ul of tris-(2-corboxyethyl) phosphine at 60°C for 1 h and alkylated with cystein blocking reagent, methyl methanethiosulfonate for 10 min at room temperature. They were then digested with sequencing grade trypsin (Promega, Madison, WI) (1:20) at 37°C for 16 h. The peptide digest from each sample type was subjected to iTRAQ labeling. Normal, hyperplasia, papilloma, and carcinoma samples were labeled with iTRAQ reagents yielding reporter ions of m/z 114, 115, 116, and 117 respectively. Labeled samples were then pooled and subjected to strong cationic exchange chromatography.

Strong cation exchange fractionation

Pooled samples were diluted with solvent A (10 mM of KH_2PO_4, 20% acetonitrile, pH 2.8). The diluted samples were acidified by adding phosphoric acid to reduce the pH to 2.8. Acidified sample was loaded on to strong cation exchange chromatography column (polyLC Inc.) at a flow rate of 250 ul/min followed by washing for 20 min. The peptides were fractionated using a 30 min gradient from 8% solvent B (350 mM KCl, 10 mM KH_2PO_4, and 20% Acetonitrile pH 2.8) to 50% solvent B, to a total of 23 fractions. Subsequently, the peptides were cleaned up using C18 zip tips. Prior to LC-MS/MS analysis, the peptide fractions were dried and stored at −20°C.

Liquid chromatography-tandem mass spectrometry analysis

Liquid chromatography-tandem mass spectrometry analysis of the iTRAQ labeled peptides was carried out using LTQ-Orbitrap Velos mass spectrometer interfaced with Agilent's 1200 Series nanoflow LC system. The chromatographic capillary columns used were packed with Magic C18 AQ (Michrom Bioresources, 5 µm particle size, pore size 100 Å) reversed phase material in 100% acetonitrile at a pressure of 1000 psi. The peptides were first loaded on to a

trap column (75 × 2 cm) at a flow rate of 5 µl/min followed by separation on an analytical column (75 × 10 cm) at a flow rate of 300 nl/min. The peptides were then eluted using a linear gradient of 7-30% solvent B (90% acetonitrile, 0.1% formic acid) over 50 min. MS analysis was performed in a data dependent manner with full scans acquired using Orbitrap mass analyzer at a mass resolution of 60,000 at 400 m/z. For each cycle, 20 most intense precursor ions from a survey scan were selected for MS/MS and detected at a mass resolution of 15,000 at m/z 400. The fragmentation was carried out using higher-energy collision dissociation with 40% normalized collision energy. The ions selected for fragmentation were dynamically excluded for 30s. The automatic gain control for full fourier transformed MS (FT MS) was set to 1 million ions and for FT MS/MS was set to 0.1 million ions with a maximum time of accumulation of 750 ms and 100 ms, respectively. For accurate mass measurements, the lock mass option was enabled. Internal calibration was enabled using the polydimethylcyclosiloxane (m/z, 445.12) ion.

Data analysis

The raw files obtained from LC-MS/MS analysis were processed using Proteome Discoverer (Version 1.3.0.339) software (Thermo Fisher Scientific, USA). MS/MS searches were carried out against NCBI RefSeq 49 rat protein database (n = 25,317) using sequest and mascot search algorithms. Oxidation of methionine, iTRAQ 4-plex modification at peptide N-terminus and lysine (K) were selected as variable modifications and methylthio of cysteine as a fixed modification. MS and MS/MS tolerance were set to 20 ppm and 0.1 Da, respectively. One missed cleavage was allowed. False discovery rate (FDR) was calculated using a decoy database. Peptide spectrum matches at 1% FDR were used for protein identification and quantitation. Relative quantification of peptides was done on the basis of relative intensity of reporter ions (115, 116, and 117 for hyperplasia, papilloma and carcinoma respectively) with respect to normal (114 for vehicle control). Protein ratios were calculated as the median of all the peptide ratios corresponding to respective proteins. A fold change of >2 was considered as upregulated while <0.5 was considered as downregulated.

Collection of human oral tumors and premalignant tissues

The tongue tumor tissues (n = 34) were collected from Tata Memorial Hospital, Mumbai, India at the time of surgery. In 14 of the cases, the adjoining histologically normal tissue was also collected. 10 paraffin embedded blocks of the biopsies collected from leukoplakia of tongue were obtained from Ragas Dental College, Chennai, India and Nair Dental Hospital, Mumbai, India. This study was approved by the Human Ethics Committees of the respective Institutional Review Boards. Informed consent was obtained from the patients before enrolling them in this study.

Immunohistochemistry

Formalin-fixed, paraffin-embedded, 5 µm thick rat and human tissue sections were mounted on poly-l-lysine coated glass slides. Sections were de-paraffinized with xylene and incubated with 3% hydrogen peroxide in methanol for 30 min in dark to quench the endogenous peroxidase activity of the tissues. After blocking with horse serum for 1 h at 37°C in humidified chamber, sections were incubated with primary antibodies, *Vim* (Sigma V 6630, mouse monoclonal, dilution 1:400), periostin (*Postn*) (Santacruz, sc 49,480, rabbit polyclonal, dilution 1:10), *Fscn1* (Pierce, MA1-20,912, mouse monoclonal dilution 1:100), Transglutaminase 3 (Tgm3) (Santacruz, sc-101,366, mouse monoclonal dilution 1:8,000), and tenascin N (*Tnn*) (Sigma, HPA-026,764 Rabbit polyclonal, dilution 1:100 [for both rat and human samples]) overnight at 4°C. Detection was done using Vectastain ABC system (Vector Laboratories, CA). Diaminobenzidine was used as the chromogen and slides were counterstained with Mayor's hematoxylin.

Ribonucleic acid isolation and quantitative real-time-polymerase chain reaction

To validate our proteomics data, we also performed quantitative real-time-polymerase chain reaction (qRT-PCR) analysis. Total cellular ribonucleic acid (RNA) was extracted from the tissue by Tri-reagent (Sigma-Aldrich, USA) as per manufacturer's protocol. RNA was estimated by measuring absorbance at 260 nm and 280 nm using nanodrop (ND-1000 Spectrophotometer, Wilmington, USA). cDNA synthesis was carried out as per the manufacturer's protocol (Fermentas, Thermo Scientific, Waltham, MA) and the obtained cDNA was used as template for qRT-PCR. Master Mix SYBR Green (Applied Biosystems, Bedford, MA) was used with 5nM of forward and reverse primers [Table 2]. Real-time quantitative PCR was performed with the ABI PRISM7700 Sequence Detection System. Beta actin gene was used as endogenous control. All amplifications were done in triplicate. Results are expressed as relative gene expression using the 2-ΔCt method.[13]

Table 2: Primer sequences used in qRT-PCR

Oligo name	5'<sequence>3'	Length
Rattus cornulin_F	CTCACGAAGCAGGAGCTGAA	20
Rattus cornulin_R	AGGATCATGGGGCTTCACTA	20
Rattus beta actin_F	ACCCGCGAGTACAACCTTCTT	21
Rattus beta actin_R	TATCGTCATCCATGGCGAACTGG	23

qRT-PCR: Quantitative real-time-polymerase chain reaction

RESULTS AND DISCUSSION

Experimental animal models have proved to be an important tool to study tumor progression.[6] In this study, Sprague Dawley rats were treated with 30 ppm of 4NQO in drinking water and sacrificed after different time points [Figure 1a]. 4NQO is potent carcinogen and 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. This activated molecule 4HAQO preferably binds to guanine residues and forms a DNA adduct. These adducts mimic ultraviolet-induced pyrimidine dimer formation. It has been proposed that the carcinogenesis process induced by 4NQO shows similar molecular alterations as in human carcinogenesis.[6,11] Figure 1b shows gross morphological alterations on the posterior dorsal of the tongue. The histopathological analysis of posterior dorsal tongue epithelium revealed no alterations in vehicle and untreated groups. However, treatment with 4NQO for 120 days resulted in the hyperplasia with hyperkeratosis while 160 days treatment resulted in the papillary growth of the squamous epithelium with increased hyperkeratosis. 200 days treatment resulted in well-differentiated SCC with marked disorganized and infiltrative growth of squamous cells [Figure 1c and Table 1]. 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.[14]

Proteomics is a promising approach for identification of markers for early detection of cancers. It has been successfully employed in studies of various tumor tissues and body fluids.[8,15] Studies on oral cancer patients to investigate possible biomarkers for early diagnosis/prognosis have been reported.[4,8] Pawar *et al.* carried out tissue proteomics on esophageal squamous cell carcinoma for novel biomarkers discovery, while Bijian *et al.* have used serum proteomics approach to discover serum biomarkers for OSCC.[8,15] Since our goal was to study sequential changes during oral carcinogenesis, we collected only tissue samples and therefore we carried out only tissue proteomics. The proteomics strategy employed in our study is shown in Figure 2.

Isobaric tags for relative and absolute quantitation labeling and liquid chromatography-tandem mass spectrometry analysis

We employed iTRAQ based quantitative proteomics to analyze differences in protein expression profiles at different stages of tongue carcinogenesis as described under material and method section. A list of proteins with identified peptides is given in the Supplementary Tables S1 and S2, respectively.

Quantitative analysis of mass spectrometry data

We identified a number of differentially expressed proteins at different stages of rat lingual carcinogenesis when compared with normal vehicle treated control. We identified a total of 2223 proteins of which 415 proteins were found to be differentially expressed in tumors when compared to normal tissues. Of these 415 proteins, 194 proteins were upregulated while 221 proteins were downregulated in premalignant and malignant lesions. Table 3 describes the details of differentially expressed proteins at each stage.

Bioinformatics analysis of the data

Bioinformatics analysis was carried out to classify proteins based on subcellular localization and biological function. We carried out classification based on Gene Ontology annotations. The distribution of proteins identified in our study based on subcellular localization and biological process is provided in Figure 3a and b, respectively. All proteins identified in the current iTRAQ-based analysis of rat lingual carcinogenesis were categorized on the basis of primary subcellular locations [Figure 3a], which resulted in 1835 proteins (83%) being localized to one of the subcellular compartments. In addition, proteins were classified on the basis of biological processes (e.g., cell signaling and communication). This resulted in the identification of 1786 proteins (80%), which were grouped into one of biological processes [Figure 3b]. The majority of the grouped proteins play a role in cellular metabolism, protein synthesis, degradation, and transport.

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. Here, we have validated some of the known candidate proteins whose differential expression in human oral carcinomas has been previously reported. These include *Vim*, *Fscn1*, Tgm3, *Postn* and cornulin (*Crnn*).

Known upregulated proteins identified in rat lingual carcinogenesis

Vimentin

Vimentin is type III intermediate filament protein, which is ubiquitously expressed in mesenchymal cells. This protein

Table 3: List of differentially expressed proteins during different stages of rat lingual carcinogenesis. Proteins showing differential expression >2-fold were reported as upregulated while proteins showing differential expression <2-fold were reported as downregulated

Stages	No. of upregulated proteins	No. of downregulated proteins	Total proteins
Hyperplasia	35	74	109
Papilloma	155	178	333
SCC	194	221	415
Total	384	473	857

SCC: Squamous cell carcinoma

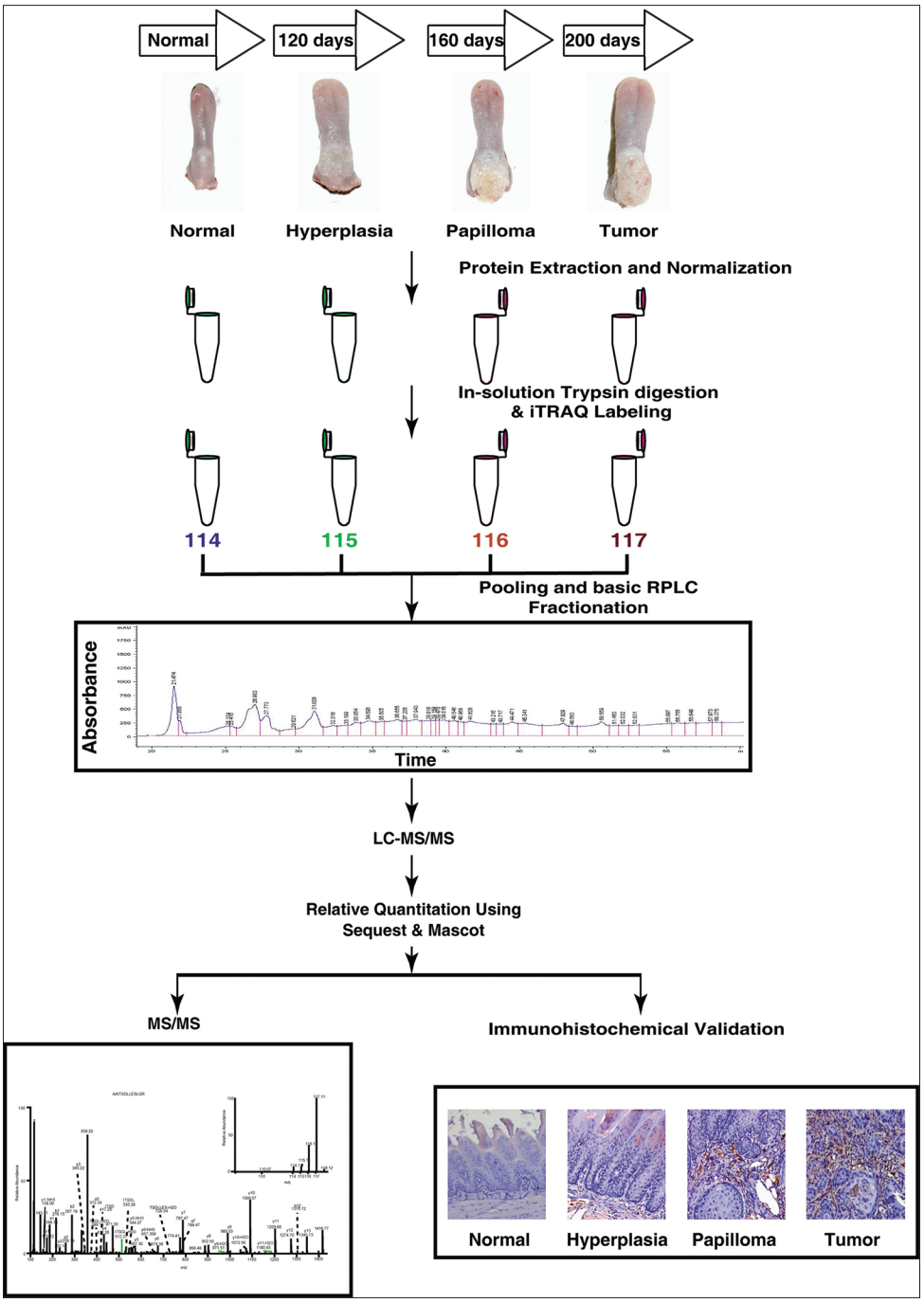


Figure 2: Work flow for quantitative tissue proteomics using isobaric tags for relative and absolute quantitation (iTRAQ) labeling and validation of biomarkers for tongue squamous cell carcinoma. For iTRAQ labeling, Proteins were isolated from 10 normals, 5 hyperplasia, 5 papilloma, and 5 tumor tissues, respectively. Proteins were subjected to trypsin digestion followed by iTRAQ labeling of peptides. Posts labeling the peptides were pooled and fractionated using strong cation exchange chromatography, followed by liquid chromatography tandem mass spectrometry/mass spectrometry on Orbitrap Velos mass spectrometer. Data were searched using Mascot and SEQEST search engines. Some of the over expressed proteins (e.g., Tnn) were validated using immunohistochemistry

not only has important role in the epithelial-mesenchymal transition of epithelial cells, but also has major role in the tumor microenvironment remodeling to facilitate the tumor cell metastasis.[16] In our proteomics study on experimental model, we have observed the sequential increase in *Vim* expression [Figure 4a]. We noted a 2-fold upregulation of *Vim* in tumor as compared with normal tissues. IHC data [Figure 5a] revealed that *Vim* expression was not detectable in normal epithelial tissues, but hyperplastic tissues demonstrated weak staining in cytoplasm and suprabasal layers. We noticed increased suprabasal and cytoplasmic expression of *Vim* in papillomas and carcinomas as compared with normal tissues [Figure 5a].

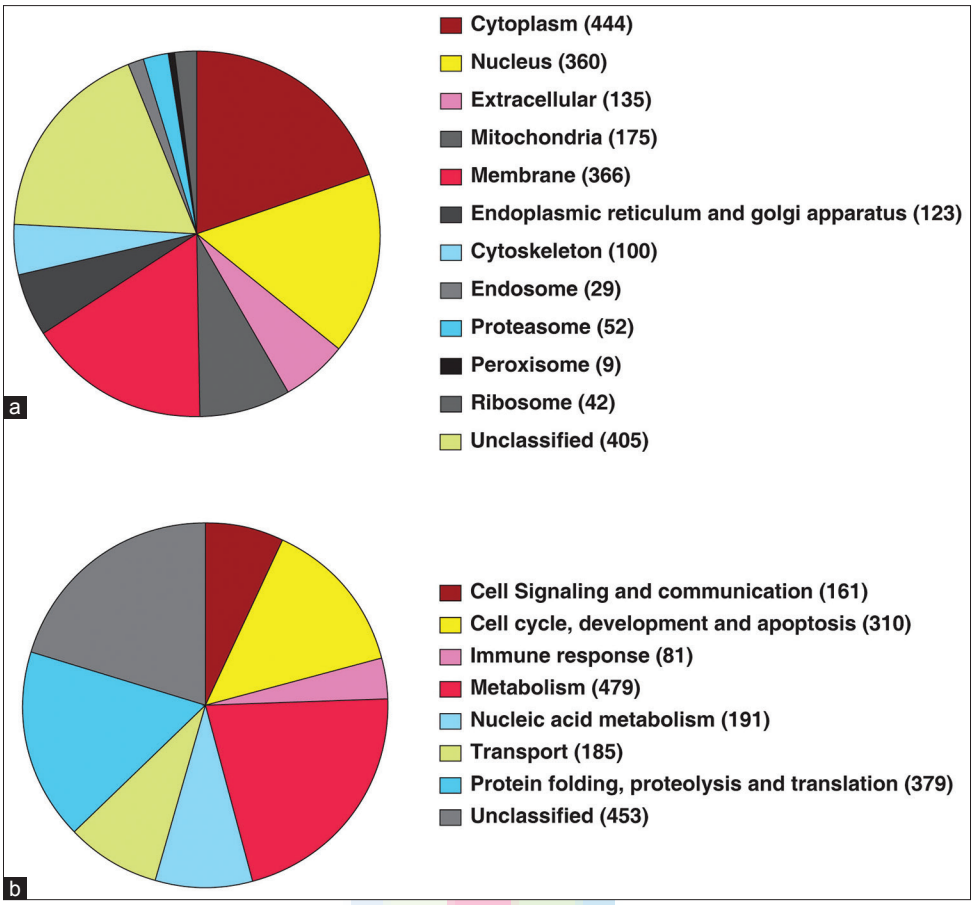


Figure 3: Classification of proteins by gene ontology based on their cellular localization and biological process. Panel (a) - Distribution of proteins based on their cellular localization using gene ontology classifier. Panel (b) - Distribution of proteins based on their biological processes using gene ontology classifier

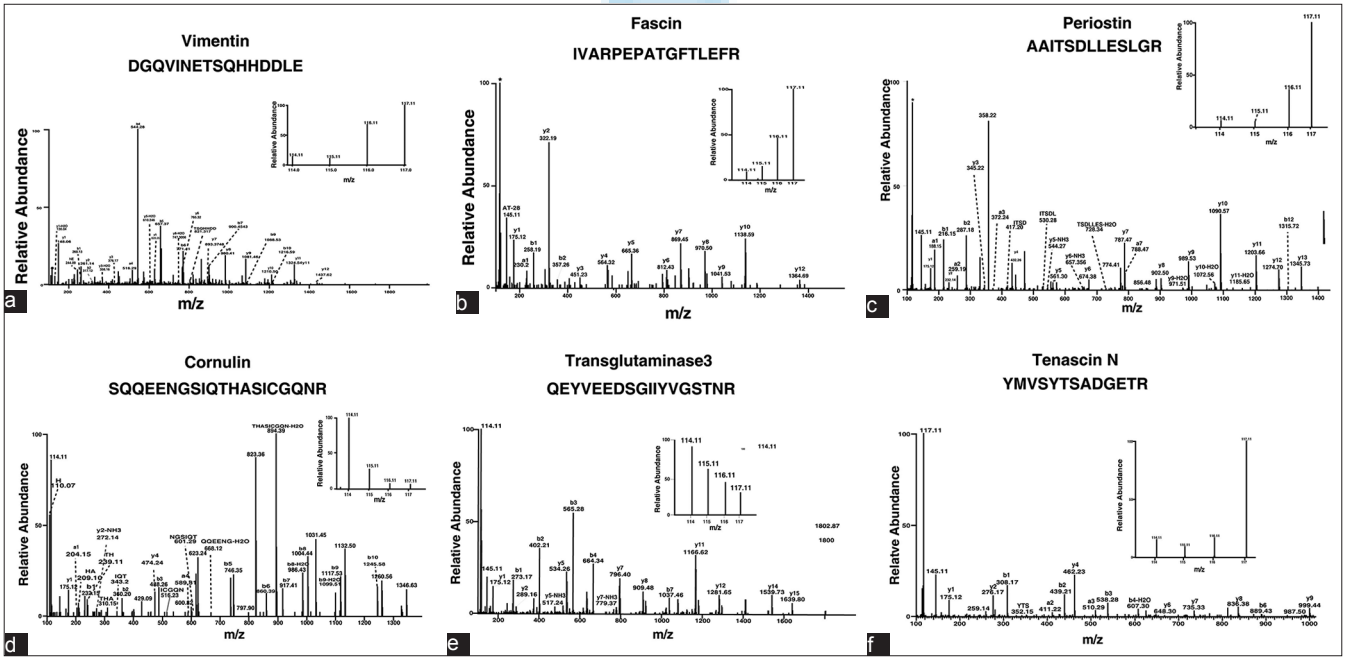


Figure 4: Mass spectrometry/mass spectrometry (MS/MS) spectra from representative differentially regulated known and novel proteins identified in this study. The inset shows the reporter ions used for quantitation. MS/MS spectra of peptide from representative differentially expressed proteins identified in this study. (a) Vimentin, (b) fascin, (c) Periostin, (d) cornulin, (e) transglutaminase 3 and (f) tenascin N

It has been shown that *Vim* expression begins in epithelial layers of variety of human cancers including head and neck,[17] prostate,[18] and breast cancers.[19] Recent study from our lab has shown aberrant *Vim* expression in precancerous lesions and SCC of oral mucosa.[20] Chaw *et al*. 2012 have proposed that aberrant expression of *Vim* may be used as a potential marker for malignant transformation in OSCC.[21]

Fascin

Fascin is an actin-bundling protein that is found in membrane ruffles, microspikes, and stress fibers.[22] It is found to be associated with tumor cell invasion and metastasis in various types of cancers including OSCC.[23,24] Our proteomics study suggests it's sequential upregulation during the process of carcinogenesis and upregulation to 3-fold in tumor as compared with normal tissues [Figure 4b]. IHC studies on rat tongue at different stages revealed that *Fscn1* expression was not detectable in the vehicle treated group while weak cytoplasmic staining was observed in the basal layer of hyperplastic tissues. Furthermore, strong cytoplasmic, and suprabasal staining was seen in papilloma and carcinoma tissues

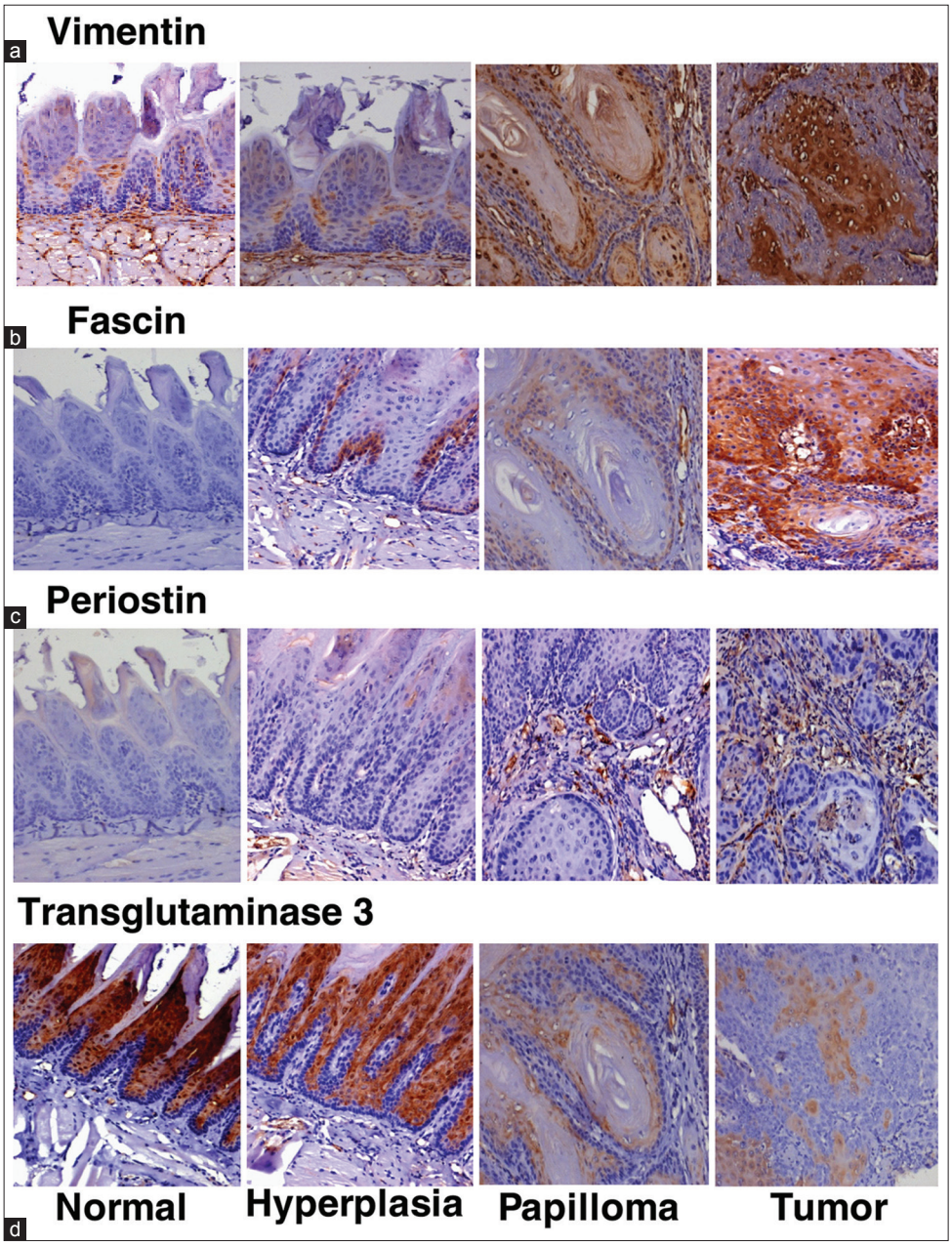


Figure 5: Validation of known proteins by Immunohistochemistry using specific antibodies. Representative photomicrographs showing immunohistochemical labeling of: (a) Vimentin, (b) Fascin, (c) Periostin, and (d) transglutaminase 3 at different stages of rat lingual carcinogenesis (magnification ×200)

respectively [Figure 5b]. Similar observations were made by Shimamura *et al.* in human oral dysplasia, who proposed that *Fscn1* overexpression in dysplastic tissue drives tumor formation.[25]

Periostin

Periostin is a matricellular protein and also reported as osteoblast-specific factor 2.[26] It is also referred as a stroma-associated protein and plays an important role in tumor development and is upregulated in a wide variety of cancers including head and neck.[27,28] Proteomics data demonstrated its sequential upregulation during rat tongue carcinogenesis and a 3.7-fold upregulation in tumors as compared with normal [Figure 4c]. Immunohistochemical analysis of *Postn* showed that *Postn* was not detectable in epithelial layers of normal and hyperplastic tissues while papillomatous lesions and tumor tissues showed *Postn* expression only in the stromal region [Figure 5c]. A study by Kyutoku *et al*. demonstrated that it plays a pivotal role in tumor progression and metastasis of murine breast cancer and proposed that this molecule can be potential drug target against breast cancer.[29] Together, these findings along with our result of progressive expression of *Postn* in 4NQO-induced rat tongue tumors demonstrate its potential candidature for early diagnostic and prognostic marker for tongue tumors.

Known downregulated proteins identified in rat lingual carcinogenesis

Transglutaminase 3

Transglutaminases are a family of calcium-dependent acyl-transfer enzymes that are widely expressed in mammalian cells.[30] Tgm3 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.[31] Marked suppression of Tgm3 is associated with various cancers like head and neck squamous cell carcinoma.[32] We obtained sequential downregulation of Tgm3 in our proteomics study and noted a ~6-fold downregulation in tumor as compared with normal [Figure 4e]. Validation by IHC indicates its strong cytoplasmic and suprabasal expression in normal tongue tissues. While, its cytoplasmic expression was sequentially downregulated during the process of tumorogenesis [Figure 5d]. Ohkura *et al*., 2005 demonstrated that Tgm3 is downregulated in OSCC and proposed that the lack of TGM-3 expression may also facilitate survival in OSCC cells.[33]

Cornulin

Cornulin is a recently identified protein also known as chromosome one open reading frame 10 (C1orf10).[34] It has conserved S100 EF-hand calcium binding motif and is highly expressed in esophagus. It also has a glutamine

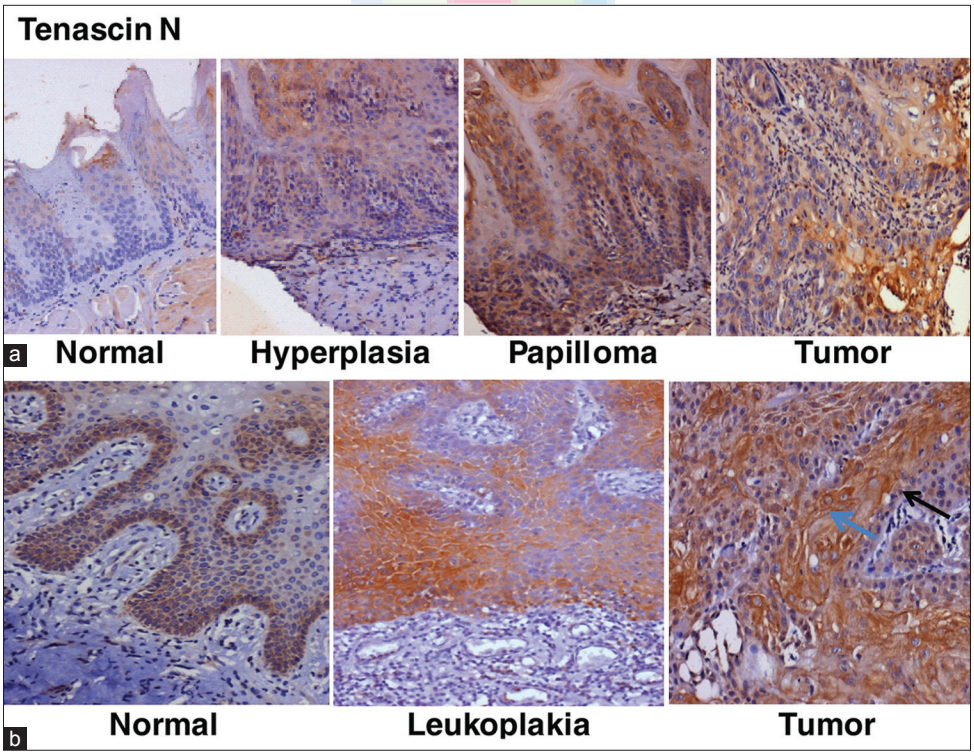


Figure 6: Validation of tenascin N (Tnn) by immunohistochemistry using specific antibody. (a) Representative photomicrographs showing immunohistochemical detection labeling of Tnn during rat lingual carcinogenesis (×200). (b) Representative photomicrographs showing immunohistochemical detection of Tnn in human normal, leukoplakia and tumor of tongue tissues. Arrows indicate the weak expression of Tnn in basal layer (black) of tumor while increased expression of tenascin N in differentiated layers (blue) (×200)

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.[35] It has been observed that forced expression of *Crnn* leads G1/S cell cycle arrest and a downregulation of cyclin D1 in OSCC.[36] It is considered as late differentiation marker of skin.[37] Due to unavailability of specific antibody for *Crnn* against rat, we validated our results of proteomics analysis using real-time quantitative PCR. Our proteomics and real-time data demonstrated marked and sequential downregulation of this protein [Figure 4d] and its messenger RNA (mRNA) in hyperplasia and papillomas and it was undetectable in tumors [Supplementary Figure 1]. Proteomics data revealed it's 14-fold downregulation in tumor as compared to normal. Real-time data revealed that *Crnn* downregulation is an early event in carcinogenesis. This indicates that *Crnn* might act as strong tumor suppressor.[35] Our data correlates with findings of Schaaij-Visser *et al*. in that *Crnn* expression was downregulated in mucosal epithelium at high risk of malignant transformation, when compared with normal oral mucosa.[38,39]

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. Our data underlines the importance of this model system for development of biomarkers. As stated earlier, we have also detected some of novel proteins whose differential expression in lingual carcinogenesis has not been documented in patients. A partial list of novel upregulated and downregulated proteins is given in Tables 4 and 5, respectively. Further, we have validated one novel upregulated protein in both rat and human systems. 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 novel over expressed protein.

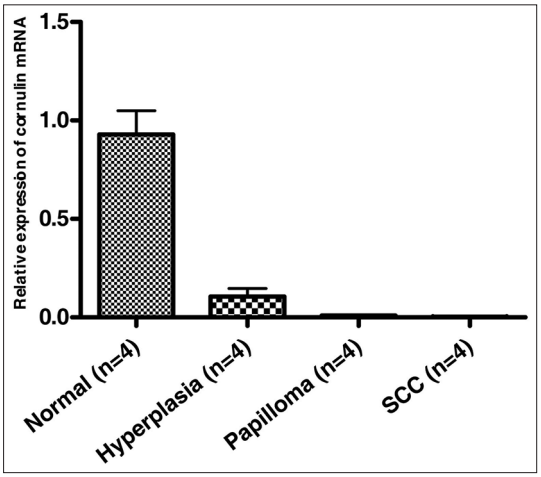
Validation of tenascin N, a novel protein in rat and human tongue tumerogenesis

Tenascin N

Tenascin is a high molecular weight extracellular matrix glycoprotein. Its expression was detected during embryogenesis, wound healing and neoplastic processes.[52] *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.[53] Our rat proteomics data demonstrated that *Tnn* was sequentially upregulated across the stages of rat lingual carcinogenesis and found to be upregulated by 2.5-fold in tumors as compared with normal tissues [Figure 4f]. To validate our proteomics results, we performed IHC on rat tissues [Figure 6a]. *Tnn* expression was not seen in the vehicle treated rat tissues (control groups) while hyperplasia tissues showed weak cytoplasmic staining in keratinized layer of epithelium. *Tnn* expression was also confined to keratinized layer in papillomas and carcinomas. Carcinomas showed higher expression of *Tnn* as compared to papillomas and hyperplastic tissues. We further validated *Tnn* expression in human tongue tissues [Figure 6b]. Immunohistochemical staining on human tissues revealed strong basal layer and cytoplasmic expression of *Tnn* in normal tissues (12/14) while upregulation was noticed in leukoplakia (9/10) in all layers. In human tongue tumors (27/32) *Tnn* was expressed in keratinized tumor cells, while its basal cell expression was weak [Figure 6b]. Strong cytoplasmic staining was detected in tumor cells. Intriguingly, *Tnn* was predominantly seen in keratinizing cells of the tumor tissues and basal layer shows very weak expression. The significance of this finding is unclear.

CONCLUSIONS AND FUTURE PERSPECTIVE

This is the most extensive quantitative proteomic study in rat model of 4NQO-induced oral carcinogenesis carried out until date. We successfully validated several known proteins like *Vim*, *Fscn1*, Tgm3, *Postn* and *Crnn*, and a novel molecule, *Tnn*, based on our proteomics findings. Using this model, we are able to show sequential alterations in expression pattern during rat tongue carcinogenesis. Furthermore, we are also able to extrapolate our rat model data to human system indicating the fact that this model has potential to be used for biomarker discovery in human oral cancer. We plan to take up validation of novel proteins on a large scale on human



Supplementary Figure 1: Real-time validation of cornulin expression during different stages of lingual carcinogenesis. Relative cornulin (Crnn) messenger ribonucleic acid (mRNA) expression during rat lingual carcinogenesis; Crnn mRNA is sequentially downregulated during rat lingual carcinogenesis and Crnn downregulation is an early event during the carcinogenesis

Table 4: Partial list of novel upregulated proteins in rat lingual carcinogenesis

Gene symbol	Protein name	Biological features	Fold change (tumor/normal) in this study
Thbs2	Thrombospondin 2 precursor	TSP are secreted multidomain glycoproteins. They are involved in various functions including modulating cell adhesion, proliferation, migration, and angiogenesis. They regulate cell proliferation induced by rac1 redox-dependent Signaling. TSP-2 inhibits tumor growth and angiogenesis of human SCCs[40]	6.9
Psmb10	Proteasome subunit beta type-10	It is a core part of the 26S proteasome complex, which is an important protein degrading system. Psmb10 gene is regulated by interferon-gamma[41]	6.2
S100a9	Protein S100-A9	It is a calcium-and zinc-binding protein and plays an important role in the regulation of inflammatory processes and immune response. It hetromerises with S100A 8 (calprotectin) and performs various intra and extracellular functions. It is strongly upregulated in many cancers including gastric, esophageal, colon, pancreatic, bladder, ovarian, thyroid, breast, and skin cancers[42]	5.2
Lrg1	Leucine-rich alpha-2-glycoprotein	It belongs to LRR family of proteins and is involved in protein-protein interactions, signal transduction, and cell adhesion and development. It is expressed during granulocyte differentiation[43]	5.0
Lgmn	Legumain precursor	It has specificity for hydrolysis of asparaginyl bonds. It may be involved in the processing of proteins for MHC class II antigen presentation in the lysosomal/endosomal system[44]	4.9
Car12	Carbonic anhydrase 12	Carbonic anhydrases are a large family of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide.[45] It is sero-diagnostic markers for lung cancer. It is also over expressed in OSCC and correlates with poor prognosis of patients.[46] It is known to be over expressed in different human cancers, such as diffuse astrocytomas, colorectal, gastrointestinal, breast, pancreatic, ovarian, and renal carcinomas	2.5
Vtn	Vitronectin	It belongs to the pexin family of proteins. It is found in serum and tissues and promotes cell adhesion and spreading.[15] It also binds to several serpin serine protease inhibitors	3.5
Hk3	Hexokinase-3	Hexokinases transfer a phosphoryl moiety from ATP to the 6-hydroxyl of glucose to produce G6P. Hexokinases I, II, and III are referred to as "low-Km" isozymes because of a high affinity for glucose even at low concentrations. HK3 also protects against cell death, and its overexpression increases ATP levels, decreases the oxidant-induced production of reactive oxygen species, attenuates the oxidant-induced reduction in mitochondrial membrane potential, and increases mitochondrial biogenesis[47]	2.0

TSP: Thrombospondins; SCC: Squamous cell carcinoma; LRR: Leucine-rich repeat; MHC: Major histocompatibility complex; OSCC: Oral squamous cell carcinoma; G6P: Glucose-6-phosphate; ATP: Adenosine triphosphate

Table 5: Partial list of novel downregulated proteins in rat lingual carcinogenesis

Gene symbol	Protein name	Biological features	Fold change (tumor/normal) in this study
Tchh	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. It may be involved in its own calcium-dependent postsynthetic processing during terminal differentiation[48]	14
Ocm	Oncomodulin	Oncomodulin is a small, ~12 kDa calcium-binding protein in the parvalbumin family. Oncomodulin, an apparently tumor-specific calcium-binding protein, has been detected in many chemically induced rat hepatomas[49]	5
Car3	carbonic anhydrase 3	It is abundantly present in skeletal muscle, adipocytes, and liver. Carbonic anhydrase III protects cells from hydrogen peroxide-induced apoptosis and its expression promotes metastasis of oral cancer via MMP2 expression[50]	5
Krt23	Keratin, type I cytoskeletal 23	Keratin 23 belongs to the acidic type I keratins. K23 was identified as a tumor-associated antigen in sera from patients with hepatocellular carcinoma. K23 is strongly upregulated in colon adenocarcinomas and in pancreatic cancer upon HDACi treatment[51]	10

MMP2: Matrix metalloproteinase-2; HDACi: Histone deacetylase inhibitor

tissues. Therefore, we are in the process of collecting SCC of tongue samples at different stages that is, from T1 to T4. We are also in the process of collecting more leukoplakia of tongue samples. Our ultimate aim is to carry out sequential analysis, so as to establish these proteins as predictive markers for human oral cancer.

REFERENCES

1. Pisani P, Bray F, Parkin DM. Estimates of the world-wide prevalence of cancer for 25 sites in the adult population. Int J Cancer 2002;97:72-81.
2. Sankaranarayanan R, Black RJ, Swaminathan R, Parkin DM. An overview of cancer survival in developing countries. IARC Sci Publ 1998;145:135-73.
3. Hunter KD, Parkinson EK, Harrison PR. Profiling early head and neck cancer. Nat Rev Cancer 2005;5:127-35.

4. Reibel J. Prognosis of oral pre-malignant lesions: Significance of clinical, histopathological, and molecular biological characteristics. Crit Rev Oral Biol Med 2003;14:47-62.
5. Kanojia D, Sawant SS, Borges AM, Ingle AD, Vaidya MM. Alterations in keratins and associated proteins during 4-Nitroquinoline-1-oxide induced rat oral carcinogenesis. J Carcinog 2012;11:14.
6. Kanojia D, Vaidya MM. 4-nitroquinoline-1-oxide induced experimental oral carcinogenesis. Oral Oncol 2006;42:655-67.
7. McDonald WH, Yates JR 3rd. Shotgun proteomics and biomarker discovery. Dis Markers 2002;18:99-105.
8. Pawar H, Kashyap MK, Sahasrabuddhe NA, Renuse S, Harsha HC, Kumar P, *et al*. Quantitative tissue proteomics of esophageal squamous cell carcinoma for novel biomarker discovery. Cancer Biol Ther 2011;12:510-22.
9. Wang Z, Feng X, Liu X, Jiang L, Zeng X, Ji N, *et al*. Involvement of potential pathways in malignant transformation from oral leukoplakia to oral squamous cell carcinoma revealed by proteomic analysis. BMC Genomics 2009;10:383.
10. Ralhan R, Desouza LV, Matta A, Chandra Tripathi S, Ghanny S, Dattagupta S, *et al*. iTRAQ-multidimensional liquid chromatography and tandem mass spectrometry-based identification of potential biomarkers of oral epithelial dysplasia and novel networks between inflammation and premalignancy. J Proteome Res 2009;8:300-9.
11. Tanaka T, Ishigamori R. Understanding carcinogenesis for fighting oral cancer. J Oncol 2011;2011:603740.
12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75.
13. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C (T) method. Nat Protoc 2008;3:1101-8.
14. Booth DR. A relationship found between intra-oral sites of 4NQO reductase activity and chemical carcinogenesis. Cell Tissue Kinet 1990;23:331-40.
15. Bijian K, Mlynarek AM, Balys RL, Jie S, Xu Y, Hier MP, *et al*. Serum proteomic approach for the identification of serum biomarkers contributed by oral squamous cell carcinoma and host tissue microenvironment. J Proteome Res 2009;8:2173-85.
16. Mendez MG, Kojima S, Goldman RD. Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition. FASEB J 2010;24:1838-51.
17. Dal Vechio AM, Giudice FS, Sperandio FF, Mantesso A, Pinto Junior Ddos S. Vimentin expression and the influence of Matrigel in cell lines of head and neck squamous cell carcinoma. Braz Oral Res 2011;25:235-40.
18. Hu S, Arellano M, Boontheung P, Wang J, Zhou H, Jiang J, *et al*. Salivary proteomics for oral cancer biomarker discovery. Clin Cancer Res 2008;14:6246-52.
19. Kokkinos MI, Wafai R, Wong MK, Newgreen DF, Thompson EW, Waltham M. Vimentin and epithelial-mesenchymal transition in human breast cancer – Observations *in vitro* and *in vivo*. Cells Tissues Organs 2007;185:191-203.
20. Sawant S, Vaidya M, Chaukar D, Alam H, Dmello C, Gangadaran P, *et al*. Clinical significance of aberrant vimentin expression in oral premalignant lesions and carcinomas. Oral Dis 2013;DOI. 10.1111/odi. 12151.
21. Chaw SY, Majeed AA, Dalley AJ, Chan A, Stein S, Farah CS. Epithelial to mesenchymal transition (EMT) biomarkers - E-cadherin, beta-catenin, APC and Vimentin - In oral squamous cell carcinogenesis and transformation. Oral Oncol 2012;48:997-1006.
22. Yamashiro S, Yamakita Y, Ono S, Matsumura F. Fascin, an actin-bundling protein, induces membrane protrusions and increases cell motility of epithelial cells. Mol Biol Cell 1998;9:993-1006.
23. Darnel AD, Behmoaram E, Vollmer RT, Corcos J, Bijian K, Sircar K, *et al*. Fascin regulates prostate cancer cell invasion and is associated with metastasis and biochemical failure in prostate cancer. Clin Cancer Res 2009;15:1376-83.
24. Alam H, Bhate AV, Gangadaran P, Sawant SS, Salot S, Sehgal L, *et al*. Fascin overexpression promotes neoplastic progression in oral squamous cell carcinoma. BMC Cancer 2012;12:32.
25. Shimamura Y, Abe T, Nakahira M, Yoda T, Murata S-i, Sugasawa M: Immunohistochemical analysis of oral dysplasia: diagnostic assessment by fascin and podoplanin expression. *Acta histochemica et cytochemica* 2011, 44:239.
26. Hamilton DW. Functional role of periostin in development and wound repair: Implications for connective tissue disease. J Cell Commun Signal 2008;2:9-17.
27. Kudo Y, Ogawa I, Kitajima S, Kitagawa M, Kawai H, Gaffney PM, *et al*. Periostin promotes invasion and anchorage-independent growth in the metastatic process of head and neck cancer. Cancer Res 2006;66:6928-35.
28. Kashyap MK, Marimuthu A, Peri S, Kumar GS, Jacob HK, Prasad TS, *et al*. Overexpression of periostin and lumican in esophageal squamous cell carcinoma. Cancers (Basel) 2010;2:133-42.
29. Kyutoku M, Taniyama Y, Katsuragi N, Shimizu H, Kunugiza Y, Iekushi K, *et al*. Role of periostin in cancer progression and metastasis: Inhibition of breast cancer progression and metastasis by anti-periostin antibody in a murine model. Int J Mol Med 2011;28:181-6.
30. Greenberg CS, Birckbichler PJ, Rice RH. Transglutaminases: Multifunctional cross-linking enzymes that stabilize tissues. FASEB J 1991;5:3071-7.
31. John S, Thiebach L, Frie C, Mokkapati S, Bechtel M, Nischt R, *et al*. Epidermal transglutaminase (TGase 3) is required for proper hair development, but not the formation of the epidermal barrier. PLoS One 2012;7:e34252.
32. Gonzalez HE, Gujrati M, Frederick M, Henderson Y, Arumugam J, Spring PW, *et al*. Identification of 9 genes differentially expressed in head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 2003;129:754-9.
33. Ohkura S, Kondoh N, Hada A, Arai M, Yamazaki Y, Sindoh M, *et al*. Differential expression of the keratin-4,-13,-14,-17 and transglutaminase 3 genes during the development of oral squamous cell carcinoma from leukoplakia. Oral Oncol 2005;41:607-13.
34. Contzler R, Favre B, Huber M, Hohl D. Cornulin, a new member of the "fused gene" family, is expressed during epidermal differentiation. J Invest Dermatol 2005;124:990-7.
35. Pawar H, Maharudraiah J, Kashyap MK, Sharma J, Srikanth SM, Choudhary R, *et al*. Downregulation of cornulin in esophageal squamous cell carcinoma. Acta Histochem 2013;115:89-99.
36. Imai FL, Uzawa K, Nimura Y, Moriya T, Imai MA, Shiiba M, *et al*. Chromosome 1 open reading frame 10 (C1orf10) gene is frequently down-regulated and inhibits cell proliferation in oral squamous cell carcinoma. Int J Biochem Cell Biol 2005;37:1641-55.
37. Liedén A, Ekelund E, Kuo IC, Kockum I, Huang CH, Mallbris L, *et al*. Cornulin, a marker of late epidermal differentiation, is down-regulated in eczema. Allergy 2009;64:304-11.
38. Schaaij-Visser TB, Bremmer JF, Braakhuis BJ, Heck AJ, Slijper M, van der Waal I, *et al*. Evaluation of cornulin, keratin 4, keratin 13 expression and grade of dysplasia for predicting malignant progression of oral leukoplakia. Oral Oncol 2010;46:123-7.
39. Chen K, Li Y, Dai Y, Li J, Qin Y, Zhu Y, *et al*. Characterization of tumor suppressive function of cornulin in esophageal squamous cell carcinoma. PLoS One 2013;8:e68838.
40. Streit M, Riccardi L, Velasco P, Brown LF, Hawighorst T, Bornstein P, *et al*. Thrombospondin-2: A potent endogenous inhibitor of tumor growth and angiogenesis. Proc Natl Acad Sci U S A 1999;96:14888-93.
41. Wu X, Yang SL, Yerle M, Zhu ZM, Wang HL, Wang H, *et al*. Genomic organization, localization and polymorphism of porcine PSMB10, a gene encoding the third beta-type proteasome subunit of 26S proteasome complex. J Anim Breed Genet 2006;123:331-6.
42. Srikrishna G. S100A8 and S100A9: New insights into their roles in malignancy. J Innate Immun 2012;4:31-40.
43. O'Donnell LC, Druhan LJ, Avalos BR. Molecular characterization and expression analysis of leucine-rich alpha2-glycoprotein, a novel marker of granulocytic differentiation. J Leukoc Biol 2002;72:478-85.
44. Chen JM, Dando PM, Rawlings ND, Brown MA, Young NE, Stevens RA, *et al*. Cloning, isolation, and characterization of mammalian legumain, an asparaginyl endopeptidase. J Biol Chem 1997;272:8090-8.
45. Kobayashi M, Matsumoto T, Ryuge S, Yanagita K, Nagashio R, Kawakami Y, *et al*. CAXII Is a sero-diagnostic marker for lung cancer. PLoS One 2012;7:e33952.
46. Chien MH, Ying TH, Hsieh YH, Lin CH, Shih CH, Wei LH, *et al*. Tumor-associated carbonic anhydrase XII is linked to the growth of primary oral squamous cell carcinoma and its poor prognosis. Oral Oncol 2012;48:417-23.
47. Wyatt E, Wu R, Rabeh W, Park HW, Ghanefar M, Ardehali H. Regulation and cytoprotective role of hexokinase III. PLoS One 2010;5:e13823.
48. Presland RB, Dale BA. Epithelial structural proteins of the skin and oral cavity: Function in health and disease. Crit Rev Oral Biol Med 2000;11:383-408.

49. Durkin JP, Brewer LM, MacManus JP. Occurrence of the tumor-specific, calcium-binding protein, oncomodulin, in virally transformed normal rat kidney cells. Cancer Res 1983;43:5390-4.
50. Räisänen SR, Lehenkari P, Tasanen M, Rahkila P, Härkönen PL, Väänänen HK. Carbonic anhydrase III protects cells from hydrogen peroxide-induced apoptosis. FASEB J 1999;13:513-22.
51. Birkenkamp-Demtröder K, Hahn SA, Mansilla F, Thorsen K, Maghnouj A, Christensen R, *et al*. Keratin23 (KRT23) knockdown decreases proliferation and affects the DNA damage response of colon cancer cells. PLoS One 2013;8:e73593.
52. Mukaratirwa S, Nederbragt H. Tenascin and proteoglycans: The role of tenascin and proteoglycans in canine tumours. Res Vet Sci 2002;73:1-8.
53. Neidhardt J, Fehr S, Kutsche M, Löhler J, Schachner M. Tenascin-N: Characterization of a novel member of the tenascin family that mediates neurite repulsion from hippocampal explants. Mol Cell Neurosci 2003;23:193-209.

How to cite this article: Soni BL, Marimuthu A, Pawar H, Sawant SS, Borges A, Kannan R, *et al*. Quantitative proteomic analysis of different stages of rat lingual carcinogenesis. Clin Commun Oncol 2014;1:2.

Source and Support: None. **Conflict of Interest:** None declared.

AUTHOR'S PROFILE

Bihari Lal Soni: TMC-Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Kharghar, Navi-Mumbai, Maharashtra, India.

Dr. Arivusudar Marimuthu: Institute of Bioinformatics, Discoverer building, International Technology Park Limited (ITPL), Whitefield, Bengaluru, Karnataka, India.

Harsh Pawar: Institute of Bioinformatics, Discoverer building, International Technology Park Limited (ITPL), Whitefield, Bangalore, Karnataka, India.

Dr. Anita Borges: Asian Institute of Oncology, S L Raheja Hospital, Mahim, Mumbai, Maharastra, India.

Ranganathan Kannan: Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai, Tamil Nadu, India.

Dr. Arvind D. Ingle: TMC-Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Kharghar, Navi-Mumbai, Maharashtra, India.

Dr. Hindahally Chandregowda Harsha: Institute of Bioinformatics, Discoverer building, International Technology Park Limited (ITPL), Whitefield, Bangalore, Karnataka, India.

Dr. Akhilesh Pandey: McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA, Department of Biological Chemistry, Department of Oncology, and Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD, USA.

Sharada S. Sawant: TMC-Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Kharghar Navi-Mumbai, Maharashtra, India.

Dr. Milind Murlidhar Vaidya: TMC-Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Kharghar, Navi-Mumbai, Maharashtra, India.