KERATIN PROFILE IN ORAL CANCER

By

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The 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 Amit G. Fulzele

August, 2013

I Dedicate this Thesis

To

The Mother Earth

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CONTENTS

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Ph. D. PROGRAMME

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SYNOPSIS

SYNOPSIS

Introduction:

Oral cancer is the fifteenth most common cancer worldwide and the second largest malignancy in the males in India [1]. The major factors responsible for incidence of oral cancer are smoking and/or chewing of the tobacco and heavy alcohol consumption. Keratins which are intermediate filament proteins are receiving intense attention in recent times due to their multifarious functions other than the role of providing structural stability to a cell and for their diagnostic potential.

Keratins are reported to be involved in regulating protein synthesis and epithelial cell growth [2] signaling [3], apoptosis [4], organelle transport [5-7] cell motility [8, 9], cell proliferation [10] and stress [11, 12]. There are 54 functional genes coding for type-I and type-II keratins, of which 28 are type-I and 26 are type-II keratins [13] which are expressed predominantly in epithelial cells. The epithelial keratins forms obligate heterodimers of one acidic (type I) and one basic (type II) keratin and exhibit tissue specific expression. Several recent, comprehensive and in-depth reviews have described the expression and role of specific keratins in different tissues [14-19]. *None of these reviews provide much information on the expression profile for the keratins in the head and neck region.*

Keratins have been used as biomarkers for diagnosis and prognosis in various cancers [18]. There are several independent reports on keratins from the head and neck region. Majority of these studies have used antibody based techniques such as immunohistochemistry without attention to subsites. A few proteomic and microarray

studies show the presence/expression of keratins in tissues from the head and neck region.

Keratin expression profile by immunohistochemistry:

K1:K10; K4:K13; K5:K14; K6:K15,K16,K17; K7:K19; K8:K18 form obligate pairs in epithelial cells [17, 19]. Analysis of the studies shows that in several of them the keratins have been reported using pan keratin antibodies, in-house antibodies and antibodies that recognize two keratins, thereby leaving an ambiguity about their expression [20-25].

Keratin expression by other methods:

Early studies by Vaidya *et. al*. [26] using 2DE and western blotting with rabbit polyclonal antibodies to all keratins have shown the presence of K4, K5, K13 and K14 in normal oral tissue and K1, K4, K14, K16, K17, K18 in oral cancer tissues. Lowered expression of K5 was reported in Leukoplakia, OSF, SCC of tongue and SCC of oral mucosa, and expression of K2, K4, K14 and K19 along with aberrant expression of K8/18 and reduced expression of K13 were also reported in oral cancer [26-29].

There are a few reports which use proteomic analyses of tissues from the head and neck region and show the differential expression or presence of keratins K1, K2, K4, K5, K6, K8, K9, K10, K13, K14, K15, K16, K17, K18 and K19 [30-35].

Genomic studies have shown the expression of K4, K13 and K15 expression in normal mucosa and its down regulation in tumor [36]. Odani *et. al.* has reported overexpression of K2 and K10 in oral leukoplakia as compared to SCC [37]. Toyoshima *et. al.* have shown the overexpression of K17 in OSCC as compared to normal mucosa [38].

Most of the studies have analyzed keratins in tissues from the head and neck region without due attention to subsites. The oral subsite, buccal mucosa is biologically and molecularly different from the tongue [39-46] which is also apparent from the K13 expression profile in tongue tissues in which K13 is overexpressed in tumor tissues [30]. Further, as the focus of the investigations was not on keratins some of the proteomic studies used tissue lysates in buffers in which keratins are not soluble. Studies reporting 2DE-mass spectrometry data have focused on regions below 50 kDa thereby overlooking the keratins.

Western blotting data from on-going studies in the laboratory investigating the status of K18 in GBC showed differential patterns in tumor and adjacent normal tissues indicating that this could be exploited as a marker. In initial studies to understand this differential pattern it became apparent that the antibody based observations were not supported by mass spectrometry indicating that there is a necessity for validation of antibody based identification of homologous proteins such as keratins by mass spectrometry. These observations and the review of literature lead to the aim of assessing the keratin profile in oral cancer using enriched preparations of keratins in normal and tumor tissues from a specific subsite of the oral cavity, the gingivo buccal complex, using mass spectrometry followed by validation of their expression by silver staining, western blotting, and immunohistochemistry with the following objectives:

Objectives:

1. *Identification of keratins in normal cut margin and tumor samples of cancer of the gingivo buccal complex by PMF- MS/MS of 1DE resolved proteins.*
2. *1DE-protein blotting with specific antibodies to keratins and post translational modifications and identification of isoelectric point /molecular weight of the keratins by 2DE-immunoblotting.*
3. *2DE-Silver staining and PMF-MS for localization and identification of keratins.*
4. *2DE-Silver staining pattern of keratins from normal and tumor tissue.*
5. *Confirmation of keratin expression by Immunohistochemistry.*
6. *Comparison of the expression pattern obtained by mass spectrometry, silver staining, western blotting and immunohistochemistry.*

Materials and Methods:

1. *Tissues*- The project was approved by the Ethics Committee of the Tata Memorial Centre. Adjacent normal, tumor and cut margin tissues were collected from patients undergoing surgery as part of their treatment for cancer of the ginigivo buccal complex after obtaining informed consent. Tissues were immediately frozen in liquid nitrogen for long term storage and for further use. The histology of the tissue samples was confirmed by the pathologist.

2. *Keratin enrichment and protein estimation*- Tissues were enriched for keratins using the Achtstaetter protocol [47]. Protein estimation was done by TCA precipitation modification of Peterson's method [48].

3. *Mass Spectrometry for 1DE and 2DE silver stained gels*- Enriched keratin preparations were resolved by 1DE or 2DE according to Laemmli [49] and stained with silver for mass spectrometry. 1DE gels containing separated silver stained proteins in the range of 72 kDa to 34 kDa were cut into small pieces. For 2DE the silver stained spots on the gel were cut out. The gel pieces were then destained; the proteins on 1DE gels were reduced using dithiothreitol and alkylated using iodoacetamide. The protein in the gel was subjected to in gel digestion with Trypsin. Reconstituted peptides were analyzed on the MALDI TOF-TOF Ultraflex-II from Brucker Daltonics, Germany, directly or after separation by liquid chromatography. Proteins identified from the gel bands/spots by mass spectrometry analysis were then assessed by immunoblotting and/or immunohistochemistry.

4. *1DE- and 2DE separation of enriched keratin preparations and immunoblotting with keratin specific and PTM specific antibodies*- Three microgram of enriched protein samples were resolved by 1DE. Thirty micrograms of protein was used for 2DE. IPG strips (17 cm, pH 4.7-5.9) were used for the separation of the keratins. Proteins from the gel were transferred to the PVDF membrane. The blotted enriched keratin proteins were probed with antibodies to keratins- K1, K4, K5, K6, K8, K10, K13, K14, K16, K17, K18 (CY90 and LDK 18) and against PTM's- Ubiquitin, O-GlcNAc, phosphoserine.

5. *Immunohistochemistry with keratin specific antibodies to differentiate the keratin expression pattern between normal and tumor tissues*- The expression pattern of the keratins obtained by mass spectrometry and verified by 2DE immunoblotting were further validated by IHC using antibodies to specific keratins. Tissues from normal

and tumor samples were fixed in formalin and embedded in paraffin blocks. Five um sections were probed with antibodies to the differentially expressed keratins K13, 14, 16 and K17 antibodies. Images were captured using Zeiss AxioImager Z1 microscope.

Results:

Keratin profile by 1DE-Mass spectrometry:

The enriched keratin preparations from fifteen sample sets (normal, tumor and cut-margin tissues) were resolved by 1DE and the proteins in the region 72 kDa to 45 kDa were identified by PMF-MS/MS. Data obtained shows that *Keratin 4 and 13 expression was confined to the normal and cut-margin tissues* and the keratin was not seen in the tumor tissues. *Keratins 14, 16 and 17 were identified predominantly in the tumor tissues and few normal/cut-margin tissues.* The expression of K1, K2, K5, K6A, K6B, K6C and K10 was observed in normal/cut margin as well as in tumor tissue samples to almost the same extent. The s*amples analyzed did not show the identity for K8 and K18.* The differential keratin expression pattern was suggestive of the molecular changes which are associated with transformation from normal to tumor cells of the cancer of the GBC.

*Evaluation of the keratin expression profile by 1DE-immunostaining***:**

Enriched keratin preparations from the fifteen sample sets were resolved by 1DE and the proteins were blotted onto the PVDF membrane. The blots were probed with antibodies specific to keratin 1, 4, 5, 6, 8, 10, 13, 14, 16, 17 and 18. Mass spectrometry results identified keratin 13 expression only in normal tissue samples and the same was confirmed by using keratin 13 specific antibody. Keratin 14, 16 and 17 expression was

observed in more tumor tissues as compared to the normal/cut-margin tissues. Antibodies to K18 (CY90) and K18 (LDK 18) also showed positive signal in the normal and tumor samples. *Mass spectrometry had not shown the presence of K8 and K18 but signals were obtained for these keratins by immunostaining.* Post translational modifications of the keratins were evaluated using specific antibodies to Ubiquitin, Phosphoserine and O-GlcNAc specific antibodies. Ubiquitination and phosphorylation was seen mainly in the tumor samples. Glycosylation was specifically observed at the ~48kDa band position which was also detected by K13 antibodies.

2DE- Mass spectrometry for identification of keratins with their respective molecular weight and isoelectric point:

To verify the data obtained by 1DE mass spectrometry and western blotting and to determine the molecular weight, isoelectric point and the relative position of the keratins 2DE-Mass spectrometry was done. Enriched keratin preparations from two sample sets consisting of normal and cancer tissue and the cut margins from same were resolved by 2DE, the gel was silver stained and the protein spots were analyzed by PMF-MS-MS. Keratin 13 was identified in normal samples at ~48kDa, pI 4.9 position. Keratin 14, 16 and 17 were identified in tumor tissue samples. K18 was not seen by 2DE-PMF-MS.

2DE- immunostaining for identification of keratins with their respective molecular weight and isoelectric point:

The results obtained by 2DE mass spectrometry were also confirmed by immunoblotting with K13, K14, K16 and K17 antibodies. The results obtained, confirmed the identity of keratins 13, 14, 16, and 17 with their respective molecular weight position and the isoelectric point.

The blots were also probed with antibodies to K18 using clone CY90 from Sigma-Aldrich, USA. A signal overlapping the position of K13 was obtained. To confirm that the observation on western blots was not due to CY-90 from Sigma, the antibody from the same clone from another manufacturer Abcam was used and same pattern of staining was obtained. Immunostaining blots with an antibody LDK18, to the C-terminal end of K18 did not give a signal at the position detected by antibody to K13 and clone CY90 to K18, indicating that clone CY90 cross reacts with K13.

To confirm that K18 was entirely absent in the spots recognized by anti K13 and CY90, enriched keratins from another sample were resolved by 2DE and the silver stained spots corresponding to the K18 signal at position ~48 kDa were subjected to trypsin digestion. The eluted peptides were resolved by liquid chromatography and analyzed by mass spectrometry. Only keratin 13 was detected and K18 identity was not obtained. Breast cancer cells (MDA MB 468) are known to express K18 and not K13. The reactivity of antibody to CY90 to K18 was also evaluated by immunostaining of blots of breast cancer cells and breast cancer tissue protein lysates resolved by 2DE. Keratin 18 (CY90) antibody detected K18 at 45 kDa as did LDK 18 which is an antibody to K18 detecting an epitope at the C-terminal end of K18. The identity of the detected spots was confirmed by mass spectrometry.

To determine if CY90 can detect K18 in the presence of K13, breast cancer cells and breast cancer tissue lysates were mixed with enriched keratin preparation from the GBC tissue samples analyzed above. The mixed protein lysates were resolved by 2DE, the proteins blotted and probed with CY90. The antibody detected the upper band corresponding to K13 indicating that it recognizes K13 in the presence of K18. The antibody signals were validated by mass spectrometry of the spots detected from a corresponding silver stained gel.

The above observations emphasized the necessity to specifically verify the antigen recognized by the antibody using mass spectrometry. Keratins are abundant proteins in epithelial cells. They have conserved rod domains and show several epitope similarities with other members of the family. It is likely that specific monoclonal/polyclonal antibodies to other keratins may also show cross reactivity with family member proteins and caution is imperative in interpretation.

2DE- immunostaining for identification of the glycosylated keratin.

1DE-imunostaining with antibody to O-GlcNAc showed a band at 48 kDa. The keratin enriched samples were resolved by 2DE and resolved proteins transferred to PVDF membrane and probed with O-GlcNAc specific antibody. The ~48kDa band which was immunoreactive for the O-GlcNAc antibody was also immunoreactive to the K13 antibody suggesting that K13 was glycosylated. *This is a first report for glycosylation of K13 in oral tissues.*

2DE- silver staining pattern for keratins from normal/cut-margin and tumor tissues:

The enriched keratin preparations from fifteen samples were resolved by 2DE, the gels were stained and keratin expression pattern was compared. There was a striking difference in the pattern of expression of the keratins between normal/cut-margin and tumor tissue samples. In particular, the protein spots for K13 at ~ 48 kDa were present in normal and cut margin tissues and absent from tumor samples. Similarly, very distinctive silver stained curved pattern below the K13 set of spots was seen for the tumor samples in which K14, 16 and 17 are located. Some of the cut margins exhibited a mixed profile with silver stained spots for K13 and the curved pattern indicating that although they were histologically normal there were molecular changes which are seen

in this 2DE silver pattern. This differential expression pattern could be used for determining early changes leading to transformation and for prognosis.

Immunohistochemistry with Keratin specific antibodies:

The keratins shown to be differentially expressed in normal and tumor tissue by mass spectrometry, western blotting and silver staining were assessed by immunohistochemistry in the same samples. Tissue sections were stained with keratin specific antibodies for K4, K13, 14, 16 and K17 and the colour developed with the Vectastain universal ABC kit. K4 and K13 staining was seen in normal tissues and the cut margins and is absent in tumor tissue sections. Keratin 14, 16 and 17 were found to be overexpressed in tumors as compared to normal tissue samples.

Comparison of the keratin profile obtained from the different techniques:

The keratin expression for the major differentiating keratins K13, K14, K16 and K17 in the samples used in the study was compared by H&E staining, the silver stained 2DE gel pattern, the signal obtained on the western blot, the IHC pattern along with the mass spectrometry data. The presence of the four keratins was validated by atleast three of the four techniques thereby confirming their definite presence and ability to distinguish between normal and transformed epithelial tissues from cancer of the gingivo buccal complex.

***Discussion*:**

Keratins are important proteins regulating several aspects of the cellular physiology. Their utility in the clinics for cancer is receiving immense attention. These proteins belong to a very large family which are very homologous. Immunohistochemistry is

routinely used to evaluate their expression in normal and tumor tissues from several different organs. There are several reports evaluating keratin expression. These studies consider the head and neck region without attention to subsites. The results emerging are therefore contradictory. To identify a well characterised set of keratins from the gingivo-buccal complex, the work in this thesis describes the evaluation of keratins in normal tissues, the cut margins and tumor tissues from cancer of the GBC using enriched preparations of keratins followed by 1DE- mass spectrometry. Keratin 1, K2, K4, K5, K6, K10, K13, K14, K16, K17 were identified. Of these keratins K4 and K13 were most often seen only in normal and/ or cut margin tissues and K14, K16 and K17 were seen in tumor tissues. Keratins 8 and 18 were not seen in any of the samples analyzed.

2DE followed by silver staining and mass spectrometry of the spots confirmed the presence of the keratins identified by 1DE-mass spectrometry. The keratin pattern seen on the silver stained gel was reflective of the histologically normal and transformed state of the tissue and a similar presentation has not been reported earlier. 2DE immunoblotting study confirmed the antibody based keratin identity with respect to molecular weight and isoelectric point of the keratins. The data in this thesis also shows that a commonly used antibody to keratin 18 detects K13 thereby recommending use of mass spectrometry to determine the identity of the protein recognized by the antibody prior to its use in antibody based techniques such as immunohistochemistry.

Immunohistochemistry confirmed the keratin expression pattern. The intracellular localization of the keratins 4, 13, 14, 16 and 17 was similar to that reported in literature. Specific observations are the down-regulation of K4 and K13 and over expression of K14, 16 and 17 in tumor tissue. The aberrant expression of these keratins 4, 13, 14, 16, and 17 may affect the tissue architecture, arrangement of the epithelial cells in the

transformed tissues, contribute to the invasiveness of the cancers and affect the structural and mechanical properties of the cell as well as their role in cell cycle and apoptosis respectively..

The keratin signature obtained by mass spectrometry and validated by 2DE, western blotting and IHC provides markers to distinguish the normal tissue from the non transformed area. The early disappearance of K4 and K13 and appearance of K14, K16, and K17 as seen in the cut margins indicate that the histologically normal tissue has already acquired molecular characteristics of the tumor. As cut margins are used to identify limits of surgical resection, evaluation of the keratin profile could be used to advantage by the clinician to monitor and/or reduce recurrence in patients. Studies to understand the mechanisms regulating the expression of these keratins will provide clues to the transformation process. The combination of K14, 16 and K17 could be tested as markers for identification of micro-metastasis in the cervical lymph nodes. Follow-up of the patient history and its clinical correlation with above findings can be instrumental for avoiding recurrence in the cancer patients.

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Doctoral Committe

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ABBREVIATIONS

1DE : One-dimensional polyacrylamide gel electrophoresis

2DE : Two-dimensional polyacrylamide gel electrophoresis

ACN : Acetonotrile

CHCA : Cyano Hydroxy Cinnamic acid

CM : Cut Margin

cm : centimeter

DTT : Dithiothreitol

ECL : Enhanced chemiluminescence

EDTA : Ethylene diamine tetra-acetic acid

GBC : Gingivo buccal complex

gm : gram

H & E : Haematoxylin and Eosin

HRP : Horseradish Peroxidase

IEF : Iso-Electric Focusing

IHC : Immunohistochemistry

IPG : Immobilized pH Gradient

K : Keratin

kDa : kilo Dalton

MALDI : Matrix-assisted laser desorption ionization

MD : Moderately Differentiated

mm : millimeter

mM : milliMolar

MS : Mass Spectrometry

MW : Molecular Weight

N : Histological Normal

NA : Not Available

nm : nanometer

No : No (Not identified/Not detected)

O-GlcNAc : O-linked N-Acetyl glucosamine

OSCC : Oral Squamous Cell Carcinoma

PAGE : Polyacrylamide gel electrophoresis

PBS : Phosphate Buffered Saline

PD : Poorly Differentiated

pI : Isoelectric point

PMF : Peptide Mass Fingerprinting

PMSF : Phenylmethanesulphonyl fluoride

PTM's : Post Translational Modifications

PVDF : Polyvinylidene difluoride

SCC : Squamous Cell Carcinoma

SDS : Sodium dodecyl sulfate

T : Tumor

TEMED : N, N, N', N' – Tetramethylene diamine

TFA : Trifluoro-acetic acid

WB : Western Blot

WD : Well Differentiated

Y : Yes (Identified/Detected)

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PREAMBLE

Cancer is a disease in which a group of cells display uncontrolled growth beyond normal limits, invasion that intrudes upon and destroys adjacent tissues, and sometimes metastasis, which spreads the cells to other locations in the body via lymph or blood. Only 5–10% of all cancer cases can be attributed to genetic defects, whereas the remaining 90–95% has their roots in the environment and lifestyle. The lifestyle factors include cigarette smoking, diet (fried foods, red meat), alcohol, sun exposure, environmental pollutants, infections, stress, obesity, and physical inactivity. Oral cancer is the fifteenth most common cancer worldwide and the second largest malignancy in the males in India.

Several biomarkers such as EGFR, bcl-2, beta 2-microglobulin, CD44, CD80, CD105, cathepsin-D, p53 and 14-3-3 sigma have been reported in literature for oral cancer.

Keratins are intermediate filament proteins. They play a major role in several cellular functions. Each tissue type expresses a specific set of keratins. In recent years the immense potential of keratins as diagnostic and prognostic markers for different cancers is emerging. However, comprehensive information on the profile of keratins in the head and neck region is not available. Several independent reports have identified keratins using only antibody based techniques like immunohistochemistry (IHC), immunofluorescence (IF), and immunoblotting which have pitfalls due to the cross reactivity of the antibodies to this set of very homologous proteins. A few recent proteomic studies have reported the identification of keratins in head and neck cancer. Majority of the studies have used tissues from the head and neck region without specifying subsites. This study reports the analysis of enriched preparations of keratins from cancer of the gingivo buccal complex (GBC) using mass spectrometry, 2DE, western blotting, silver staining of 2DE gels and IHC. It provides a well characterized

profile of keratins in normal and tumor tissues from the gingivo buccal complex with potential for use in the clinics.

The **Introduction** provides an overview of cancer, oral cancer and keratins and the lacunae in literature leading to the objectives. The **Materials and Methods** section covers reagents, instruments and experimental procedures used in the study. The data obtained are given in the **Result** section which is divided into subsections as per the objectives. The **Discussion** section presents the data obtained in this work in the light of literature and gives a take home message regarding the keratin expression profile in cancer of the gingivo buccal complex with a precautionary note for the use of antibody based methods in the study of very homologous proteins such as the keratins.

Chapter 1

INTRODUCTION

Chapter 1. INTRODUCTION

1.1 Oral Cancer

Overview:

Oral cancer is the fifteenth most common cancer worldwide and the second largest malignancy in the males in India [1] and is more common in less developed countries [50, 51]. Tobacco chewing is associated with the high risk for oral cancer incidence. Tobacco and heavy alcohol consumption increases the risk for the cancer. HPV is associated with cancer of the oropharynx.

Ninety five percent of the oral cancers are squamous cells carcinoma arising from the epithelial cells. Oral cancer is classified into different stages depending on the size of the tumor, its nodal involvement and extent of metastasis referred to as TNM classification.

The major problem in the treatment of the oral cancer is that most patients present at late stage due to lack of awareness regarding the symptoms particularly in less developed and developing countries like India.

Signs and symptoms of oral cancer:

The major reason for the incidence of oral cancer is that the early stage cancer may not have detectable symptoms. Smokers and drinkers are at greater risk for oral cancer. Typical signs and symptoms for oral cancer includes patches on the lining of the mouth or tongue, usually red or red and white in color, mouth ulcers that do not go away, a sore that does not heal, a swelling in the mouth that persists for over three weeks, a lump or thickening of the skin or lining of the mouth, pain during swallowing of food, loosening teeth (tooth) for no clear reason, jaw pain are the few reasons to name.

Epidemiology:

The average incidence of lip and oral cavity cancers worldwide in males is 5.2 per 100,000 and among females, it is 2.5 per 100,000 [1]. Lip and oral cavity cancer is more prevalent in less developed countries as compared to developed countries and the reason for this bias could be due to the life style, access to medical amenities, eating habits, lack of public education etc. In India, for year 2010, the age-standardised cancer mortality rate per 100 000 individuals for lip, oral cavity, and pharynx cancer have been found to be 22.1 for men and 14.7 for females [52]. The incidence rate for cancer of lip and oral cavity ranks second (only after lung cancer) for males and fourth for females in India [1].

Etiology:

The etiological factors responsible for oral cancer are very well documented in literature.

• **Smoking**- Smoking is a practice in which a substance, most commonly tobacco, is burned and the smoke is tasted or inhaled. Smoking is practised by the use of cigarettes, bidis, hookahs, vaporizers, and bongs. The major components of tobacco are alkaloids, with nicotine as the main compound (85-95% of total compounds) [53]. There are strong evidences to associate tobacco smoking with the oral, pharyngeal and oesophageal cancer [54-59]. In a study comprising of 247 oropharyngeal and 148 oral cavity cancer cases from the Population-Based Cancer Registry records and 260 controls randomly selected from a tobacco survey conducted in the Bhopal population, Dikshit *et. al*. [60] have reported 71.6% population-attributable risk per cent (PARP) for smokers for the development of oropharyngeal cancer.

• **Chewing tobacco**- Smokeless tobacco is consumed without burning the product, and can be used orally or nasally. Chewing tobacco can be classified as loose leaf (made

from cigar leaf tobacco that is air-cured, sweetened, and loosely packed), plug (made from heavier grades of tobacco leaves harvested from the top of the plant, immersed in a mixture of licorice and sugar and pressed into a plug), or twist (air-cured or fire-cured burley tobacco leaves, flavoured and twisted in form of a rope) [53]. Several studies have shown the association of chewing of tobacco with oral cancer in India [58, 60-62].

• **Using snuff**- Snuff is a product made from ground or pulverised tobacco leaves. Smokeless tobacco can also be consumed as a snuff which is either moist or dry. Sankaranarayanan *et. al.* [63] in a case-control study of cancer of the gingiva comprising of 187 cases and 895 hospital-based controls have shown significant positive associations of risk of cancer of the gingiva with use of snuff ($P<0.05$).

• **Alcohol consumption**- The study for the effect of alcohol consumption with a dose-response relationship among non-smokers [64-67] has shown that alcohol can be regarded as an individual risk factor for the development of oral cancer. The combined effect of alcohol consumption with the tobacco use can increase the risk of oral cancer.

• **HPV (human papillomavirus) infection**- Human papillomavirus (HPV) is a virus from the papillomavirus family that is capable of infecting humans. Like all papillomaviruses, HPVs establish productive infections only in keratinocytes of the skin or mucous membranes. The evidence for the association of the HPV in oral cancer was first reported by Scully et. al. [68, 69] by demonstrating the presence of viral nucleic acids. HPV is especially associated with cancers in immune-compromised persons. **Fig. 1** shows the diagrammatic summary of main OSCC risk factors.

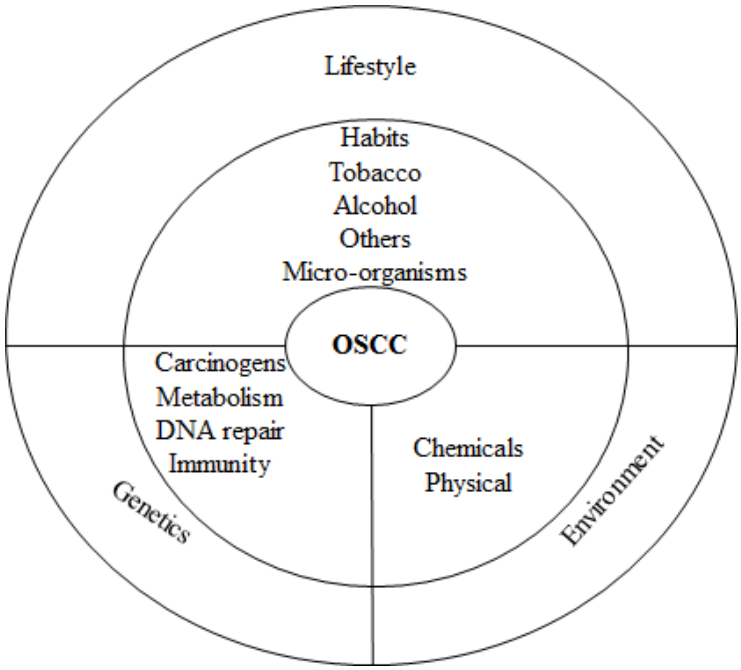


Figure 1. Diagrammatic summary of risk factors for OSCC. (Adapted and modified from C. Scully [70]).

Molecular biology

Oral cancer begins with an altered stem cell in the basal layer expanding rapidly and clonally to replace the successive upper layers of normal epithelia. Normal epithelium undergoes neoplastic progression through series of steps from hyperplasia to dysplasia to carcinoma in situ and invasive carcinoma [71]. The precise nature of the genetic alterations occurring at each step is evolving. Oral carcinogenesis requires cumulative gene alterations which deregulate and adaptively utilize the cell homeostatic mechanism. The genetic insult in the oral cancer may occur because of tobacco-associated intra-oral carcinogens, genetic susceptibility of individuals and external agents, such as alcohol, dietary factors and human papilloma virus (HPV) [72]. There are reports which show genetic instability in oral cancer such as loss of several areas in a chromosome. The frequently occurring alterations are loss of 3p, 4q, 5q21-22, 8p21-

23, 9p21-22, 11q13, 11q23, 13q, 14q, 17p, 18q and 22q [73-84]. These aberrations show the critical involvement of tumor suppressor genes such as p16 (9p21), APC (5q21-22), and p53 (17p13) in HNSCC.

Up-regulation of several oncogenes such as Epidermal growth factor receptor (EGFR) and its ligands [85], c-erb B-2 [86], int-2 (FGF-3) [87], hst-1 (FGF-4) [88], Cyclin D1 [89, 90], c-myc/N-myc [91], K-ras/N-ras [91, 92], Stat-3 [93] has been observed in the oral cancer. There are several tumor suppressor genes which are reported to be deregulated/down-regulated/inactivated/modified in oral cancer, some of which are p53 [94-96], CDKN2A [97, 98], MTS2 [99, 100] and p27 [101]. A comprehensive sequence of events from a normal cell to tumor has been described by Leemans *et al.* [102].

Oral Pre-cancerous lesions (OPL)

According to World Health Organization (WHO) definition, the precancerous lesion is defined as any morphological alteration in a tissue which makes it more susceptible to malignant transformation as compared to its adjacent apparently normal tissue. Many oral SCCs develop from premalignant conditions of the oral cavity [103, 104] which includes leukoplakia, erythroplakia, oral lichen planus, oral submucous fibrosis, discoid lupus erythematosus, and hereditary disorders such as dyskeratosis congenital and epidermolysis bullosa [105].

• **Leukoplakia:** The term leukoplakia describes a white patch or plaque that cannot be characterized clinically or pathologically as any other disease [106]. Leukoplakia occurs most often in middle-aged and older men and arises most frequently on the buccal mucosa, alveolar mucosa, and lower lip. Several studies have shown the probability of leukoplakia to turn into malignant transformation ranging from 0.16 % to 6% which rises to 14% when dysplasia is present [107].

• **Erythroplakia:** Erythroplakia is a clinical term used to describe a red patch that cannot be clinically or pathologically distinguished as any other definable disease. The occurrence of erythroplakia is not common as leukoplakia but it has a more chance to turn into dysplasia or carcinoma. Studies with the histological analysis have shown 51% of erythroplakic lesions to demonstrate invasive squamous cell carcinoma (SCC), with 40% demonstrating carcinoma in situ, and 9% exhibiting mild-moderate dysplasia [108].

• **Submucous Fibrosis:** Oral Submucous Fibrosis (or OSF) is a chronic, complex, irreversible, highly potent pre-cancerous condition characterized by juxta-epithelial inflammatory reactions and progressive fibrosis of the submucosal tissues. OSF is associated with areca nut chewing. Pindborg *et. al.* [109] have suggested that the submucous fibrosis as an important precancerous condition in Southeast Asia.

Subsites of the Oral Cavity

Oral cavity includes the lips, the front two-thirds of the tongue, the gums, the lining inside the cheeks and lips, the floor of the mouth under the tongue, the bony top of the mouth (hard palate), and the small area behind the wisdom teeth, the retromolar trigone.

1. **Tongue and Floor of the mouth**- The tongue has several divisions: The oral part that includes the front 2/3 that is considered to be in the mouth and the back 1/3 that sits in the throat and is called the base of tongue. The tongue and Floor of the mouth are the most common sites of origin for primary squamous cell carcinomas in the oral cavity in the western world. The predominant tumour of the tongue is squamous cell carcinoma, most of which are well differentiated.

2. **Gingivo buccal complex (GBC)-** comprises of buccal mucosa (buccal area is the inner lining of the cheeks), retromolar trigone, lower gingivo buccal sulcus and lower alveolus/gum (consists of the mucosal tissue that lies over the mandible inside the mouth). The cancer of this site is more prevalent in India as compared to western countries. In this study we have concentrated only on GBC subsite of the oral cancer. **Figure 2** shows the various sites of oral cavity.

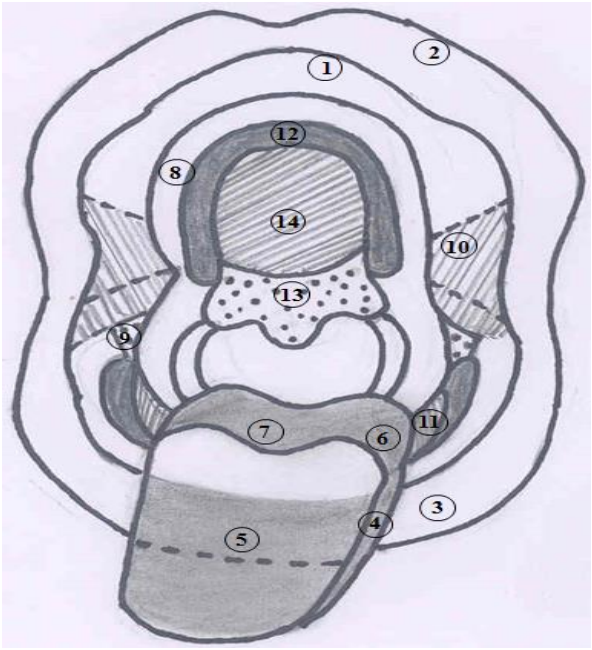


Figure 2. Schematic representation of subsites of the oral cavity. 1. lip-inner aspect, 2. external upper lip. 3. external lower lip, 4. border of tongue, 5. anterior two third tongue, 6. lingual tonsil, 7. base of tongue, 8. vestibule of mouth, 9. retromolar area, 10. cheek mucosa (buccal mucosa), 11. floor of mouth, 12. gum (gingiva), 13. soft palate, 14. hard palate.

Treatment modalities

Treatment of oral cancers is ideally a multidisciplinary approach involving the efforts of surgeons, radiation oncologists, chemotherapy oncologists, dental practitioners, nutritionists, and rehabilitation and restorative specialists. The commonly utilised

curative treatment modalities are usually surgery and radiation, with chemotherapy added to decrease the possibility of metastasis, to sensitize the malignant cells to radiation, or for those patients who have confirmed distant metastasis of the disease. The use of treatment modalities depends on the size, location, and stage of the primary tumour, the patient's ability to tolerate treatment, and the patient's consent.

1. **Surgery:** Surgery is often the first line treatment option for large tumors of the oral cavity.
2. **Radiotherapy**: Radiotherapy is the treatment of cancer with ionizing radiation. Ionizing radiation deposits energy that injures or destroys cells in the area being treated (the target tissue) by damaging the genetic material (DNA) in the individual cells, making it impossible for them to continue to grow. Conventional radiotherapy consists of one fraction daily for three to seven weeks with a total dose varying between 50 -70 Gy.
3. **Chemotherapy:** Chemotherapy is the use of chemicals that uses drugs to stop the growth of cancer cells, either by killing the cells or by stopping the cells from dividing. . Chemotherapy alone does not have an evidence of increased survival [110] but combination therapy with use of radiotherapy is the current treatment of choice in most cases. Several randomised trials have shown that combination of chemotherapy with radiotherapy reduces mortality by 11% [111]. The widely used chemotherapy agents are Cisplatin, 5-fluorouracil (5-FU), Carboplatin, Bleomycin, Methotrexate, and Docetaxel (Taxotere). Combination of drugs has often been found to more effective as compared to a single drug.

   Fig. 3 shows the pictorial presentation of treatment modalities and its complication.

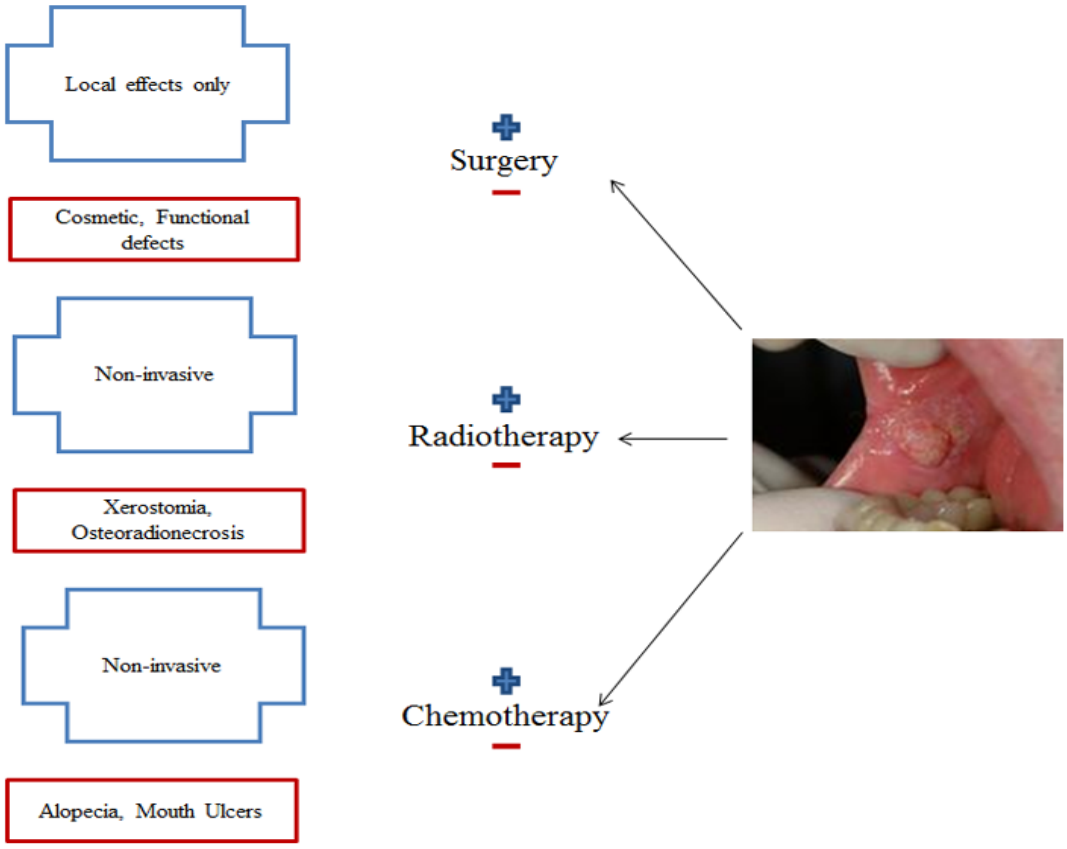


Figure 3. Treatment modalities for Oral Squamous cell carcinoma and the main complications. (Adapted and modified from C. Scully [70]).

1.2 Keratins

Overview

Intermediate filaments (IFs)

Intermediate filaments (IFs) are cytoskeletal components found in almost all vertebrates. Most types of intermediate filaments are cytoplasmic, but one type, the lamins, are nuclear. IFs have an average diameter of 10 nanometres and they are designated with the name 'intermediate' because their average diameter is between those of other cytoskeletal components narrower (7 nm) microfilaments and wider (25 nm) microtubules [112, 113].

IF proteins have been divided into six different types based on their characteristic molecular features. Type I to IV IF proteins form cytoplasmic IFs, type V IF proteins, the lamins, constitute a filamentous network inside the nuclear membrane [114] and type VI IF proteins, the nestins are expressed mostly in nerve cells. Type I IFs in humans comprises of epithelial acidic keratins (keratin's 9 to 28) and hair keratins (K31–K40) whereas type II group comprises of epithelial basic keratins (1–8 and 71–80) and hair keratins (K81–K86) [13]. Type III IFs comprises of i) desmin IFs- which are structural components of the sarcomeres in muscle cells, ii) GFAP (glial fibrillary acidic protein) – which are found in astrocytes and other glia, iii) peripherin – which are found in peripheral neurons and iv) Vimentin- which are most widely distributed of all IF proteins. Type IV IFs comprises of i) α-Internexin ii) neurofilaments iii) Synemin and iv) Syncoilin. Type V IFs comprises of lamins. Type VI IFs are the nestins which are expressed mostly in nerve cells where they are implicated in the radial growth of the axon.

Keratins

Keratins (also known as cytokeratin's) are the intermediate filament (IF) forming proteins of epithelial cells but there are reports suggesting keratin expression in muscle cells [115] and stromal cells in the Wharton's jelly [116]. They are heterogeneous in size (40–70 kDa) and charge (pI 4.7–8.4), are generally very inert, not easily disrupted by many drugs and are insoluble in commonly used buffers. There are 54 functional keratin genes of which 28 are type I (acidic) and 26 are type II (basic or neutral) keratins [13] and each of these are expressed in a specific defined sequence during differentiation. The 28 type I keratin genes except one are organized as a cluster on the long arm of human chromosome 17 (mouse chromosome 11), whereas all 26 type II keratin genes along with the type I K18 gene are clustered on the long arm of human chromosome 12 (mouse chromosome 15). Keratin expression pattern is unique for different epithelia and therefore it has been used in identification of various carcinomas. Keratins are also associated with various diseases collectively called as "**keratinopathies**" which includes Epidermolysis Bulbosa Simplex (EBS) [117], White sponge nevus (WSN) [118-120], Familial cirrhosis [121, 122], Pachyonychia congenita [123] etc. Previously keratins were thought to provide merely mechanical strength to the cells but recent studies have shed light on different functional aspects of keratins such as regulation of protein synthesis, organelle transport, stress response, apoptosis, tissue differentiation, immune regulation, signalling and growth control [124-127].

Nomenclature and classification of keratins

First attempt to name keratins based on the properties of molecular weight and iso-electric point observed by 2D isoelectric focusing and SDS-PAGE from a large number of normal human epithelia, tumors, and cultured cells was reported by Moll *et. al.* [128].

They grouped the basic-to-neutral type II keratins as K1–K8 and the acidic type I keratins as K9–K19. With the advent of Human Genome Project, genome sequencing in recent years uncovered the existence of several novel keratin genes and their encoded proteins. Their naming could not be adequately handled by the Moll's nomenclature system. In another nomenclature, proposed by Hess *et. al.* [129], all human type I keratins were named Ka9 to KaX and all type II keratins were named Kb1 to KbY, thus, enabling type I and II keratins of other mammalian species to be added consecutively into this open-ended system.

The Human Genome Nomenclature Committee (HGNC) and the Mouse Genome Nomenclature Committee and Schweizer *et. al.* [13] in 2006 proposed a new consensus nomenclature for mammalian keratins. To structure the new nomenclature system, the 54 human keratins and their genes were divided into three categories: (1) epithelial keratins/genes, (2) hair keratins/genes, and (3) keratin pseudogenes. The nomenclature also allowed inclusion of a fourth category of nonhuman epithelial and hair keratins of other mammalian species, whose genes are either absent or occur as pseudogenes in the human genome [13].

The numbering scheme of keratins is shown in **Table 1 to 3.**

Table 1. The numbering scheme of keratins. (Adapted from Schweizer *et. al*. [130]).

Category	Number range
Human type I epithelial keratins	9–28
Human type I hair keratins	31–40
Nonhuman type I epithelial and hair keratins	41–70
Human type II epithelial keratins	1–8 and 71–80
Human type II hair keratins	81–86
Nonhuman type II epithelial and hair keratins	87–120
Type II keratin pseudogene	121–220
Type I keratin pseudogenes	221 →

According to the new nomenclature system, there are 17 (number 9-28) type I human epithelial keratins and 20 different (number 1–8 and 71–80) type II human epithelial keratins.

Table 2. Type I Human epithelial keratins (Adapted and modified from Schweizer *et. al*. [130]).

Current protein designation	*Current gene designation*	*Protein designation according to Hesse et al., 2004*[a] [129].	*New protein designation*	*New gene designation*
K9	KRT9	Ka9	K9	KRT9
K10	KRT10	Ka10	K10	KRT10
K12	KRT12	Ka12	K12	KRT12
K13	KRT13	Ka13	K13	KRT13
K14	KRT14	Ka14	K14	KRT14
K15	KRT15	Ka15	K15	KRT15
K16	KRT16	Ka16	K16	KRT16
K17	KRT17	Ka17	K17	KRT17
K18	KRT18	Ka18	K18	KRT18
K19	KRT19	Ka19	K19	KRT19
K20	KRT20	Ka20	K20	KRT20
K23	KRT23	Ka23	K23	KRT23
K24	KRT24	Ka24	K24	KRT24
K25irs1[b],K10C[c],hIRSa1[d]	KRT25A	Ka38	K25	KRT25
K25irs2[b], K10D[c]	KRT25B	Ka39	K26	KRT26
K25irs3[b], K10B[c], hIRSa3.1[d]	KRT25C	Ka40	K27	KRT27
K25irs4[b], hIRSa2[d]	KRT25D	Ka41	K28	KRT28

[a]Hesse *et al*., [129]; [b]Rogers *et al*. [131]; [c]Hesse *et al*. [132]; [d]Bawden *et al*. [133].

Table 3. Type II Human epithelial keratins (Adapted and modified from Schweizer *et. al*. [130]).

Current protein designation	*Current gene designation*	*Protein designation according to Hesse et al., 2004*[a] [129].	*New protein designation*	*New gene designation*
K1	KRT1	Kb1	K1	KRT1
K2e	KRT2A	Kb2	K2	KRT2
K3	KRT3	Kb3	K3	KRT3
K4	KRT4	Kb4	K4	KRT4
K5	KRT5	Kb5	K5	KRT5
K6a	KRT6A	Kb6	K6a	KRT6A
K6b	KRT6B	Kb10	K6b	KRT6B
K6e/h,[a,b]	no designation	Kb12	K6cc	KRT6C
K7	KRT7	Kb7	K7	KRT7
K8	KRT8	Kb8	K8	KRT8
K6irs1	no designation	Kb34	K71	KRT71
K6irs2	no designation	Kb35	K72	KRT72
K6irs3	no designation	Kb36	K73	KRT73
K6irs4	no designation	Kb37	K74	KRT74
K6hf	no designation	Kb18	K75	KRT75
K2p	KRT2B	Kb9	K76	KRT76
K1b[c]	no designation	Kb39	K77	KRT77
K5b[c]	no designation	Kb40	K78	KRT78
K6l[c]	no designation	Kb38	K79	KRT79
Kb20[a,b]	no designation	Kb20	K80	KRT80

[a]Hesse *et al*. [129]; [b]Rogers *et al.* [131]; [c]Hesse *et al*. [132].

Structure of keratins-

Keratin proteins can be divided into three functional groups: '**simple**' keratins, expressed in embryonic and one-layered epithelia, such as liver, lung intestine and glandular secretory cells (e.g., K8/18, K19 and K20);'**barrier**' keratins, expressed in stratified epithelia (e.g., K5/14, K1/10, K3/12, K4/13, K6a/16, K6b/17, K19); and '**structural**' keratins that form hair, nail, horn, and reptilian scales [16, 125]. All Intermediate filaments have a common "tripartite" structure; i) non-helical head region , ii) central helical rod domain featuring long range, coiled-coil forming heptad repeats of

apolar residues and iii) non helical tail region. Head and tail regions are susceptible for post translational modifications whereas rod domain is highly conserved in nature. The rod domain is further subdivided into coil 1 (sub domains 1A and 1B) and coil 2 (2A and 2B), that are interrupted by non- α -helical 8–17 amino acid linker (L1, L12 and L3) regions. **Figure 4** shows the schematic presentation of typical intermediate filament depicting tripartite nature of keratins, the hotspot regions for post translational modifications, trigger sites (TrS) that mediate nucleation of keratin heterodimerization via coiled coil interactions and the caspase sensitive cleavage sites (Casp).

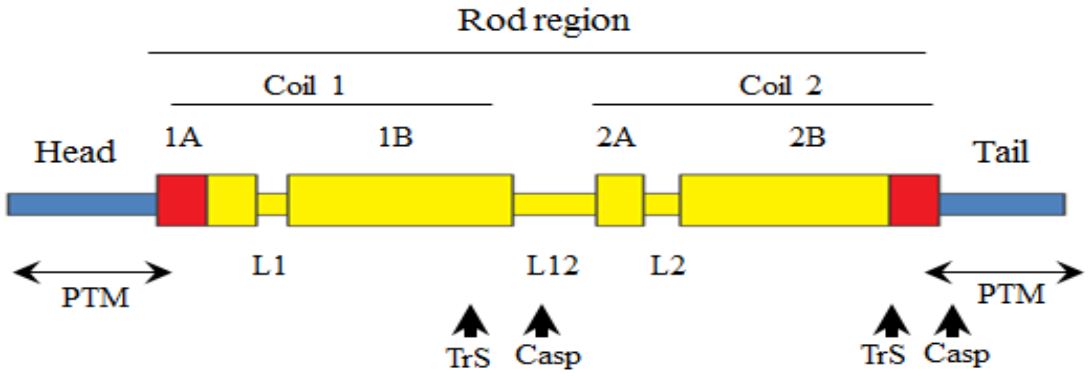


Figure 4. Schematic structure of keratin intermediate filament. The red boxes depict the position of 15-20 highly conserved amino acid segments (Adapted and modified from Coulombe *et. al*. [134]).

Keratins assemble as heterodimer of one type I and one type II keratin monomer with rod domains aligned in parallel and in register. In the next step of filament organization, the dimers form tetramers by overlapping the N-terminal half of their rod domains in antiparallel manner. Tetramers then assemble into 60 nm length filament. These unit length filaments then assemble in end-to-end fashion to form nanofilaments that are 10 nm thick. Due to the antiparallel nature of filament assembly, they do not exhibit the characteristic of polarity generally shown by molecular motors. **Figure 5** shows the schematic representation of intermediate filament assembly.

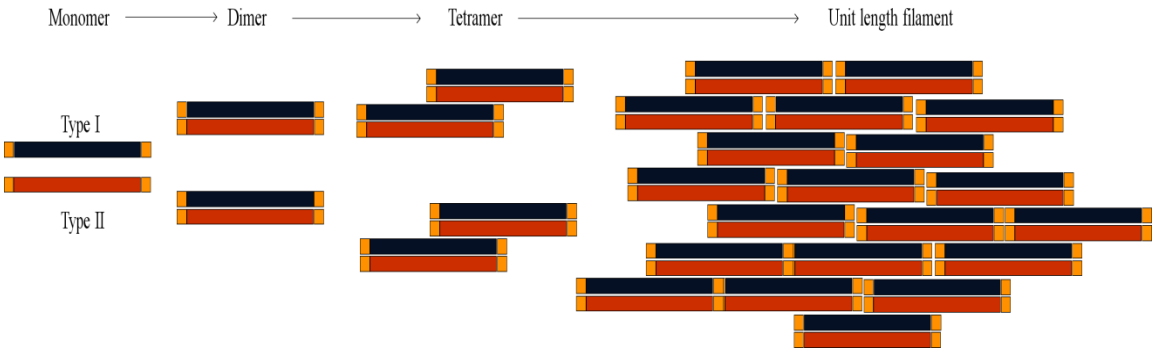


Figure 5. Schematic representation for assembly of keratin filament. (Adapted and modified from Haines et. al. 2012 [125]).

Keratin expression in normal and cancer tissues-

Keratins are known to be expressed in cell type specific and differentiation dependent manner. All the epithelial tissues express at least 2-8 different keratins [19]. In the following, expression pattern for different keratin pairs has been discussed together with their cell type and tissue distribution.

K1/10: In the epithelial cells the transition of keratin expression occurs from basal layer (K5, K14, K15) to intermediate layer (K4, K13) to more differentiated layer that comprises K1 and K10. This pair is co-expressed during differentiation of simple and stratified epithelial tissues. The differentiation specific expression pattern of keratins exemplifies their tight and careful regulation. K1/K10 form particularly dense bundles which are characteristic of suprabasal epidermal keratinocytes. In addition to their expression in terminal epidermal differentiated and keratinized cells, K1/K10 may be focally expressed in suprabasal cells of internal noncornifying stratified squamous epithelia [135]. Human Protein Atlas (HPA) database (http://www.proteinatlas.org) shows K1 expression by IHC in oral mucosal squamous epithelial cells with moderate staining and in vagina and cervix squamous epithelial cells with intense staining for normal tissues. The tumor tissues from head and neck region showed moderate staining with an antibody CAB002153 for K1. Staining for K10 was observed in tonsil, oral, and esophageal normal squamous epithelial tissues and in colorectal, breast, ovarian,

prostate, cervical, head and neck, thyroid, lung, skin, pancreas, stomach and liver cancer tissues with an antibody HPA012014.

K4/13: Mucosal stratified squamous epithelial cells express K4/13 pair. MAbs against K13 revealed the presence of K4 and K13 in the entire suprabasal compartment of mucosal stratified squamous epithelia, whereas the basal compartment is positive for K5/K14 moreover K4/K13 is completely absent in the epidermis and adnexal structures [19]. IHC studies in the HPA initiative, report the moderate expression of K4 in tonsil, gallbladder, nasopharynx, cervix, fallopian tube, placenta, epididymis, prostate, seminal vesicle, kidney, urinary bladder, and skin cells whereas strong staining is seen in liver and esophageous cells of normal tissue. The tumor tissues from colorectal, breast, prostate, stomach, pancreatic and liver cancer showed more than 50 % staining for K4 with an antibody CAB002154. Staining for K13 was observed in vaginal, tonsilar, oral and esophageal normal squamous epithelial cells whereas the tumor tissues from cervical cancer showed strong positivity with an antibody CAB000133.

K5/14: Keratin 5/14 are the major keratins of basal keratinocytes of stratified squamous epithelia, including the epidermis as well as mucosal non-keratinizing stratified squamous epithelia [128]. Fuchs and Green [136] reported strong expression of K5/14 in undifferentiated basal cell layer containing the stem cells and down-regulation in the differentiating suprabasal cell layers. K5/14 are uniformly expressed throughout all layers in the widely well stratified follicular outer root sheath. HPA database shows K5 expression by IHC in esophagus, lung, and breast and vagina tissues with a moderate staining whereas intense staining was observed in tonsil, oral mucosa, nasopharynx, bronchus, cervix, normal squamous epithelial cells. The tumor tissues from cervical, head and neck, skin and urothelial cancer showed more than 50% staining with an antibody CAB000129.

K6/16: Keratin 6 and 16 are major keratins of hyper-proliferative keratinocytes that are inducible in "activated" epidermis. Keratin 6 isoform K6a pairs with K16 while K6b pairs with K17 [125]. Moll *et. al.* [128] identified K6/16 in normal epithelia of foot sole, anal canal, hair follicle (outer sheath), cornea, exocervix, tongue, epiglottis and esophagus based on 2DE based electrophoretic mobility of keratins with respect to molecular weight and isoelectric point. K6/16 are reported to express constitutively in nail epithelia [137, 138] and in non-keratinizing stratified squamous epithelia [128]. HPA database shows K6a and K6b expression by IHC in normal squamous epithelia of tonsil, oral, esophagus, vagina and cervix tissues. Tumor tissues from cervical, lung, head and neck, skin and urothelial cancer also showed high staining by using HPA045697 antibody.

K8/18: Keratin 8/18 are primary keratins of simple epithelial cells such as hepatocytes pancreatic acinar and islet cells, and proximal tubular kidney epithelial cells including various parenchymatous epithelia [18]. These are the first keratins to appear in embryogenesis, as early as in pre-implantation embryos [139]. In some epithelial cell types, K8 and K18 are the only keratins present. K8 and K18 are also found in pseudostratified and urothelial epithelia, where they are expressed mainly in luminal cells [140]. K8/18 are over-expressed in many cancers of simple epithelia expressing tissue/organs such as biliary duct, bladder, colon, kidney, liver, lung, ovary, pancreas, stomach and uterus. This pair is also expressed in normal breast mixed epithelia and is over-expressed in breast cancer. Various studies have reported aberrant expression of K8/18 pair in oral cancer where it is not present in normal stratified squamous epithelia as shown by immunohistochemistry [22, 141-143] and by 2D electrophoresis followed by western blotting using laboratory generated rabbit polyclonal antibodies to total

keratins [26, 27]. The aberrant expression has been correlated with poor prognosis in squamous cell carcinoma of the oral cavity and in oral leukoplakia [20].

K7/19: Keratin 7/19 are secondary keratins of simple epithelial cells. These are widely distributed simple-epithelial keratins which are frequently but not always co-expressed. Toivola *et. al.* [144] reported the K8 and K18 localization preferentially within cytoplasmic filaments, whereas K7 and K19 were reported to be expressed primarily in the membrane-proximal apicolateral compartment. K7/19 typically occurs in simple ductal epithelia such as bile and pancreatic ducts. In absence of K7 as intestinal epithelium, the type I keratin K19 forms a pair with K8 which is otherwise a pair of K18. K7/19 expression is observed in various cancers such as colon, esophagus, stomach, pancreas, ovary and lung carcinoma [140].

K2, K9, 15 and K17: Keratin 2 is a marker of highly differentiated, advanced epidermal keratinocytes [19]. K2 is widely distributed over most body sites and is expressed late, at an advanced stage of differentiation, in the uppermost epidermal layers (upper stratum spinosum, stratum granulosum) to a variable extent [145]. HPA database shows K2 IHC staining in normal tissues of tonsil, oral, esophagus, vagina and cervix squamous epithelial cells whereas K2 staining is observed in almost all cancer tissues. Keratin 9 is a marker of palmoplantar epidermal differentiation [19]. K9 is a marker of terminally differentiating keratinocytes of palmoplantar epidermis where it is abundantly, but heterogeneously expressed [146, 147]. However, no reports are available for association of K9 in cancer but its gene mutation is associated with a disorder of the skin of the palms and soles and epidermolytic palmoplantar keratoderma [148, 149].

Keratin 15 is a marker of basal keratinocyte and hair follicle stem cells [19]. Moll *et. al.* [128] reported for the first time the expression of K15 in normal epithelia of epidermis,

anal canal epithelium, hair follicle, sebaceous gland, exocervix, tongue, epiglottis and esophagus and in the cancer's such as basal cell epithelioma, SCC of epiglottis, esophagus and rectal anal canal using the 2DE approach. K15 is restricted to the basal cell component of the epidermis and other stratified squamous epithelia. K15 pairs with K5 in basal cell layer of stratified squamous epithelia [150, 151]. HPA database shows the presence of K15 in normal tissues from tonsil and vagina squamous epithelial cells and tumor tissues from breast, prostate, cervical, endometrial, thyroid, lung, skin, stomach and pancreatic cancer using the HPA023910 antibody.

Keratin 17 is a marker of basal/myoepithelial cells and is inducible in "activated" keratinocytes [19]. It was first identified by Moll *et. al*. [152] in their early gel electrophoretic studies as a major keratin of basal cell carcinomas of the skin which was also present in the normal pilosebaceous tract but not in normal epidermis. Several proteomic studies have shown presence of K17 in squamous cell carcinomas of various origin such as breast cancer [153], lung squamous cell carcinoma, lung adenocarcinoma, malignant mesothelioma, salivary gland mucoepidermoid carcinoma and salivary gland adenoid cystic carcinoma [14].

Post translational modifications

Keratins undergo several post translational modifications such as phosphorylation, glycosylation, sumoylation, ubiquitination, proteolysis, and trans-glutamination. These PTMs for the keratins are discussed below-

i) **Phosphorylation-** Head and tail regions of the keratin are the major sites for PTM and phosphorylation is the widely studied modification amongst all. Several functions are associated with the phosphorylation of keratins, for example; K18 Ser52 phosphorylation is associated with filament re-organization and solubility

[154, 155]. Role of K8 Ser 73 [156] and K18 Ser 53 [157] is associated with the apoptosis as shown by induction of apoptosis in cultured cells. Toivola *et. al*. [158] have reported association of hyper-phosphorylation of K8/18 with progression of human liver disease. Ku *et. al*. [159] have reported the phosphorylation of human keratin 18 serine 33 which in turn regulates binding to 14-3-3 proteins which are cell cycle associated proteins. Thus, phosphorylation of keratins plays a very pivotal role in regulating various cellular processes.

ii) **Glycosylation**- Glycosylation of keratins was first reported in human K13 that was isolated from cultured new-born human foreskin in keratinocytes. The glycosylation modification was identified by metabolic labelling with ^{3}H-glucosamine [160], and the modification was found to be 0-glycosidically linked on K13 [161]. Other keratins with characteristic glycosylation includes K8 and K18 [162]. For K18, three glycosylation sites are Serine 29, 30 and 48 residues [163] and the PTM is restricted to only head and tail regions of the keratin. The O-GlcNAcylation has been reported to determine the solubility, filament organization, and stability of keratins 8 and 18 in immortalized (Chang) and transformed hepatocyte (HepG2) cell lines [164]. Recent studies have shown that K18 glycosylation provides a unique protective role in epithelial injury by promoting the phosphorylation and activation of cell-survival kinases [165].

iii) **Sumoylation**- Sumoylation is a very important regulatory modification of proteins implicated in various human diseases, including cancer, neurodegeneration, and cardiovascular disease. Sumoylation of keratin regulates filament organization and solubility. The addition or removal of **S**mall **U**biquitin-like **Mo**difier (SUMO) polypeptides (SUMO-1, -2, or -3) has shown to affect protein localization, interactions with binding partners, and degradation [166]. Sumoylation typically

modifies lysine residues located within a classical SUMO consensus motif ΨKX (D/E), where Ψ is a hydrophobic amino acid and X is any residue. In a recent study, Omary *et. al*. have demonstrated that K8, K18, and K19 undergo oxidative and other stress-induced sumoylation on rod domain lysines within SUMO consensus sites and also demonstrated the role of phosphorylation in regulation of keratin sumoylation [167].

iv) **Ubiquitination**- Ubiquitination is a post-translational modification carried out by a set of three enzymes, E1, E2 and E3. Ubiquitination usually leads to the proteasome mediated degradation of the labelled protein but is however not always fatal for the protein. Several non-proteolytic functions are also associated with the addition of a single ubiquitin molecule or specific cases of polyubiquitination such as mono-ubiquitination alters the fate of the protein in a less terminal fashion, potentially affecting its cellular sub-location, function or its degradation though lysosomes. Keratin polypeptides 8 and 18 have been reported to turn over by ubiquitination in a phosphorylation-modulated fashion [168]. Jaitovich *et. al.* [169] have reported ubiquitin-proteasome-mediated degradation of keratin intermediate filaments in a mechanically stimulated A549 cells and have also shown the phosphorylation of K8 Ser-73 to be required for the shear stress-mediated ubiquitination, disassembly, and degradation of the keratin IF network.

v) **Proteolysis**- Proteolysis is the breakdown of proteins into smaller polypeptides or amino acids. This generally occurs by the hydrolysis of the peptide bond, and is most commonly achieved by cellular enzymes called proteases, but may also occur by intramolecular digestion, as well as by non-enzymatic methods such as the action of mineral acids and heat. Chan *et. al*. [170] have identified the proteolytic derivatives of keratins 18 and 19 in the culture medium of MCF-7 cells using a

panel of monoclonal antibodies and peptide mapping. Keratins 18 and 19 are reported to undergo caspase mediated cleavage during epithelial cell apoptosis [157, 171-173]. There are strong evidences for release of cytokeratin-18 and -19 fragments (TPS and CYFRA 21-1) into the extracellular space during apoptosis [174]. Caspase 3 is reported to be involved in the production of cytokeratin 18 (TPS) and 19 (CYFRA21-1) fragments [175, 176]. The antibody M30 which recognizes the caspase cleaved neoepitope of K18 [177-180]. Circulating full-length and caspase-cleaved K18 are considered biomarkers of chemotherapy-induced cell death and are measured using a combination of the M30 and M65 ELISAs [181].

vi) **Trans-glutamination-** Transglutamination is the reaction of formation of a covalent bond between a free amine group (e.g., protein- or peptide-bound lysine) and the gamma-carboxamide group of protein- or peptide-bound glutamine which is catalysed by a group of enzymes called transglutaminase. Keratin transglutamination occurs in epidermal and simple epithelial keratins under physiologic and pathologic states, respectively. In the physiological context, the role of this modification is clear in terms of providing a compact protective structure, while in the pathologic context of liver disease the role remains ambiguous [182].

Functions

i) **Mechanical function-** Role of keratin intermediate filament to provide mechanical resilience to the cells was realised with the study of skin fragility disorders involving mutations in epidermal keratins. The well-known example is Epidermal bullosa simplex (EBS) disorder in which the keratin pair K5/14 which is generally expressed in the basal layer is mutated and is characterised by cytolysis of

keratinocytes and loss of structural integrity [183]. K1/K10, a marker of suprabasal layers of the epidermis is mutated in epidermolytic hyperkeratosis (EHK) disease which also confers similar phenotypes [184, 185]. Keratin mutation-based fragility phenotypes have been closely correlated with alteration in the micro-mechanical properties of the cytoskeleton [184, 186, 187].

ii) **Keratins and stress response-** Several intrinsic (genetic, and endoplasmic reticulum) and extrinsic/ environmental (heat, radiation, mechanical, wound, infection, hypoxia) lead to stress on the cells and tissues. The well-studied example is the phosphorylation in *cis* of K8 and K18 in liver hepatocytes which confers protection against a variety of metabolic, oxidative, and chemical stresses [188, 189]. A recent study by Ku *et. al*. [165] has shown the protective role of K18 glycosylation in epithelial injury of mice by promoting the phosphorylation and activation of cell-survival kinases.

iii) **Keratins and organelle transportation-** Mutational analysis of some keratins has revealed a role for these proteins in certain membrane trafficking events [6, 7, 190]. Mutation in K5 identified in Dowling–Degos disease leads to a skin pigment phenotype which is distinct from skin blistering [191]. K5 mutation, leads to the defect in the transfer of melanosomes, or their arrangement in keratinocytes, that can lead to changes in skin pigmentation. Although the exact mechanism by which K5 mutation leads to the alteration in melanosome pigmentation is not known, it might involve an interaction of K5 with the chaperone HSC70, which is involved in vesicle uncoating [192].

iv) **Regulatory functions-** The regulation of the cell cycle and of cell size provides the basis for the diversity of mammalian tissues and their enormous adaptability to environmental cues. A study with K17-null mouse embryos has shown that a delay

in the closure of surface wounds in the embryonic ectoderm [193] correlates with a failure of wound-proximal epithelial cells to undergo the expected large increase in size [127]. K17-null skin keratinocytes are also smaller while in primary culture, where they reproducibly show an ~18% depression in translation paralleled with reduced activity of Akt and mTOR kinases, selectively [127]. This phenotype could be a due to involvement of K17 in regulating the cell growth through binding to the adaptor protein 14-3-3sigma [127] which belongs to the family of 14-3-3 proteins that are conserved regulatory molecules expressed in all eukaryotic cells. 14–3–3 proteins are reported to activate protein synthesis since they somehow negate TSC1/TSC2's ability to negatively regulate mTOR [194, 195]. Most likely, site-specific phosphorylation on the head domain of K17 creates a composite binding site for 14–3–3σ, causing its partial retention in the cytoplasm, where it can foster a full activation of mTOR in wound or serum-activated keratinocytes [127].

Regulation of apoptosis represents an additional mechanism through which keratin IFs are able to coordinate cell and tissue growth [196]. Keratins can accomplish this by 'sequestering' death-promoting effector molecules such as tumor necrosis factor receptor 2 (TNFR2) [197] and TNF receptor-associated death domain-containing protein (TRADD) [198-200].

v) **Keratins and tissue differentiation-** Keratins are known to be expressed in tissue-specific and differentiation-dependent manner and their expression is tightly controlled. Keratins have long been used as a marker in epithelial tumours to identify the tissue of origin [201] and also have been used as a prognostic markers [18]. Studies of 'unnatural' keratin pairs, such as K5 paired with K16 or K18 rather than its normal partner, K14, suggest that the assembly and mechanical properties of the filaments can be drastically altered by changing the type I or type II keratins

present [202, 203]. Change in the keratin expression pattern might lead to a subsequent alteration in physical properties which may benefit tumor cells to invade and expand in dimensions.

Keratin expression in Head and Neck cancer

Normal oral mucosa is composed of stratified non-keratinized epithelial layers. Most stratified epithelia express the keratin pair K5/ K14 in the proliferative basal layer that lies adjacent to the basement membrane and this layer is normally the only mitotic cell layer [204]. The subsequent (suprabasal) cell layers are normally post-mitotic and undergo a program of differentiation, expressing different pairs of keratin proteins. Epidermal and gingival tissues express keratins K1 and K10 in the spinous (prickle) layer, while non-keratinizing tissues such as buccal and soft palate produce K4 and K13 in the suprabasal layers. Additional keratin layers of these tissues are K2e in the epidermis, and K6, K16, and K2p in the differentiating upper layers of gingiva and hard palate tissues [204].

Several recent, comprehensive and in-depth reviews have described the expression and role of specific keratins in different tissues [14-19]. None of these reviews provide much information on the expression profile for the keratins in the head and neck region. There are independent reports on keratins from the head and neck region. Majority of these studies have used antibody based techniques such as immunohistochemistry, immunofluorescence and immunoblotting. A few proteomic and microarray studies show the presence/expression of keratins in tissues from the head and neck region.

i) Keratin expression profile by immunohistochemistry

K1:K10; K4:K13; K5:K14; K6:K15,K16,K17; K7:K19; K8:K18 form obligate pairs in epithelial cells [17, 19]. In several of these studies the keratins have been

reported using in house generated or pan keratin antibodies or those that recognize two keratins thereby leaving an ambiguity about their expression. Keratin 1 has been shown to be expressed in normal oral tissues [20-22]. Ranganathan *et. al.* [22] have used the monoclonal antibody 34 β B4 and reported the down regulation of K1 from normal tissues (40%), OSF (32%) to oral cancer (20%) and Lalli *et. al.* [23] have used an in house antibody and shown its over expression in oral submucous fibrosis. Its pair, Keratin 10 is overexpressed in OSF [23], lesions of oral mucosa [205] and in oral cancer [206]. Keratin 4 expression is observed in normal, OSF and oral cancer tissues to nearly the same extent [22]. Its pair K13 is reported to be expressed in normal [20, 21, 24, 207, 208] and OSF tissues [23] and the expression is down regulated in lesions of oral mucosa [205], and the oral cancer [207-209] while it is reported to be same as normal using the antibody CK 8.12 which recognises K13/K16 [206]. On the contrary, Xu *et. al.* [24] have reported its expression in normal, dysplastic as well as in HNSCC. Keratin 5 expression has been reported in normal and oral cancer tissues [20-22, 142] and Keratin 14 is over-expressed in oral cancer [22, 206, 210]. Keratin 15 expression is less studied by IHC and Lalli *et. al.* [23] have shown its expression in OSF tissue samples using an in-house generated antibody. Keratin 6 is expressed in normal tissues [20, 21] and is overexpressed in OSF [23]. Comparison of the status of K6 in normal and oral cancer tissue is not very well documented by IHC. Keratin 16 expression is reported in normal [20, 23, 206] and OSF samples and its expression in tumors is not well studied. Keratin 17 is overexpressed in OSF [23] and oral cancer [208, 211]. Keratin 8 [20, 22, 24, 141, 143, 212, 213] and K18 [22, 141, 143, 214] are reported to be aberrantly expressed in oral cancer. Xu *et. al.* [24] have shown K19 overexpression from normal (13%) dysplastic (71%) to HNSCC (82%) while Crowe

et. al. [25] have reported its down regulation in oral SCC cell lines as compared to normal tissues.

The other keratins less reported in oral epithelial cells are K2, K7, and K9. Gires *et. al.* [20] have used a pan keratin antibody while Mai *et. al.* [21] have reported the expression of K2 in normal oral tissues with a specific antibody, CK-E3. Keratin 7 expression is reported in the study by Gires *et. al.* [20] wherein it is expressed in normal tissues while Su *et. al.* [141] have reported no expression in normal tissues and weak expression in dysplastic and SCC of oral cavity. Keratin 9 is reported to be overexpressed from leukoplakia to SCC [143].

ii) Keratin expression by other methods

Early studies by Vaidya *et. al*. [26] using 2DE and western blotting with rabbit polyclonal antibodies to total keratins have shown the presence of K4, K5, K13 and K14 in normal oral tissue and K1, K4, K14, K16, K17, K18 in oral cancer tissues. Lowered expression of K5 was reported in Leukoplakia, OSF, SCC of tongue and SCC of oral mucosa, and expression of K2, K4, K14 and K19 along with aberrant expression of K8/18 and reduced expression of K13 were also reported in oral cancer [26-29]. There are a few reports which use global proteomic analyses of tissues from the head and neck region and show the differential expression or presence of keratins K1, K2, K4, K5, K6, K8, K9, K10, K13, K14, K15, K16, K17, K18 and K19 [30-34].

Genomic studies have shown the expression of K4, K13 [215] and K15 in normal mucosa and its down regulation in tumor [36]. Odani *et. al.* has reported overexpression of K2 and K10 in oral leukoplakia as compared to SCC [37]. Toyoshima *et. al.* have shown the overexpression of K17 in OSCC as compared to normal mucosa [38].

Most of the studies have analysed keratins in tissues from the head and neck region without due attention to subsites. The oral subsite, buccal mucosa is biologically and molecularly different from the tongue [39-46] which is also apparent from the K13 expression profile in tongue tissues in which K13 is overexpressed in tumor tissues [30]. Further, as the focus of the investigations was not on keratins some of the proteomic studies used tissue lysates in buffers in which keratins are not soluble. Studies reporting 2DE-mass spectrometry data have focussed on regions below 50 kDa thereby overlooking the keratins.

Western blotting data from on-going studies in the laboratory investigating the status of K18 in GBC showed differential patterns in tumor and adjacent normal tissues indicating that this could be exploited as a marker. In initial studies to understand this differential pattern it became apparent that the antibody based observations were not supported by mass spectrometry indicating that there is a necessity for validation of antibody based identification of homologous proteins such as keratins by mass spectrometry. These observations and the review of literature lead to the aim of assessing the keratin profile in oral cancer using enriched preparations of keratins in normal and tumor tissues from a specific subsite of the oral cavity, the gingivo-buccal complex, using mass spectrometry followed by validation of their expression by silver staining, western blotting, and immunohistochemistry with the following objectives:

Objectives-

i) *Identification of keratins in normal cut margin and tumor samples of cancer of the gingivo buccal complex by PMF- MS/MS of 1DE resolved proteins.*

ii) *1DE-protein blotting with specific antibodies to keratins and post translational modifications and identification of isoelectric point /molecular weight of the keratins by 2DE-immunoblotting.*

iii) *2DE-Silver staining and PMF-MS for localization and identification of keratins.*

iv) *2DE-Silver staining pattern of keratins from normal, cut margin and tumor tissue.*

v) *Confirmation of keratin expression by Immunohistochemistry.*

vi) *Comparison of the expression pattern obtained by mass spectrometry, silver staining, western blotting and immunohistochemistry*

Chapter 2

MATERIALS AND METHODS

Chapter 2. MATERIALS AND METHODS

2.1 MATERIALS

This study was approved by the Tata Memorial Centre, Scientific Review and Hospital Ethics committees and all samples were collected with informed consent. Adjacent normal (N), cut margin (CM) and tumor (T) tissues were collected from patients with cancer of gingivo buccal complex undergoing surgery as part of their treatment. Tissues were quick frozen in liquid nitrogen till further use.

2.1.1 Reagents for enrichment of keratins

The chemicals, the concentration used and source are:

Homogenization buffer: 96 mM NaCl (Fisher Scientific- 1591M), 8 mM KH_2PO_4 (S. D. Fine Chem. Pvt. Ltd.- 39608), 5.6 mM $Na_2HPO_4 \cdot 2H_2O$ (S. D. Fine Chem. Pvt. Ltd.- 20383), 1.5 mM KCl (Sigma P-9333), 10 mM EDTA (Sigma-ED4SS) and 0.1 mM Dithiothrietol (DTT) (Sigma-D9779) and the pH was adjusted to 6.8 with HCl. Yellow^{Line} DI 25 basic homogenizer was used for the homogenization of the tissue samples.

Detergent buffer: 10 mM Tris-HCl (Tris-Sigma-T1378, HCl-Fisher Scientific-29505), 140 mM NaCl, 5mM EDTA, 1% Triton-X-100 (Sigma-T9284) and the final pH was adjusted to 7.6 with HCl.

High salt buffer: 10 mM Tris-HCl, 140 mM NaCl, 1.5 M KCl, 5 mM EDTA, 0.5% (w/v) Triton-X-100. The final pH was adjusted to 7.6 with HCl.

2.1.2 Reagents for protein estimation using the TCA precipitation, modification of Peterson's method [48] and for Bio-Rad Protein Assay, based on the method of Bradford.

For protein quantitation of the total tissue lysates and enriched keratins, the protein standard used was 1 mg/ml Bovine Serum Albumin (Sigma-A7906) solubilized in 1:10 diluted 2DE buffer.

Other chemicals, their concentration and source used were 20% Sodium carbonate (Qualigen-15955), 0.2% Copper sulphate (Sarabhai M Chemicals-2790), 0.4% Potassium-Sodium tartarate (USB-80088), 10% Sodium dodecyl sulphate (Sigma-L3771), 0.8N Sodium Hydroxide (Ranbaxy-S0270), 1.5% Deoxycholic acid, sodium salt (USB-21026), 72 % Trichloro acetic acid (Sisco Research Laboratories 20482), Folin and Ciocalteau's Phenol Reagent (FC reagent) (Sisco Research Laboratories 62015).

Copper Tartarate Carbonate (CTC) solution:

0.1 % copper sulphate, 0.2 % potassium tartarate, 10 % Sodium carbonate:

A] 20 % Sodium carbonate: 20 gm sodium carbonate was dissolved in deionized water to a final volume of 100 ml.

B] 0.2 gm copper sulphate dissolved in 40 ml deionized water and 0.4 gm potassium tartarate dissolved in 40 ml deionized water were mixed together and the volume made up to 100 ml to a final concentration of 0.2 % and 0.4 % respectively.

Equal volume of solution A] and B] was added to the mixture to obtain the final CTC solution.

10% Sodium Dodecyl Sulphate (SDS):

SDS (Sigma L3771) solution in deionized water. 10 gm of SDS was dissolved in 80 ml of deionized water and final volume was made to 100 ml with deionized water.

Working solution A:

Equal volumes of CTC solution, 10 % SDS, 0.8 N NaOH and were mixed together and the mixture was diluted 1:1 (v/v) with deionized water.

Working solution B:

2 N Folin and Ciocalteau's Phenol Reagent (FC reagent) (Sisco Research Laboratories 62015) diluted 1:5 with deionized water.

BioRad Protein Assay Dye Reagent Concentrate (catalog number 500-0006) contains 450 ml of solution containing dye, phosphoric acid, and methanol.

2.1.3 Reagents for One Dimensional Polyacrylamide Gel Electrophoresis (1DE) and Two Dimensional Polyacrylamide Gel Electrophoresis (2DE)

For One Dimensional Polyacrylamide Gel Electrophoresis (1DE) and Two Dimensional Polyacrylamide Gel Electrophoresis (2DE) the reagents used were Acrylamide (USB-75820), Bisacrylamide (USB- 75821), SDS (Sigma L-4509), Tris (USB-75825), Ammonium persulphate (S.D. Fine Chem. Pvt. Ltd- 20032), TEMED (USB-76320), Iodoacetamide (Sigma- I1149), Urea (Sigma-U5378), Thiourea (Sigma-T7875), CHAPS (USB-13361), DTT (Sigma-D9779), Glycerol (Merck- 87 % GR), Mineral oil (BIO-RAD).

Stock acryl amide solution:

30 % acrylamide solution: 29.2 gm acrylamide and 0.8 gm bisacryl amide were dissolved to a final volume of 100 ml in deionized water.

Separating gel for SDS-PAGE:

10% Acrylamide, 375 mM Tris-Cl (pH 8.8), 0.1% SDS:

The composition for 7 cm mini gels, is given as follows-

Separating Gel composition: For mini gel (6 ml):

Component's	**10% gel**
30% Polyacrylamide	2 ml
Tris pH 8.8	2.24 ml
10% SDS	60 µl
Deionized water	1.637 ml
10% APS	60 µl
TEMED	3 µl

The composition for 17 cm maxi gels, is as follows-

Separating Gel composition: For maxi gel (40 ml/gel):

Component's	**10% gel**
30% Polyacrylamide	13.333 ml
Tris pH 8.8	14.933 ml
10% SDS	0.4 ml
Deionized water	10.913 ml
10% APS	0.4 ml
TEMED	20 µl

Stacking gel for SDS-PAGE:

5 % Acrylamide, 0.175 M Tris-Cl (pH 6.8), 0.2% SDS:

For two 7X7 cm mini gels, 600 µl of 30 % acrylamide, 672 µl of 1 M Tris (pH 6.8), 18 µl of 10 % SDS and 1.71 ml deionized water were mixed and 60 µl of 10 % APS and 3 µl TEMED were added just before pouring the gel..

SDS Sample buffer:

0.189 M Tris-Cl (pH 6.8), 30% Glycerol, 6 % SDS:

2.4 ml 1 M Tris-Cl (pH 6.8), 3 ml 20 % SDS, 3 ml 100 % glycerol, and 1.6 ml β-mercaptoethanol were mixed and the final volume made up to 10 ml with deionized water.

3X Sample loading buffer for 1DE-gels:

Tris pH 6.8	187mM
SDS	6%
Glycerol	15%
Bromophenol Blue	0.009%
ß-mercaptoethanol	2.7%

Electrode buffer:

0.025 M Tris, 0.19 M Glycine and 0.2 % SDS:

3 gm Tris, 14.3 gm glycine (Sigma-G8898) and 2 gm SDS (Sigma L3771) were dissolved in deionized water to make final volume up to 1 liter.

Ampholines and IPG strips:

Ampholines of pH 4.7 to 5.9 (Cat. No. 163-2097) and IPG strips pH 4.7 to 5.9, 17 cm (Cat. No. 163-2021) and pH 5 to 8, 17 cm (Cat. No. 163-2011) were purchased from Bio-Rad Laboratories, USA.

2DE buffer:

8 M Urea (480 mg/ml), 2 M Thiourea (152 mg/ml), 2% CHAPS (200 µl from the stock solution made of 10% CHAPS in deionized water) and 1% DTT (100 µl from the stock solution made of 10% DTT in deionized water) were dissolved in deionized water to the final volume of 1 ml.

Rehydration buffer:

8 M Urea (480 mg/ml), 2 M Thiourea (152 mg/ml), 2% CHAPS (200 µl from the stock solution made of 10% CHAPS in deionized water) and 1% DTT (100 µl from the stock solution made of 10% DTT in deionized water), 2 µl ampholine and 2 µl of 0.1% bromophenol blue were dissolved in deionized water to make the final volume to 1 ml.

Equilibration buffer 1:

6 M Urea, 0.375 M Tris (pH 8.8), 2% SDS, 20% Glycerol, 2% DTT:

Component's	For 5ml	For 10ml	For 15ml	For 20ml
Urea	1.8gm	3.6gm	5.4gm	7.2gm
1M Tris pH 8.8	1.875ml	3.75ml	5.625ml	7.5ml
SDS	0.1gm	0.2gm	0.3gm	0.4gm
Glycerol	1ml	2ml	3ml	4ml
DTT	0.1gm	0.2gm	0.3gm	0.4gm

Above mentioned components were added into the T50 tube and final volume was made up with deionized water.

Equilibration buffer 2:

6 M Urea, 0.375 M Tris (pH 8.8), 2% SDS, 20% Glycerol, 2.5% Iodoacetamide:

Component's	For 5ml	For 10ml	For 15ml	For 20ml
Urea	1.8gm	3.6gm	5.4gm	7.2gm
1M Tris pH 8.8	1.875ml	3.75ml	5.625ml	7.5ml
SDS	0.1gm	0.2gm	0.3gm	0.4gm
Glycerol	1ml	2ml	3ml	4ml
Iodoacetamide	0.125gm	0.25gm	0.375gm	0.5gm

Above mentioned components were added into the T50 tube and final volume was made up with deionized water.

2.1.4 Reagents for transfer of proteins to Polyvinylidene difluoride (PVDF) membrane

PVDF membrane was purchased from Millipore (Cat. No. IPVH00010). Other regents used for transfer of proteins were, methanol (Merck), Glycine (Sigma-G8898) and Tris (Sigma-T1378).

The composition of transfer buffer is as follows-

0.025 M Tris, 0.19 M Glycine, and 20 % Methanol:

3.03 gm Tris, 14.4 gm glycine and 200 ml methanol were dissolved in deionized water and the final volume made up to 1 liter.

2.1.5 Reagents for Immunostaining

For immunostaining, ECL plus detection system (GE Healthcare-RPN2132) was used. For blocking of the PVDF membrane, 3 % BSA (Sigma-A7906) was used and for antibody dilution 1 % BSA was used. TBST was used for the washing of the blots.

Blocking buffer - Tris Buffered Saline with Tween 20 (TBST):

20 mM Tris-Cl (pH 7.6), 150 mM NaCl containing 0.1 % Tween 20 (USB- 20605).

Blocking solution:

3 % BSA (Sigma-A7906) in blocking buffer.

Washing buffer (TBST):

20 mM Tris-Cl (pH 7.6), 150 mM NaCl containing 0.05 % Tween 20.

X-Ray films:

X-ray films were supplied from Kodak India Pvt Ltd (MXB 4908703).

Antibodies used in the study:

List of antibodies and their dilutions used in the study for western blotting and immunohistochemistry is given in Table 4.

Table 4. Details of primary antibodies used for western blotting and Immunohistochemistry-

Sr. No.	Antibody	Clonality	Clone	Catalogue No.	Company	Dilution for WB (Primary Ab)	Dilution for WB (Secondary Ab)	Dilution for IHC (Primary Ab)
1.	Cytokeratin 1	Mouse MAb	AE1	ab9286	Abcam	1:8000	1:2000	
2.	Cytokeratin 4	Rabbit MAb	EP1599Y	1994-1	Epitomics	1:1000	1:5000	1:1000
3.	Cytokeratin 5	Rabbit MAb	EPR1600Y	2290-1	Epitomics	1:20,000	1:5000	
4.	Cytokeratin 6	Rabbit MAb	EPR1603Y	1951-1	Epitomics	1:10,000	1:5000	
5.	Cytokeratin 8	Mouse MAb	M20	C5301	Sigma	1:2000	1:2000	
6.	Cytokeratin 10	Mouse MAb	RKSE60	Ab9025	Abcam	1:1000	1:2000	
7.	Cytokeratin 13	Rabbit PAb	Polyclonal	Ab58744	Abcam	1:2000	1:5000	1:300
8.	Cytokeratin 14	Rabbit MAb	EP1612Y	2001-1	Epitomics	1:20,000	1:5000	1:3500
9.	Cytokeratin 16	Rabbit MAb	EP1615Y	Ab76416	Abcam	1:10,000	1:5000	1:2500
10.	Cytokeratin 17	Rabbit MAb	EPR1624Y	1960-1	Epitomics	1:10,000	1:5000	1:1500
11.	Cytokeratin 18	Mouse MAb	CY90	C 8541	Abcam	1:2000	1:2000	
12.	Cytokeratin 18	Mouse MAb	LDK18	Ab31844	Abcam	1:2000	1:2000	
13.	O-Linked N-Acetylglucosamine	Mouse MAb	RL2	MA1-072	Affinity Bio Reagents	1:3000	1:2000	
14.	Ubiquitin	Mouse MAb	1B4-UB	Ab122	Abcam	1:1000	1:2000	
15.	Cytokeratin 18 (Phospho S33)	Rabbit PAb	Polyclonal	Ab51149	Abcam	1:2000	1:5000	
16.	Phosphoserine	Mouse MAb	PSR-45	P3430	Sigma	1:3000	1:2000	

Secondary Antibodies:

Anti-mouse IgG, HRP conjugated secondary antibody from sheep (Cat. No. NA931V), anti-rabbit IgG, HRP conjugated secondary antibody from donkey (Cat. No. NA934V) were purchased from Amersham Biosciences (GE Healthcare), UK.

Negative control for IHC:

Primary monoclonal antibody was replaced by rabbit IgG from Epitomics (Cat. ISO-3855) at 1:1000 dilution.

2.1.6 Reagents for staining of PVDF membrane

BIO-RAD's Colloidal Gold total protein stain (170-6527).

2.1.7 Reagents for Immunohistochemistry

Tris-EDTA for antigen retrieval:

10 mM Tris, 2 mM EDTA, (pH-9.0).

5 X Tris-EDTA solution:

3.03 gm Tris and 1.86 gm EDTA were dissolved in deionized water and pH of the solution was adjusted to 9.0 with HCl and volume was made up to 500 ml with deionized water. 1 X solution was made by diluting 5 X solution at the time of use.

Tris buffered saline (TBS)

150 mM NaCl, 50 mM Tris (pH 7.6):

8.77 gm NaCl and 20 ml 1 M Tris (pH 7.6) were dissolved in deionized water to make final volume up to 1 liter.

Vectastain ABC kit (Vector lab PK-6102)

Diaminobenzidene substrate

0.025 % diaminobenzidene and 0.03 % H_2O_2:

25 mg diaminobenzidene was dissolved in deionized water to make final volume up to 100 ml and 30 µl H_2O_2 was added to the solution.

Poly-L-Lysine coated slides

1/100 diluted 0.1 % Poly-l-lysine (Sigma P-8920):

0.1 % poly-l-lysine was 1:100 diluted in deionized water and slides were kept in this solution for 30 min. and then dried by keeping at 37 °C overnight.

2.1.8 Reagents for silver staining

For Silver staining the reagents used were:

Destainer:

40 % Methanol and 10 % Acetic acid.

Solution 1:

0.02 % Sodium thiosulphate (Sisco Laboratories- 14455):

0.02 gm Sodium thiosulphate was dissolved in deionized water to a final volume of 100 ml.

Solution 2:

0.2 % Silver nitrate (Qualigens-15804), 0.075 % formaldegyde (Merck- 1/17837):

0.2 gm silver nitrate and 75 µl of formaldehyde were dissolved in deionized water to a final volume of 100 ml.

Note: 75 µl of formaldehyde was added at the time of staining.

Solution 3:

2 % Sodium carbonate (Qualigens- 15955), 0.05 % formaldehyde (Merck- 1/17837):

2 gm sodium carbonate, 50 µl of formaldehyde and 2 ml solution 1 were mixed in deionized water to a final volume of 100 ml.

Note: 50 µl of formaldehyde was added at the time of staining.

Stop solution:

10 % Acetic acid.

2.1.9 Reagents for Mass-spectrometry

For mass spectrometry all plastic wares used were not autoclaved.

Buffer composition for destaining of silver stained gel pieces:

i) Ammonium bicarbonate (50 mM NH_4HCO_3):

40 mg of NH_4HCO_3 was dissolved in 10 ml deionized water in an unautoclaved T50 tube.

ii) Potassium ferricyanide (K_3 [Fe (CN) $_6$]):

100 mg of K_3 [Fe (CN) $_6$] was dissolved in 10 ml deionized water in a unautoclaved T50 tube.

iii) Sodium thioslphate ($Na_2S_2O_3$):

158 mg of $Na_2S_2O_3$ dissolved in 10 ml deionized water in a unautoclaved T50 tube.

Trypsin for in-gel digestion:

10 ng/µl: 20 µg trypsin (Sigma T-6567) 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 -80 °C for further use.

Extraction buffer for extraction of peptides:

50 % Acetonitrile (Sigma 34851) and 5 % Trifluoro acetic acid (TFA) (Applied Biosystems-400003): 500 µl of 100 % acetonitrile, 450 µl of deionized water and 50 µl 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.

Matrix for loading of peptides on to the MALDI plate:

Saturated solution of α-Cyano Hydroxy Cinnamic acid (CHCA) (Brucker-201344) (Approx. 20 mg/ml) in 50 % acetonitrile and 0.1 % TFA.

All other chemicals used for the study were obtained from local companies and were of analytical grade. Deionized water was used for making the solutions.

2.2. METHODS

2.2.1 Tissue sample collection, Storage and Histology

The project was approved by the Hospital ethics Committee, Tata Memorial Centre. Prior informed consents were taken from the patients. The adjacent Normal (N), Cut margin (CM) and Tumor (T) tissues from cancer of buccal mucosa were collected from ACTREC Hospital, Navi- Mumbai. The tissue samples were snap frozen in liquid Nitrogen and kept at -80°C till further use.

Tissues were sectioned (5 µm thick) on a cryostat. The histology of the tissue samples was confirmed by the pathologist and histologically normal and tumor tissues were taken for further processing.

2.2.2 Enrichment of keratins (insoluble proteins) from tissues

Enriched keratin fractions were made from the collected tissues using essentially the protocol of Achtstaetter *et. al.* [47]. Briefly, tissues were thoroughly washed with chilled 10 mM Tris HCl and then homogenized in 10 ml buffer (96 mM NaCI, 8 mM KH_2PO4, 5.6 mM $Na_2HPO_4 \cdot 2H_20$, 1.5 mM KCl, 10 mM EDTA, 0.1 mM DTT, pH 6.8) using the Yellow^{Line} DI 25 basic homogenizer, Germany. The homogenate was centrifuged at 4,500 rpm for 30 min, 4°C in a Rota 4R centrifuge from Plasto Crafts, India. The supernatant (sup.1) was collected in another tube. Chilled detergent buffer A (10 mM Tris-HCl, 140 mM NaCI, 5 mM EDTA, 1% Triton X-100, pH 7.6) was added to the pellet and the mixture was incubated for 30 min at 4°C. It was then centrifuged for 30 min, at 4°C at 4,500 rpm. The supernatant (sup.2) was collected in another tube and the pellet was suspended in high salt buffer B (10 mM Tris-HCl, 140 mM NaCl, 1.5 M KCI, 5 mM EDTA, 0.5% (w/v) Triton X-100, pH 7.6). The

homogenate was stirred for 1h at 4°C. Homogenate was then centrifuged at 4,500 rpm at 4°C. The final pellet was dissolved in 2DE buffer and kept frozen at -20 ºC.

2.2.3 Protein estimation by TCA precipitation a modification of Peterson's method [48] or by BioRad Protein Assay based on the method of Bradford.

Protein was estimated using TCA precipitation a modification of Peterson's method. BSA (1 mg/ml) was prepared in 1:10 diluted 2DE buffer. Aliquots of these standards were taken to provide a concentration range of 20 µg to 100 µg with 20 µg intervals. Samples which were enriched for keratin and solubilized in (2DE buffer) were also diluted with water to reduce the concentration of urea in the buffer to 0.8M and to avoid excess hindrance by urea during protein estimation. The standard and test protein solutions were taken in duplicate. 1.5% of Sodium deoxycholate was added to each tube, the mixture was vortexed and kept at RT for 10 min. Hundred µl of 72% TCA was added to each tube and mixture shaken gently. The tubes were then spun at 3000 rpm for 1h at RT. The supernatants was discarded and 1 ml of 1:10 diluted sodium deoxycholate was added to each tube. The contents were gently tapped and mixture kept at RT for 10 min. Hundred µl of 72% TCA was again added to each tube and mixture spun at 3000 rpm for 1h at RT. The supernatant was discarded and pellate was dissolved in 2 ml of deionized water : CTC solution. The mixture was vortexed and kept at RT for 10 min. Five hundred µl of 1:5 diluted FC reagent was added to each tube and mixture kept at RT in dark place for 30 min. The optical density (OD) reading at 750 nm was recorded using UV spectrophotometer. Readings for standard BSA were plotted and concentration for protein in test sample was extrapolated from the graph.

Protein estimation by Bradford method was done as per BioRad protein Assay manual.

2.2.4 One Dimensional Polyacrylamide gel electrophoresis (1DE)

The enriched keratin preparations dissolved in 2DE buffer were resolved by 1DE essentially according to Laemmli [49]. After protein quantitation the normal and/or cut margin and tumor samples were diluted in 3X sample loading buffer in 2:1 ratio to make final loading concentration of loading buffer to 1X. Three µg of each sample was loaded in each well of the 10 % poly acrylamide gel (7 cm length and 1 mm of thickness). Five µl of prestained protein ladder (Fermentas- #SM0671) was also loaded in one of the wells in each gel to monitor the gel run and to identify the molecular weight of the proteins. Electrode buffer was poured in to the electrophoresis tank. A constant voltage 200V was applied and time was set to 45 min.

2.2.5 Two Dimensional Polyacrylamide gel electrophoresis (2DE)

2-DE of the proteins was done essentially according to Laemmli [49]. For 2-DE, 17 cm (pH 4.7-5.9) or 17 cm (pH 5-8) immobilized pH gradient (IPG) dry strips (BIO-RAD) were rehydrated with 30 µg of proteins enriched for keratins in 300 µl rehydration buffer. The rehydration buffer containing the protein sample was dispensed along the length of the rehydration tray, the plastic cover of IPG strip was removed and the strip was placed on the sample in the rehydration tray (gel side facing down) and incubated for 20 min at RT. After incubation, 2 ml of mineral oil was overlaid on each of the IPG strips to prevent evaporation during the process of rehydration. The IPG strips were rehydrated overnight at RT. After overnight rehydration (14-16 h), small paper wicks were cut from Whatman filter paper (Cat-1441150) and placed on both ends of electrodes in the Protean IEF focusing tray (BIO-RAD). Paper wicks were wet with 8 µl of deionized water. IPG strips were carefully removed from the rehydration tray and excess mineral oil was drained out by placing the tip of the IPG strip on a tissue paper.

The IPG strip was then placed in the IEF focusing tray (gel side facing down) and the strips were covered with 2 ml of mineral oil. After removing air bubbles beneath the IPG strips, the IEF focusing tray was placed in the Protean IEF cell (BIO-RAD) and the proteins were focused using the following program.

For 17cm IPG strip:

	Start voltage	End voltage	Time	Final Volt-Hours	
Step 1	0 V	250 V	20 min	___	Linear
Step 2	250 V	10000 V	2 h, 30 min	___	Linear
Step 3	10000 V	10000 V	___	40000 V-h	Rapid

Temperature was set at 20ºC and maximum current was set at 50 µA/strip. Separation in the second dimension was carried out in the PROTEAN® II xi Cell (BIO-RAD) on a 17 cm, 10% SDS polyacrylamide gel. After IEF, IPG strips were placed in a washed rehydration tray (gel side facing up) and equilibrated with equilibration buffer-1 and 2 one after another for 10 min each. IPG strips were then dipped in electrode buffer and placed on the 10% SDS-PAGE for the second-dimension separation in BIO-RAD PROTEAN® II xi Cell and fixed into position with 1% agarose solution. Second dimension separation was carried out at a constant voltage of 200 V. The reference dye (phenol blue) was allowed to run out and then the instrument was switched off.

Cup-loading method was used for separation of basic keratins. The IPG strip used was of pH 5-8 (17 cm). IPG strips were rehydrated overnight with 330 µl rehydration buffer. The rehydrated IPG strips were transferred from rehydration tray to the focusing tray positioning gel side up. The movable electrode assemblies were positioned on top of the strips at anode and cathode end. Sample loading cups were positioned near the anode. Enriched keratin preparation (30 µg) dissolved in rehydration buffer was loaded in the sample cup and overlaid with 50 µl mineral oil. The tray was placed in the

cooling platform in PROTEAN IEF cell and isoelectric focusing was carried out as described earlier.

2.2.6 Immunoblotting with keratin specific and post-translational modification specific antibodies

For 7 cm PVDF membrane:

After electrophoresis, proteins in the gel were transferred to the Polyvinylidene difluoride (PVDF) membrane using a Bio-Rad Minigel transfer apparatus at a constant voltage of 100 V for 1 h When using the Bio-Rad Minigel transfer apparatus the transfer buffer to be used was cooled to 4°C for at least 5 h. After transfer, membrane was rinsed with TBST once. The membranes were incubated for 1 h in blocking solution (3 % BSA dissolved in TBST). The membranes were then incubated for 1 h at RT with keratin specific antibodies dissolved in 1 % BSA. For 7 cm PVDF membrane, 2 ml of the diluted antibody was used. After three washes (10 min each) with washing buffer (TBST), each membrane was placed in 2 ml of diluted horseradish peroxidase-conjugated secondary mouse or rabbit antibodies according to the origin of primary antibodies and incubated for 1 h at RT. The dilution for mouse secondary antibody was 1:2000 while for the rabbit secondary antibody was 1:5000. After incubation, membranes were washed with washing buffer three times (10 min each). After washing, the signals were developed using the enhanced chemiluminescence ECL plus kit (GE Healthcare-RPN2132) and the same captured on X-ray film (Kodak).

For 17 cm PVDF membrane:

The proteins in the gel were transferred to the Polyvinylidene difluoride (PVDF) membrane using a Bio-Rad Maxigel transfer apparatus at a constant voltage of 70 V for 3 h. When using the Bio-Rad Maxigel transfer apparatus the transfer buffer to be used

was cooled to 4°C for at least 5 h. After transfer, membrane was rinsed with TBST once. The membranes were incubated for 1 h in blocking solution (3 % BSA in TBST). The membranes were then incubated for 1 h. at RT with keratin specific antibodies (as per the antibody dilution table in section 2.1.5) dissolved in 1 % BSA. For 17 cm PVDF membrane, 5 ml of the diluted antibody was used. After three washes (10 min each) with washing buffer (TBST), each membrane was placed in 5 ml of diluted horseradish peroxidase-conjugated secondary mouse or rabbit antibodies according to the origin of primary antibodies and incubated for 1 h at RT. The dilution for mouse secondary antibody was 1:2000 while for the rabbit secondary antibody was 1:5000. After incubation, membranes were washed with washing buffer three times (10 min each). After washing, signals were developed using the enhanced chemiluminescence ECL plus kit (GE Healthcare-RPN2132) and the same captured on X-ray film (Kodak).

2.2.7 Silver staining of 1DE and 2DE gels

Silver staining of 17 cm gel:

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 RT. The washing was repeated three times. After washing, the gel was incubated with 100 ml Solution 1 for 1 min on the shaker followed by a quick deionized water wash. The gel was then incubated with 500 ml Solution 2 for 20 min on the shaker followed by a quick deionized water wash. 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. The stained gel was scanned on the BIO-RAD GS-800 densitometer and the image was filtered using PD-QUEST software.

2.2.8 Mass spectrometry for identification of proteins from 1DE and 2DE gel plugs

Proteins in the silver stained spots were identified by MALDI-TOF-TOF analysis using Ultraflex II, MALDI-TOF-TOF instrument from Brucker, Germany. The in-gel digestion of proteins with trypsin in the silver stained spots was carried out essentially according to Shevchenko *et.al*. [216]. 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. Proteins in the 1DE resolved gel plugs were reduced using 10 mM DTT and then alkylated with 55 mM iodoacetamide. For protein in 2DE silver stained gels reduction and alkylation is done in the equilibration buffers used for 2DE [49]. The proteins in the plugs were then trypsinized overnight with 10 ng/µl trypsin in 25 mM ammonium bicarbonate (10 µl/plug) and the peptides were recovered by extraction with 40 µl of 50% ACN and 5% TFA (2-3times). Tryptic protein digests were reconstituted in 10% ACN with 0.1% TFA solvent before subjecting them to mass spectrometry analysis.

Mass calibration was carried out using peptide mixture of five known peptides spanning mass range of 757-3147 m/z and error was kept to less than 10 ppm. Accelerating voltage of 25 kV was applied to the first TOF tube. The MS data was acquired in an automated manner using a solid state NdYAG laser at 337 nM. The resulting MS data was analysed using Flex analysis 3.0 (Brucker Daltonik, Germany) software and was acquired using Biotools 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 0.7 to 1 Da for MS/MS analysis.

2.2.9 Immunohistochemistry (IHC)

Frozen tissues from normal, cut margin and tumor area were collected and fixed in buffered formalin (10 % formalin, 0.025 M sodium dihydrogen phosphate and 0.046 M disodium hydrogen phosphate in deionized water) overnight. Fixative was removed by keeping tissue in tap water followed by dehydration of tissues in 70 % ethanol, 80 % ethanol, 95 % ethanol and 100 % ethanol (5 min each). Tissues were kept in xylene for 30 min twice and then in mixture of xylene and paraffin (1:1) for 1h. Tissues were paraffinized in fresh paraffin for 2 h twice and then embedded in paraffin blocks. Sections from these blocks were examined by the pathologist and areas identified for generating a tissue microarray. Cores of 1 mm size from the donor blocks were transferred in an array format on to the recipient block made of low melting paraffin wax, (Gold Standard Peel-A-Way Micro Cut Paraffin from Polysciences, Inc. USA (Cat. 24198). Five micron sections from the array were placed on poly-L-lysine coated slides. The sections were deparaffinised and rehydrated prior to endogenous blocking of peroxidase with 3% H_2O_2 in methanol. The nonspecific antigenic sites were blocked using horse serum and the sections were then incubated at 4 $^{\circ}$C overnight with specific antibodies for keratins K4, K13, K14, K16 and K17. The bound primary antibody was detected using Vectastain Universal ABC kit (PK-6200). The signal was developed using 0.025% diaminobenzidene with 0.03% H_2O_2. The stained sections were counterstained using Mayer's haematoxylin and finally mounted with DPX (mixture of distyrene, a plasticizer, and xylene). In the negative control, primary monoclonal antibody was replaced by rabbit IgG from Epitomics (Cat. ISO-3855) with 1:1000 dilution. Images were captured using Zeiss AxioImager Z1 microscope.

Chapter 3

RESULTS

Chapter 3. RESULTS

3.1 Identification of keratins in normal, cut margin and tumor samples of cancer of the gingivo buccal complex by PMF- MS/MS of 1DE resolved proteins

Fifteen sets of tissue samples of gingivo buccal complex containing normal/cut margin and tumor area were collected and snap frozen in liquid nitrogen for further mass spectrometry analysis. The tissue samples chosen were such that there were five samples each of a well differentiated (WD), moderately differentiated (MD) and poorly differentiated (PD) tumor for the analysis. Five micron sections of the frozen tissues were stained with Haematoxylin and Eosin (H&E). The histology of the stained sections was evaluated by the pathologist for the grade of differentiation and for marking the tumor / normal/stromal areas. Details of the samples used for the mass spectrometry analysis and pictures of the H&E stained sections are given in the **Table A1** and **Fig. A1** respectively in the Appendix.

The remaining tissues were processed for making enriched keratin preparations. The keratin preparations were resolved by 1DE and visualized by silver staining. **Figures 6 to 9** show the silver staining pattern of the enriched keratin preparations and mass spectrometry identity obtained for the samples. Each of the gels was sliced from Mr 72 kDa to 34 kDa as shown in the figure and the gel pieces used for in-gel digestion to obtain peptides. The peptides so obtained were subjected to MS. The proteins identified by PMF were confirmed by MS-MS by taking at least 5 most abundant peaks for that keratin. Acceptable mass spectrometry information was obtained from 12 N/CM and 13 T tissue samples. The detailed mass spectrometry data is given in **Table A2** in the Appendix.

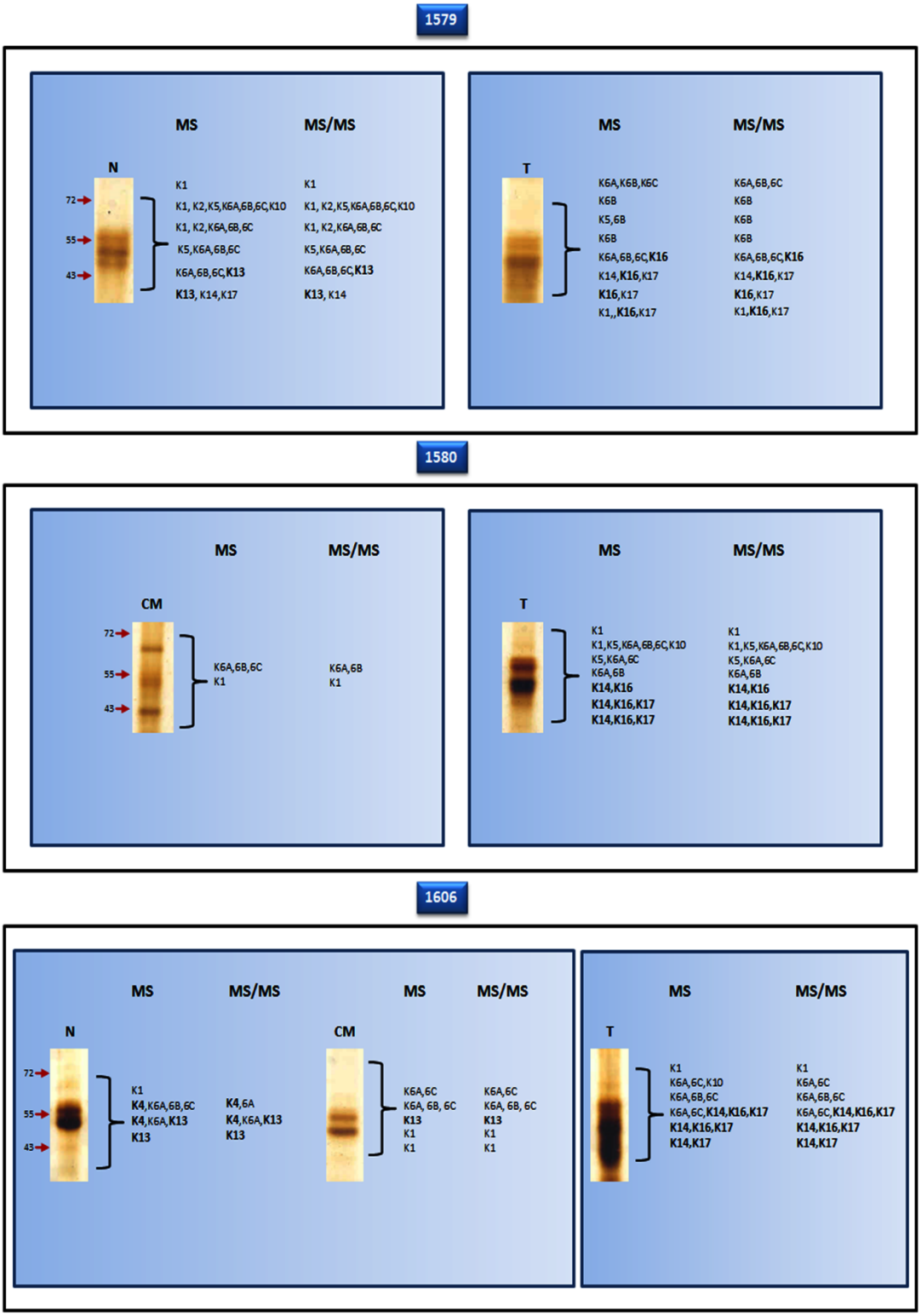


Figure 6. 1DE-Silver stained gel images for the enriched preparations of keratins from samples 1579, 1580 and 1606 used for mass spectrometry analysis. Figure shows the 1DE silver stained gel profile for normal (N)/cut margin (CM) and tumor (T) samples The sample numbers are given above each panel and the lanes corresponding to the normal (N)/ cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa and the proteins identified by MS/MS-MS analysis.

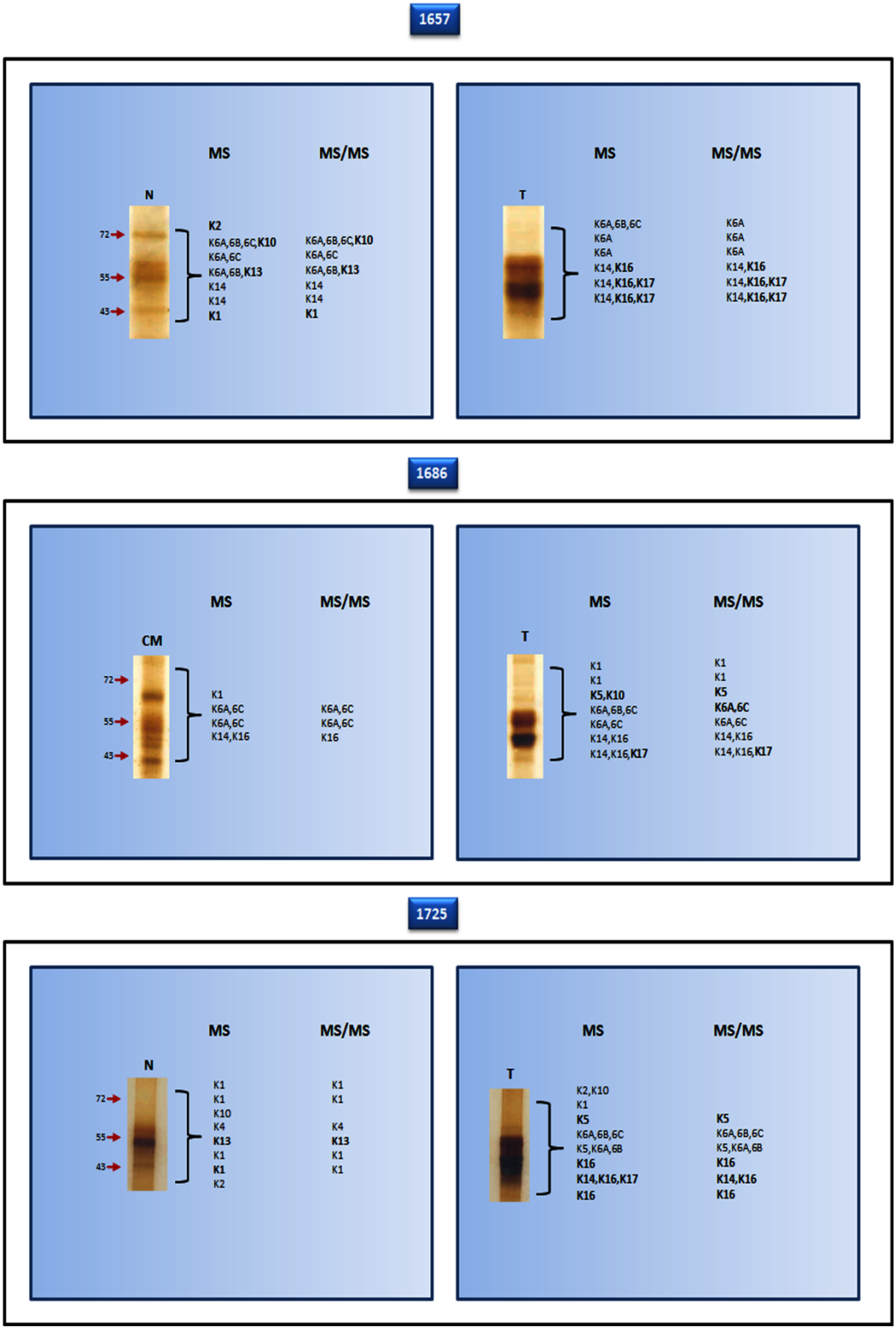


Figure 7. 1DE-Silver stained gel images for the enriched preparations of keratins from samples 1657, 1686 and 1725 used for mass spectrometry analysis. Figure shows the 1DE silver stained gel profile for normal (N)/cut margin (CM) and tumor (T) samples The sample numbers are given above each panel and the lanes corresponding to the normal (N)/ cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa and the proteins identified by MS/MS-MS analysis.

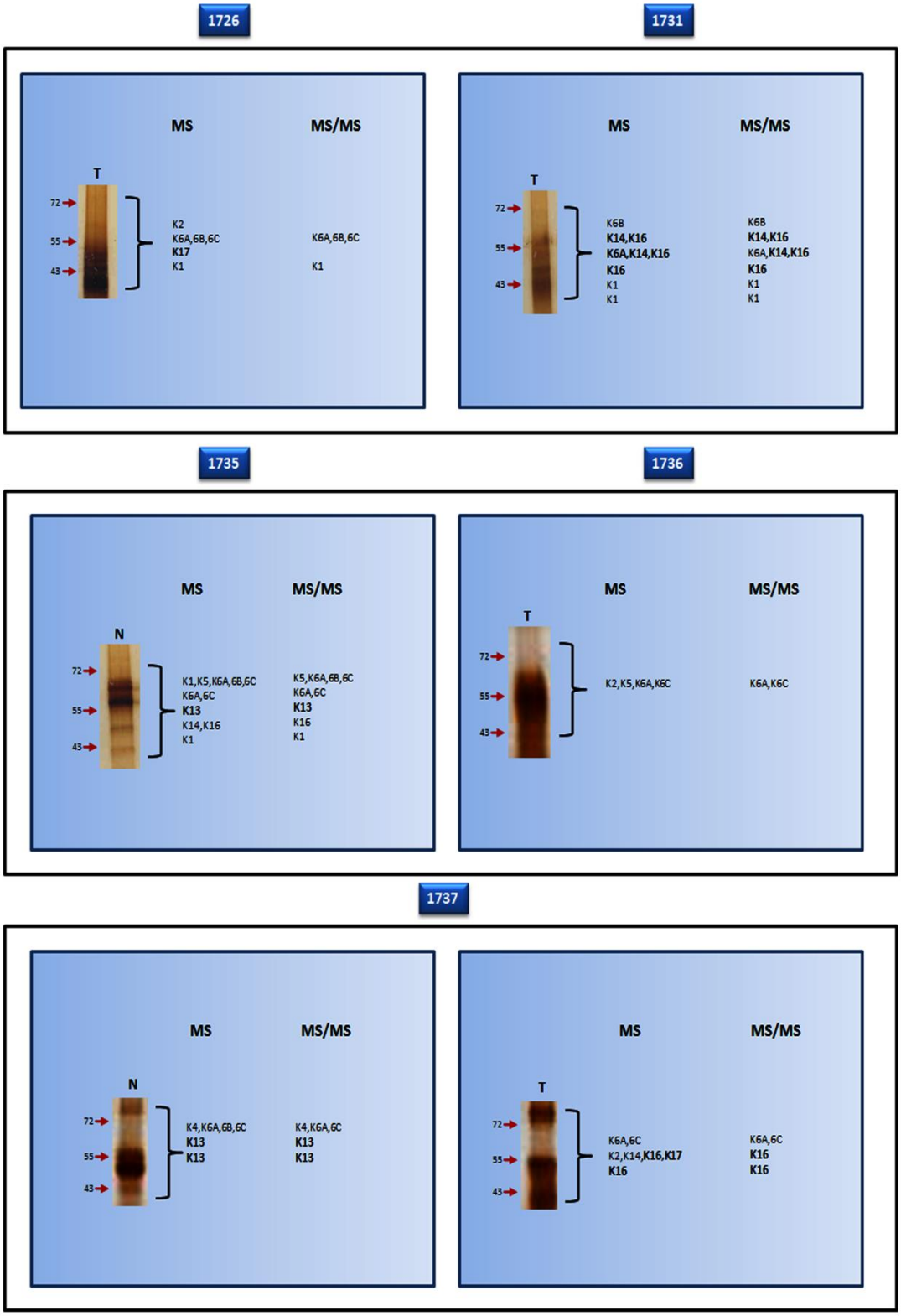


Figure 8. 1DE-Silver stained gel images for the enriched preparations of keratins from samples 1726, 1731, 1735, 1736 and 1737 used for mass spectrometry analysis. Figure shows the 1DE silver stained gel profile for normal (N)/cut margin (CM) and tumor (T) samples The sample numbers are given above each panel and the lanes corresponding to the normal (N)/ cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa and the proteins identified by MS/MS-MS analysis.

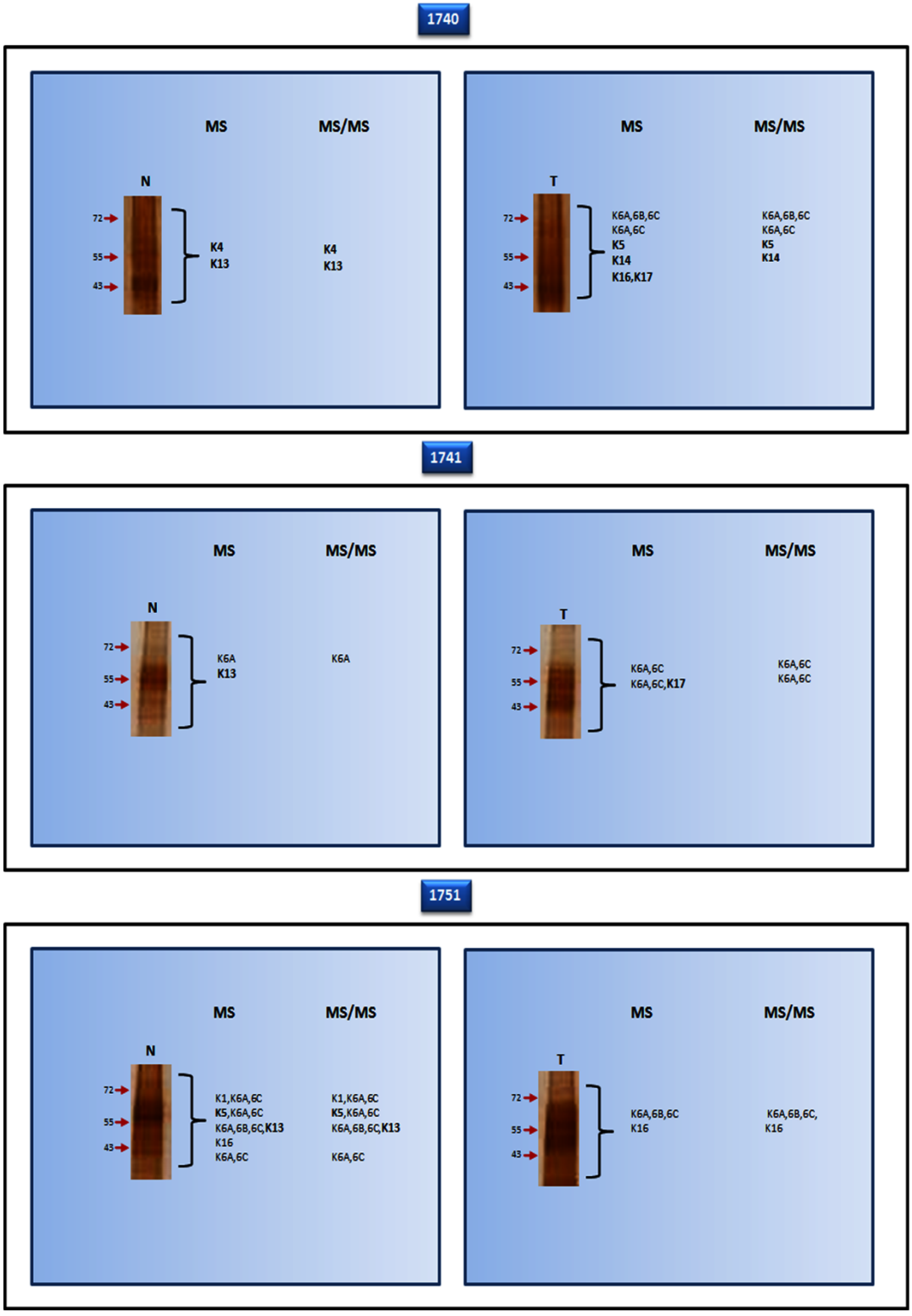


Figure 9. 1DE-Silver stained gel images for the enriched preparations of keratins from samples 1740, 1741 and 1751 used for mass spectrometry analysis. Figure shows the 1DE silver stained gel profile for normal (N)/cut margin (CM) and tumor (T) samples The sample numbers are given above each panel and the lanes corresponding to the normal (N)/ cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa and the proteins identified by MS/MS-MS analysis.

Figure 10 summarizes the expression of keratins in the samples from which MS data was obtained. Detailed MS analysis is given in Table A2 in the Appendix.

Keratins	1579N	1580CM	1606N	1606CM	1657N	1686CM	1725N	1735N	1737N	1740N	1741N	1751N	1579T	1580T	1606T	1657T	1686T	1725T	1726T	1731T	1736T	1737T	1740T	1741T	1751T
K1	□●	□●	□	□●	□●	□	□●	□●				□●	△●	△●	△●		△●	△	△●	△●					
K2	□●				□		□											△	△		△	△			
K4			□●				□●		□●	□●															
K5	□●							□●				□●	△	△●			△●	△●			△		△●		
K6A	□●	□●	□●	□●	□●	□●		□●	□●		□●	□●	△●	△●	△●	△●	△●	△●	△●	△●	△●	△●	△●	△●	△●
K6B	□●	□●	□	□●	□●			□●	□			□●	△●	△●	△●	△	△	△●	△●	△●			△●		△●
K6C	□●	□	□	□●	□●	□●		□●	□●			□●	△●	△●	△●	△	△●	△●	△●		△●	△●	△●	△●	△●
K10	□●				□●		□							△●	△		△	△							
K13	□●		□●	□●	□●		□●	□●	□●	□●	□	□●													
K14	□●				□●	□		□					△●	△●	△●	△●	△●	△●		△●		△	△●		
K16						□●		□●				□	△●	△●	△●	△●	△●	△●		△●		△●	△		△●
K17	□												△●	△●	△●	△●	△●	△	△			△	△	△	

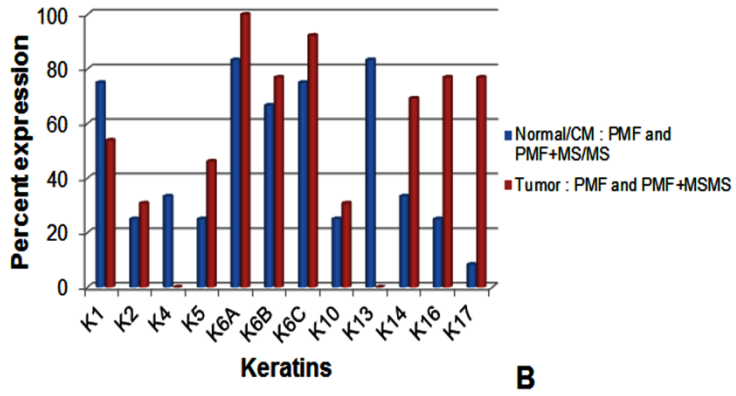
□ MS identity confirmed in N/CM △ MS identity confirmed in T ● MS/MS identity confirmed

Figure 10. Summary of keratin expression in the tissue samples from the gingivo buccal complex as identified by mass spectrometry. The normal (N), cut margin (CM), and tumor (T) samples are given in the upper panel and the keratins identified are shown on the left hand side panel. Symbol □ indicates the keratins identified by PMF in normal/ cut margin (N/CM) samples; Δ indicates the keratins identified by PMF in tumor (T) tissue samples and ● indicates the MS/MS confirmation of the keratins.

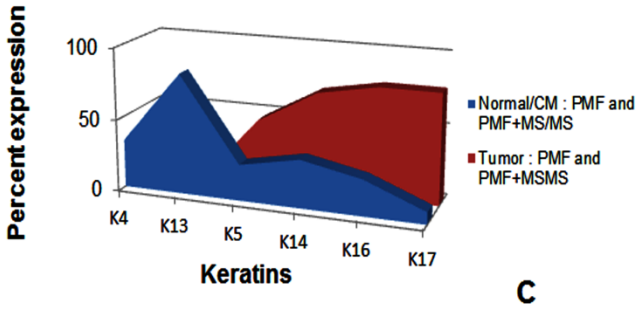
Figure 11 shows the percentage of samples in which different keratins were identified. It is seen that K4 and K13 were identified mainly in normal/cut margin tissues while it was absent in all 13 tumor tissues. Keratin 5 was identified in 25% (3/12) normal/cut margin tissues and in 46% (6/13) tumor tissue samples. K13 was identified in 83 % (10/12) adjacent normal/cut margin tissues while it was absent in all 13 tumor tissues. The expression in normal/cut margin tissues for K14, K16 and K17 was identified in 33 % (4/12), 25 % (3/12) and 8 % (1/12) samples for respective keratins while its expression in tumor was identified in 69 % (9/13), 77 % (10/13) and 77 % (10/13) samples for respective keratins. There is a clear difference in the expression pattern for K4, K13, K14, K16 and K17 in the normal/cut margin and tumor samples. The expression of K1, K2, K5, K6A, K6B, K6C and K10 was observed in normal/cut margin as well as in tumor tissue samples to almost the same extent.

Keratins	Normal/Cut Margin (N=12)			Tumor (N=13)		
	PMF	PMF-MS/MS	%	PMF	PMF-MS/MS	%
K1	2	7	75	1	6	53
K2	2	1	25	4	0	30
K4	0	4	33	0	0	0
K5	0	3	25	2	4	46
K6A	0	10	83	0	13	100
K6B	2	6	66	2	8	76
K6C	2	7	75	1	11	92
K10	1	2	25	3	1	30
K13	1	9	83	0	0	0
K14	2	2	33	1	8	69
K16	1	2	25	1	9	77
K17	1	0	8	5	5	77

A



B



C

Figure 11. Comparison of keratin expression in N/CM and T tissues as obtained by mass spectrometry. (A) Table shows the percentage expression of different keratins in normal/ cut margin (n=12) and tumor (n=13) samples given in Figure1. (B) Bar diagram shows the percentage (%) expression of different keratins in normal/ cut margin and tumor samples. (C) Graphical representation of the major differentially expressed keratins K4, K13, K14, K16 and K17 between normal/ cut margin and tumor samples. K5 has been shown as a keratin which is near equally expressed in normal and transformed tissues

3.2 Evaluation of the keratin expression profile by 1DE-immunostaining

Mass spectrometry results have identified keratin 1, 2, 4, 5, 6A, 6B, 6C, K10, K13, K14, K15, K16 and K17, but K8 and K18 were not seen although they have been reported in literature using immunohistochemistry. In order to check the status of all these keratins by another technique western blotting was performed using antibodies specific to each of the keratins. Antibodies to post translational modifications (PTMs), O-GlcNAc,

Ubiquitin, Phosphoserine and Phosphoserine-33 were also used. Enriched keratin preparations from the **f**ifteen samples were resolved by 1DE, the proteins were blotted onto the PVDF membrane and expression of each of the proteins/PTMs was assessed by immunostaining. Details of the antibodies used are given in **Table 4**. The data obtained is given below-

Figure 12 shows the 1DE profile for K1 (Mr 66 kDa) using K1 specific mouse monoclonal antibody **AE1** (ab9286) from Abcam. K1 expression is seen in 8 of 15 normal/cut margin tissue samples and in 9 of 15 samples. There is no significant difference in the keratin 1 expression between normal/cut margin and tumor samples as also seen by mass-spectrometry experiments.

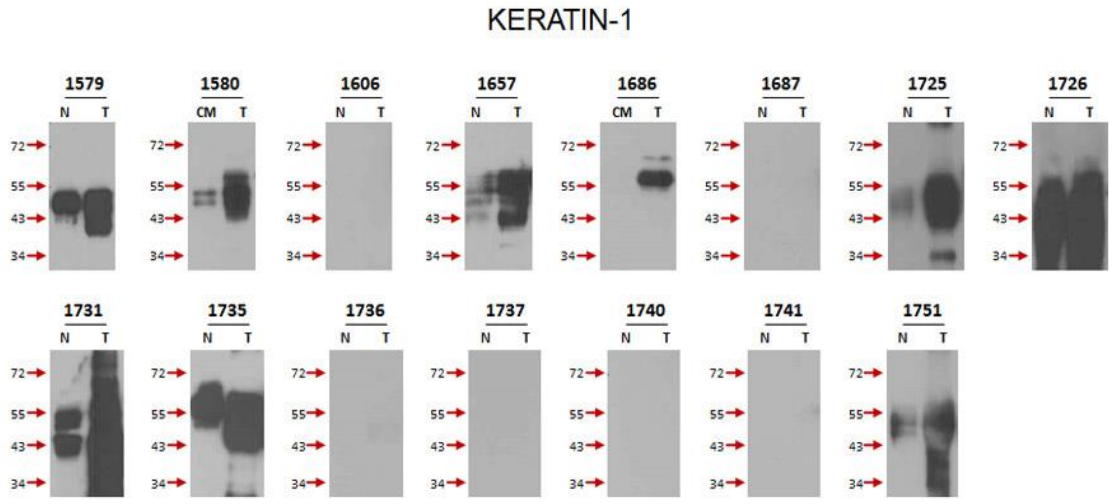


Figure 12. 1DE-western blotting profile for enriched keratin preparations using keratin 1 specific antibody. The sample numbers are given above each panel and the lanes corresponding to the normal (N), cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa.

Figure 13 shows the 1DE expression profile for K4 (57.7kDa) using K4 specific rabbit monoclonal antibody **EP1599Y** from Epitomics. K4 is seen in all 15 samples in the

normal/cut margin tissues, while it was seen in only 5 of 15 tumor samples. The trend of K4 downregulation in tumor matches with the trend with mass spectrometry data.

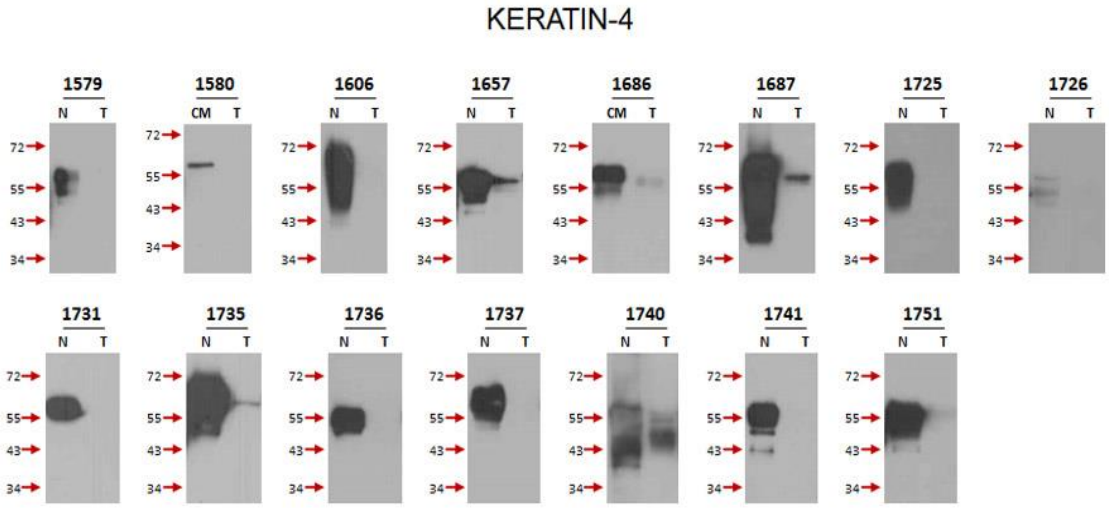


Figure 13. 1DE-western blotting profile for enriched keratin preparations using keratin 4 specific antibody. The sample numbers are given above each panel and the lanes corresponding to the normal (N), cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa.

Figure 14 shows the 1DE expression profile of Keratin 5 (62.6 kDa) using a rabbit monoclonal antibody **EPR1600Y** from Epitomics. K5 is seen in 5 of 15 normal/cut margin tissues and 10 of 15 tumor tissue samples. This data matches with the mass spectrometry data.

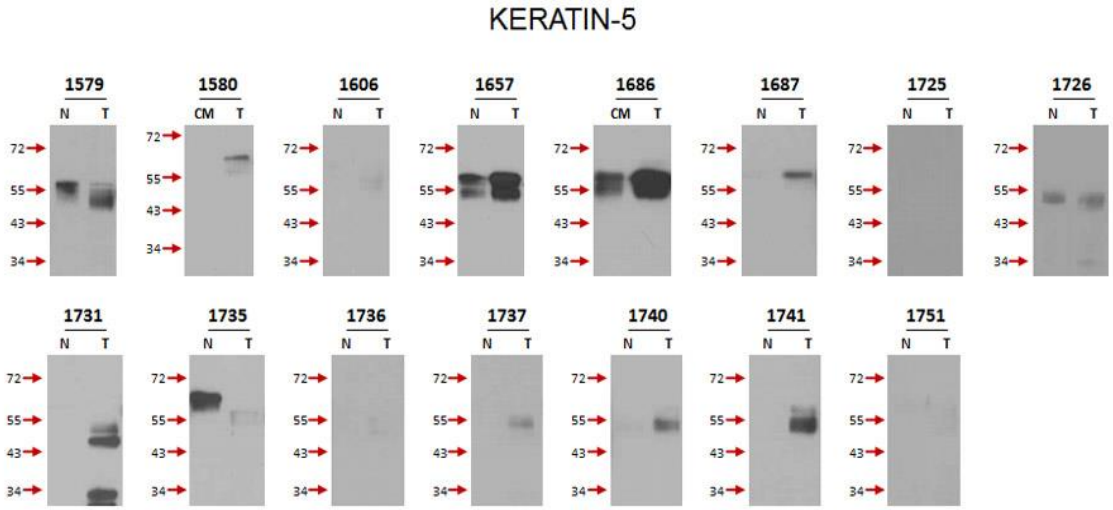


Figure 14. 1DE-western blotting profile for enriched keratin preparations using keratin 5 specific antibody. The sample numbers are given above each panel and the lanes corresponding to the normal (N), cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa.

Figure 15 shows the expression profile of keratin 6A (60kDa) using K 6A specific rabbit monoclonal antibody **EPR1603Y** from Epitomics. K 6A is seen in 14 of 15 samples in the normal/cut margin tissues and 13 of 15 tumor samples. These results match those with the mass spectrometry data in majority of samples.

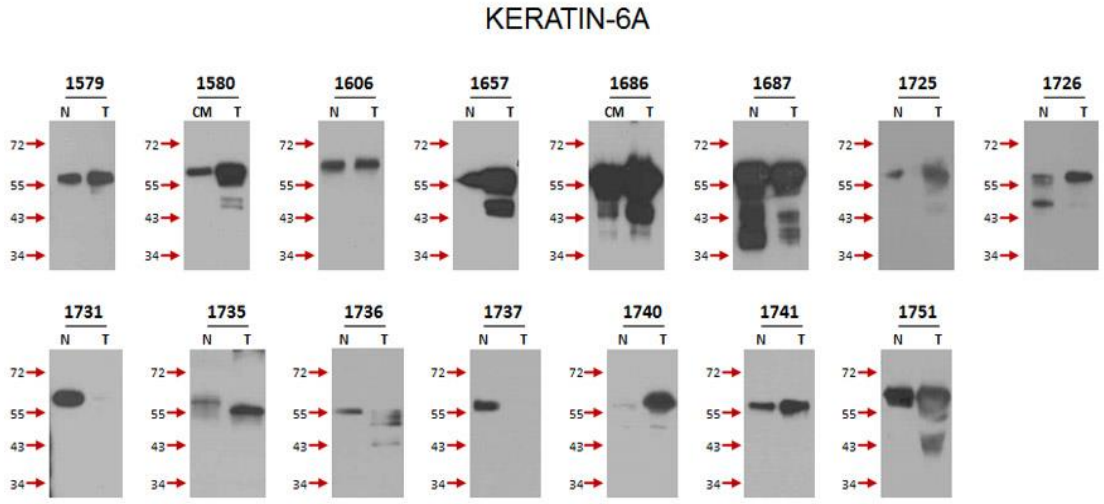


Figure 15. 1DE-western blotting profile for enriched keratin preparations using keratin 6 specific antibody. The sample numbers are given above each panel and the lanes corresponding to the normal (N), cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa.

Figure 16 shows 1DE profile of keratin 8 (53.7 kDa) using K8 specific mouse monoclonal antibody M20 from Sigma. K8 is seen in 6 of 15 normal/cut margin tissues and in 13 of 15 tumor tissue samples. K8 was not identified either in normal/cut margin or tumor tissues by mass spectrometry.

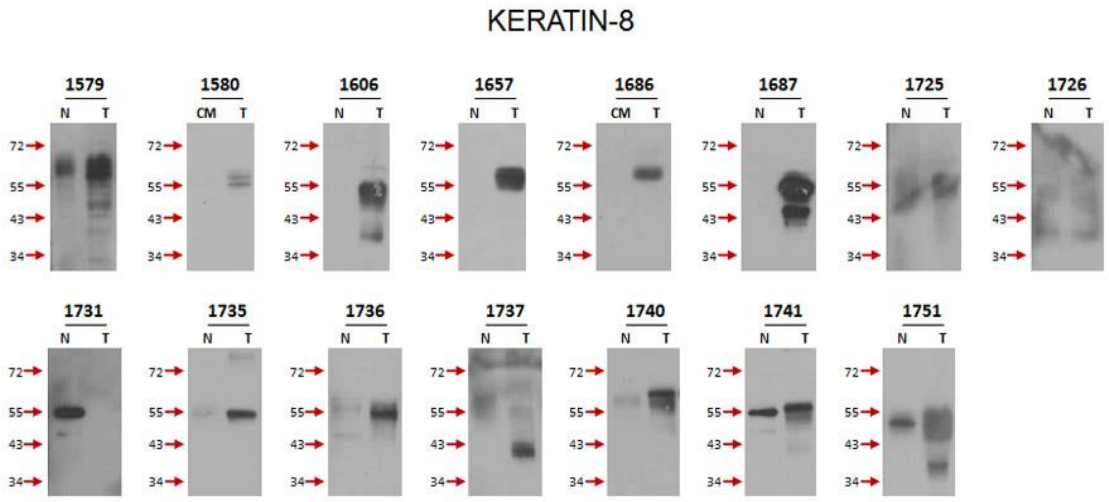


Figure 16. 1DE-western blotting profile for enriched keratin preparations using keratin 8 specific antibody. The sample numbers are given above each panel and the lanes corresponding to the normal (N), cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa.

Figure 17 shows the 1DE expression profile of keratin 10 (59.kDa) using K10 specific mouse monoclonal antibody **RKSE60** from Abcam. Expression of K10 was seen in 3 of 15 in normal/cut margin tissues and 7 of 15 tumor samples. The results show that K10 is expressed in more number of tumor samples. K10 has been reported to be overexpressed in lesions of the oral mucosa and oral squamous cell carcinoma by immunohistochemistry [23, 205, 206]. K10 was seen in only a few samples by mass spectrometry.

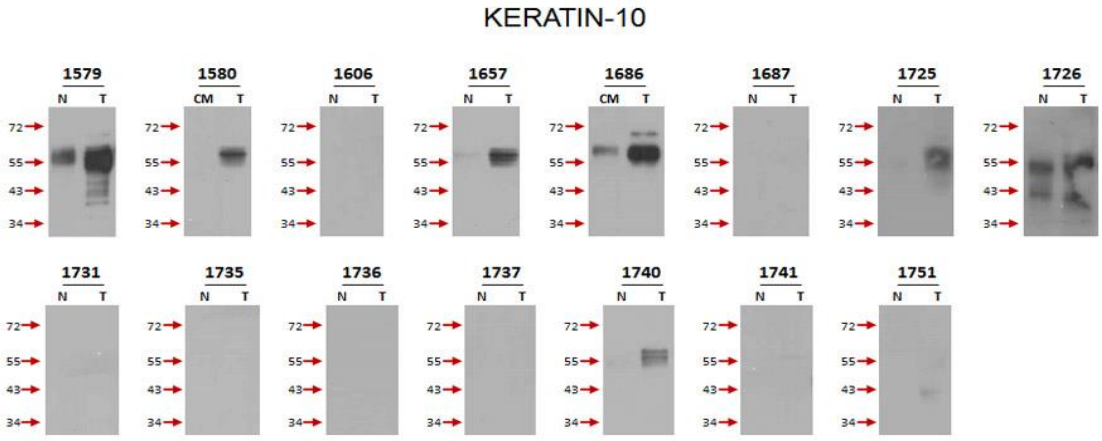
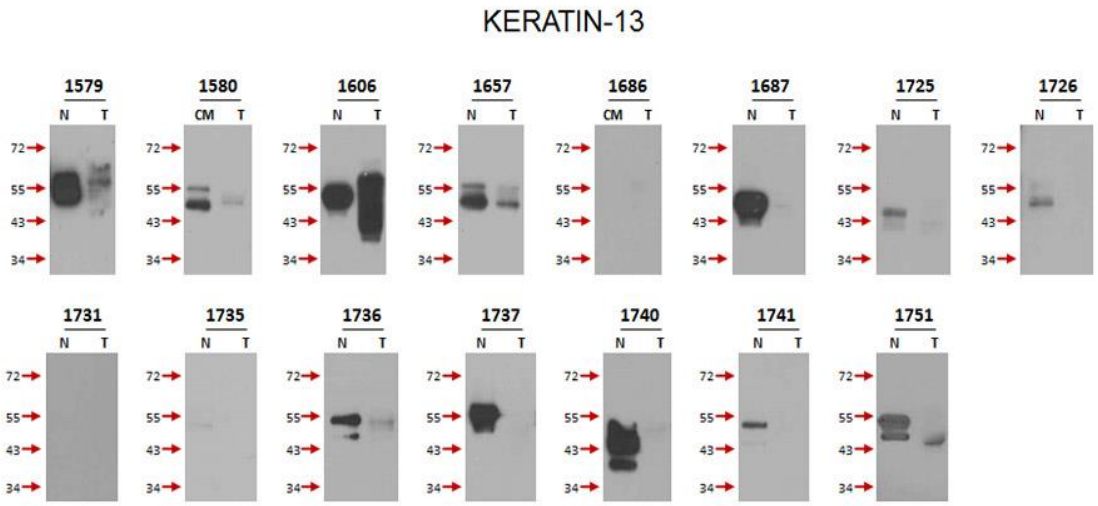


Figure 17. 1DE-western blotting profile for enriched keratin preparations using keratin 10 specific antibody. The sample numbers are given above each panel and the lanes corresponding to the normal (N), cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa.

Figure 18 shows the 1DE expression profile of Keratin 13 (49.kDa) using K13 specific rabbit polyclonal antibody from Abcam. K13 was seen in 12 of 15 samples in the normal/cut margin tissues and there were faint signals in 6 of 15 tumor samples. The result suggests loss/ downregulation of K13 in tumor cells. K13 pairs with K4 and both are found in suprabasal layer of oral mucosa. The mass spectrometry results given in



the previous section also supports the western blotting data.

Figure 18. 1DE-western blotting profile for enriched keratin preparations using keratin 13 specific antibody. The sample numbers are given above each panel and the lanes corresponding to the normal (N), cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa.

Figure 19 shows the 1DE expression profile of keratin 14 (51.9 kDa) using K14 specific rabbit monoclonal antibody **EP1612Y** from Epitomics. K14 expression is seen in 13 of 15 normal/cut margin tissues and 8 of 15 tumor samples. The western blotting result shows more K14 staining in normal samples and less in tumor samples as opposed to the mass spectrometry results.

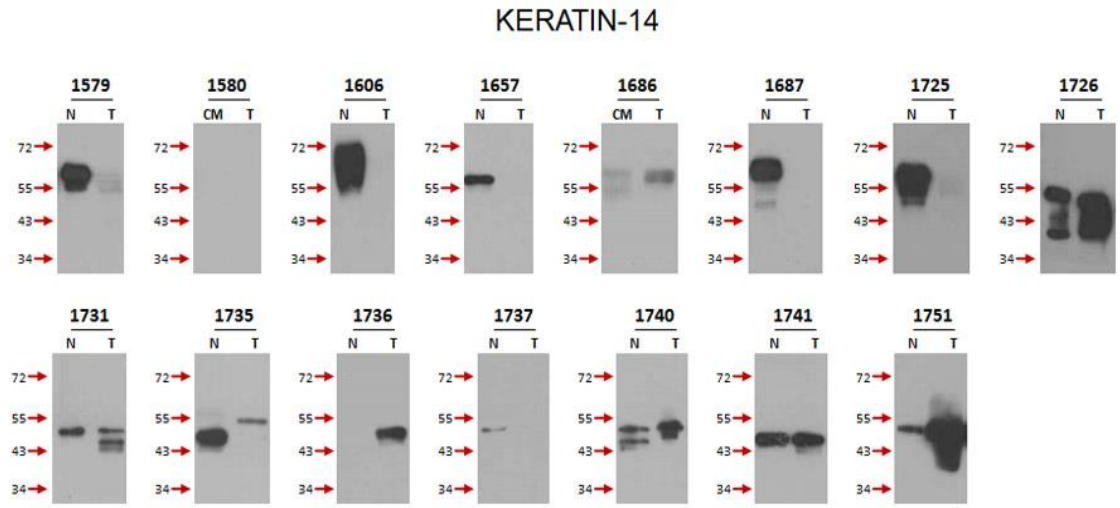


Figure 19. 1DE-western blotting profile for enriched keratin preparations using keratin 14 specific antibody. The sample numbers are given above each panel and the lanes corresponding to the normal (N), cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa.

Figure 20 shows the 1DE profile of keratin 16 (51.6 kDa) using K16 specific rabbit monoclonal antibody **EP1615Y** from Epitomics. K16 is seen in 3 of 15 samples in the normal/cut margin tissues and in 12 of 15 tumor samples. Results suggest that there is increased expression of K16 in cancer of gingivo buccal complex similar to that seen by mass spectrometry results.

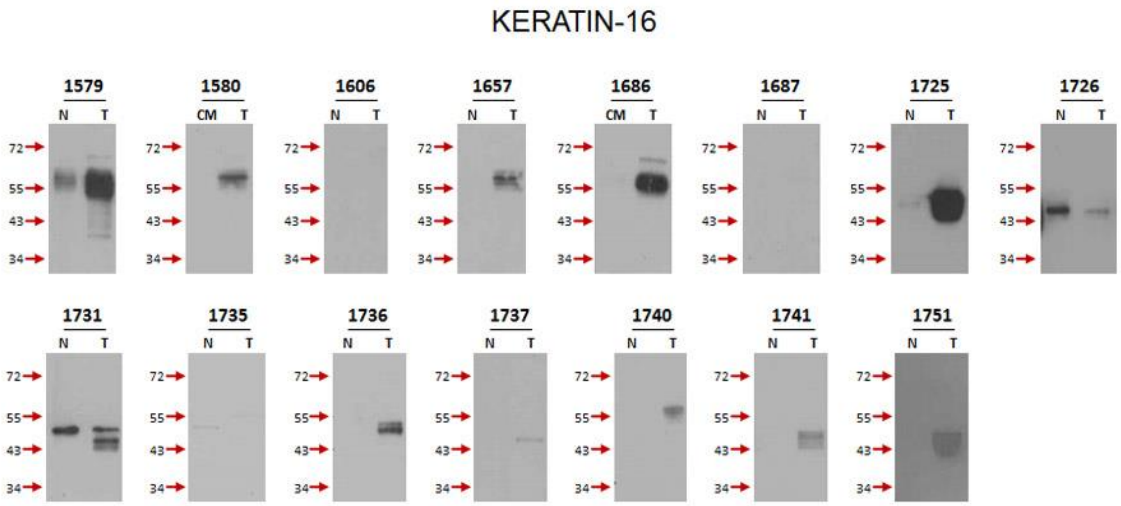


Figure 20. 1DE-western blotting profile for enriched keratin preparations using keratin 16 specific antibody. The sample numbers are given above each panel and the lanes corresponding to the normal (N), cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa.

Figure 21 shows the 1DE profile of keratin 17 (48.4kDa) using K17 specific rabbit monoclonal antibody **EPR1624Y** from Epitomics. K17 is seen in 8 of 15 normal/cut margin samples and in 12 of 15 samples, suggesting its increased expression in cancer of gingivo buccal complex. K17 partners with K6 to form keratin heterodimers.

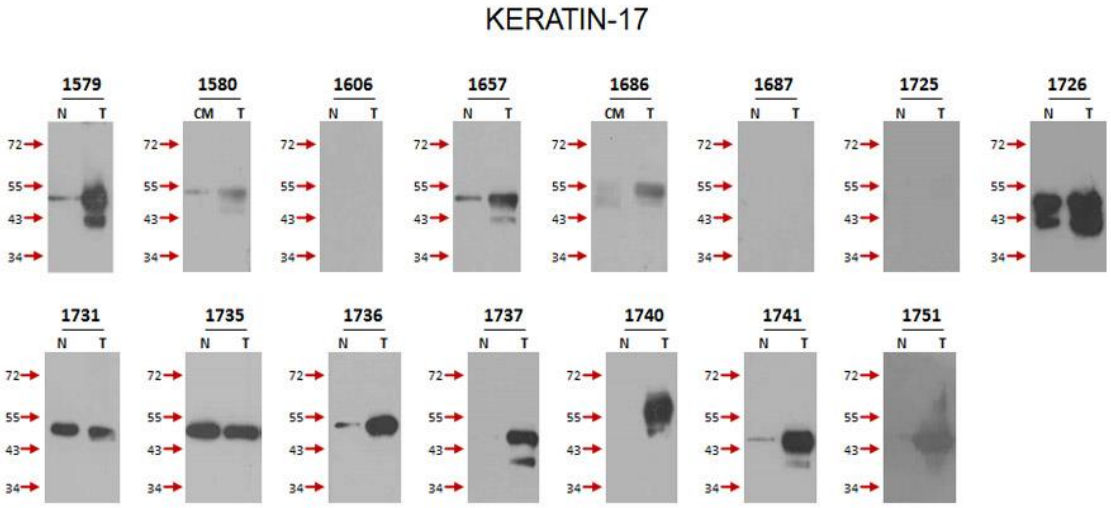


Figure 21. 1DE-western blotting profile for enriched keratin preparations using keratin 17 specific antibody. The sample numbers are given above each panel and the lanes corresponding to the normal (N), cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa.

Figure 22 shows the 1DE expression profile of keratin 18 (48 kDa) using K18 specific mouse monoclonal antibody **CY90** from Sigma. K18 is seen in 13 of 15 normal/cut margin tissues and in all the 15 tumor samples. The western blotting result shows aberrant expression of K18 in gingivo buccal complex tumor as previously reported in literature [26, 27]. The 1DE data did not match the mass spectrometry results wherein K18 was not identified in any of the normal or tumor samples analysed. K18 forms a heterodimer with K8 protein and the pair is regarded as a marker for simple epithelia. It has been shown to be aberrantly expressed in oral mucosa which is stratified epithelium.

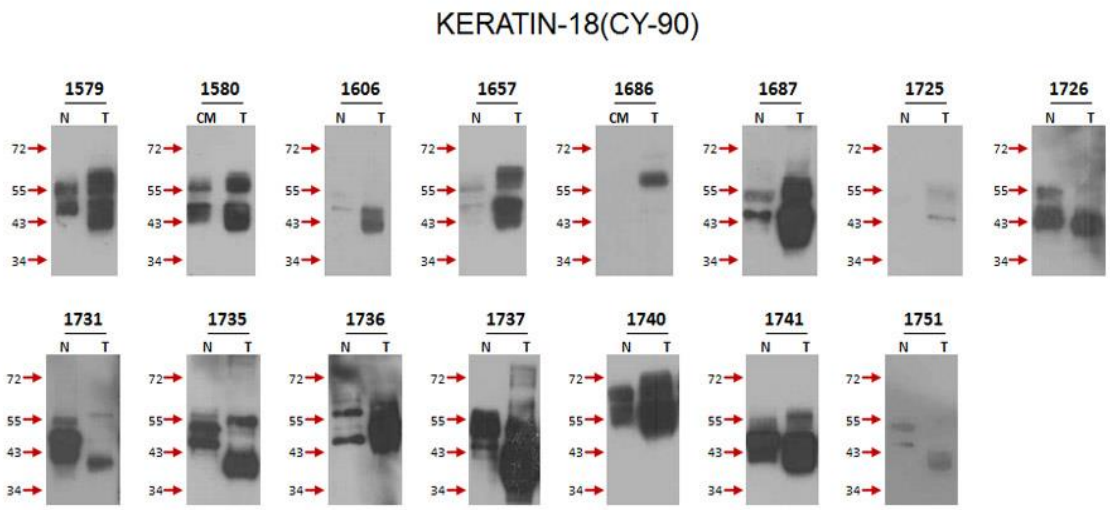


Figure 22. 1DE-western blotting profile for enriched keratin preparations using keratin 18 specific antibody (CY90). The sample numbers are given above each panel and the lanes corresponding to the normal (N), cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa.

To further check if the clone of K18 is showing cross reactivity with a related protein, another antibody for K18 (LDK18) was used for western blotting.

Figure 23 shows the 1DE expression profile for keratin 18 (48.kDa) using K18 specific mouse monoclonal antibody LDK18 from Abcam. K18 was seen in 7 of 15 samples in the normal/cut margin tissues and in 14 of 15 tumor samples. The western blotting results show the aberrant expression of K18 in tumor as shown above using K18 (CY90) antibody.

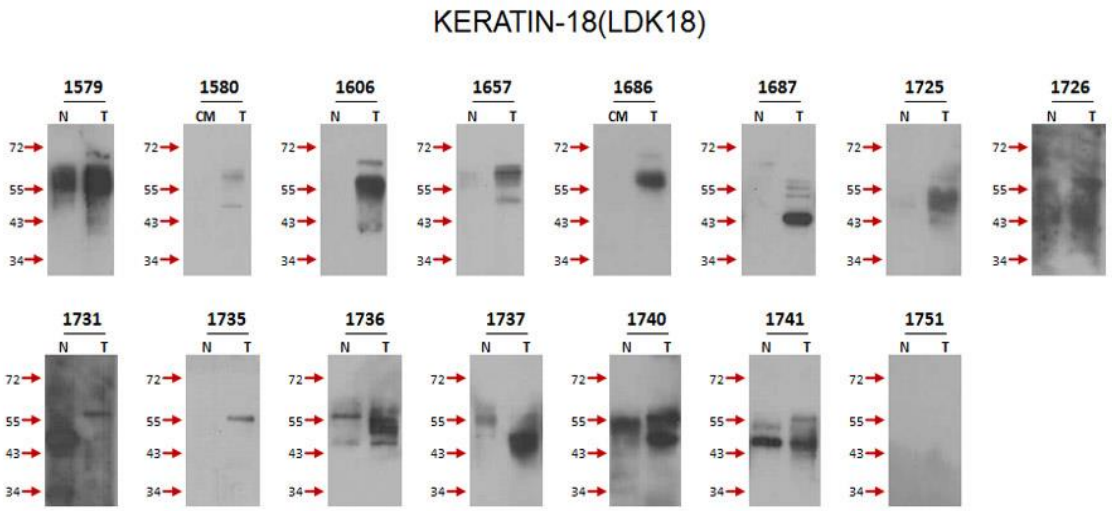


Figure 23. 1DE-western blotting profile for enriched keratin preparations using keratin 18 specific antibody, (LDK18). The sample numbers are given above each panel and the lanes corresponding to the normal (N), cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa.

Figure 24 shows the 1 DE expression pattern of O-GlcNAc in the samples using O-GlcNAc specific mouse monoclonal antibody **RL2** from Affinity Bioreagents. Signals for O-linked glycosylation were seen in 14 of 15 samples in the normal/cut margin tissues and in 8 of 15 tumor samples.

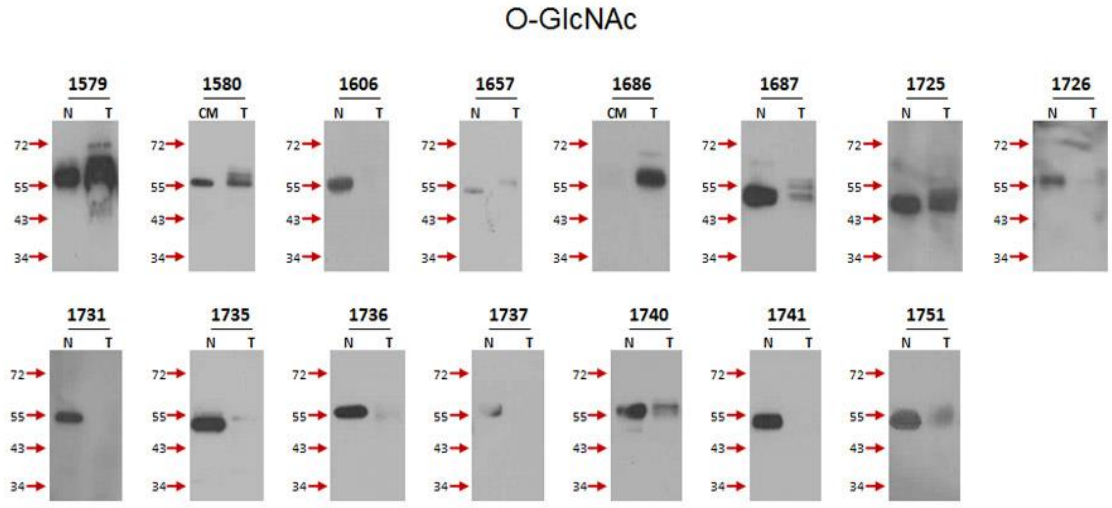


Figure 24. 1DE-western blotting profile for enriched keratin preparations using keratin O-GlcNAc specific antibody. The sample numbers are given above each panel and the lanes corresponding to the normal (N), cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa.

Figure 25 shows the profile of signals obtained for ubiquitinylation using ubiquitin specific mouse monoclonal antibody **1B4-UB** from Abcam. Signals were seen in 4 of 15 normal/cut margin tissues and in 12 of 15 tumor samples. Ubiquitinylation is observed in more number of tumor samples but no ubiquitinylation trail is seen as frequently seen during ubiquitinylation mediated protein degradation.

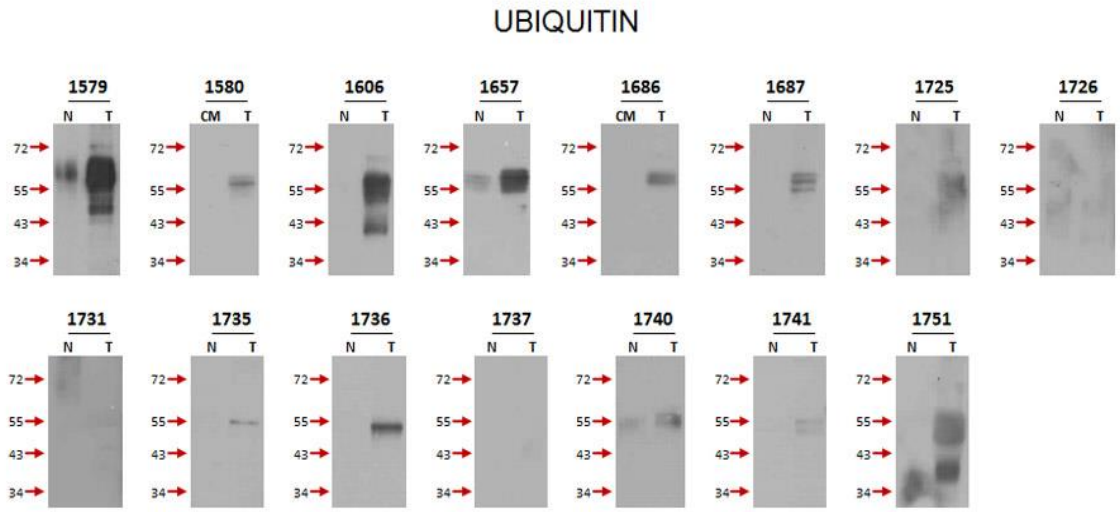


Figure 25. 1DE-western blotting profile for enriched keratin preparations using ubiquitin specific antibody. The sample numbers are given above each panel and the lanes corresponding to the normal (N), cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa.

Figure 26 shows the phosphorylation at serine residues in the samples using phosphoserine specific mouse monoclonal antibody **PSR-45** from Sigma. Phosphorylation is seen in 9 of 15 samples in the normal/cut margin tissues and in 14 of 15 tumor samples. The results show the serine phosphorylation is higher in keratins from tumor samples.

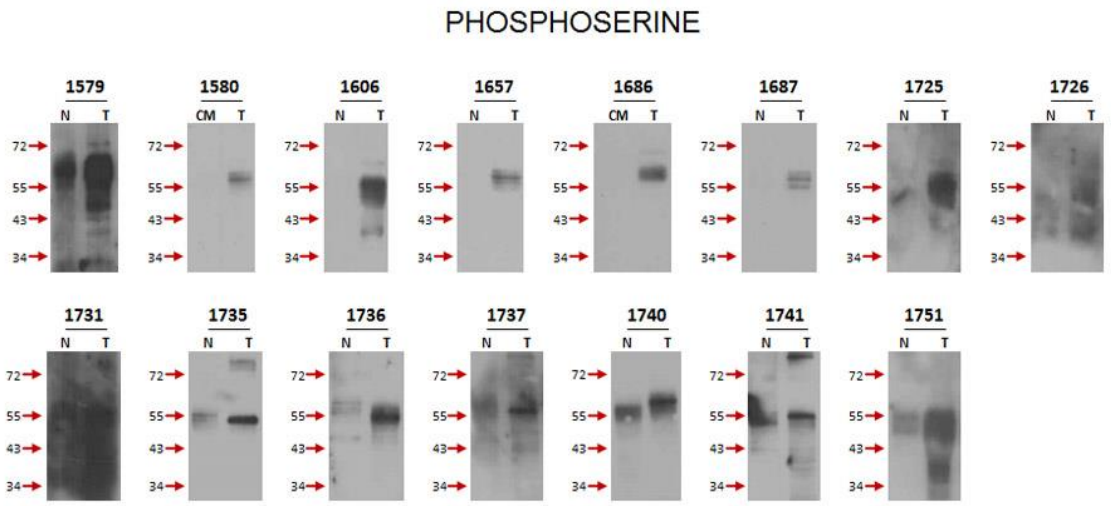


Figure 26. 1DE-western blotting profile for enriched keratin preparations using phosphoserine specific antibody. The sample numbers are given above each panel and the lanes corresponding to the normal (N), cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa.

K18 is known to be serine phosphorylated at S33 and S52 residue. Phosphorylation on S33 residue was therefore assessed. **Figure 27** shows the signals for phosphorylation of serine-33 residue of K18 in the samples using PS-33 specific rabbit polyclonal antibody from Abcam. Signals were seen in 2 of 15 normal/cut margin tissue samples and in 3 of 15 tumor samples. The antibody is raised against the S33 residue of K18 protein and the results shows the PS33 staining for only few samples.

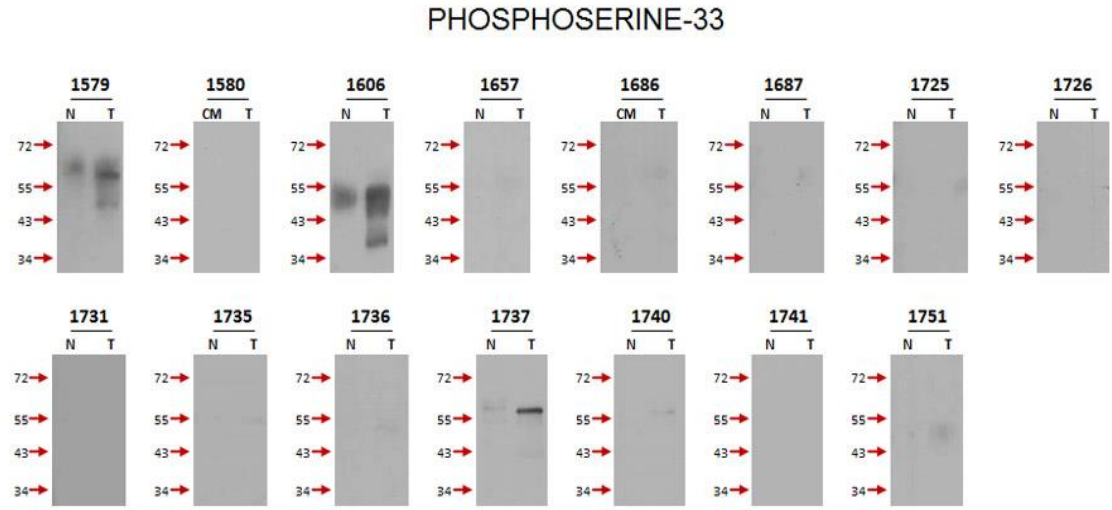


Figure 27. 1DE-western blotting profile for enriched keratin preparations using phosphoserine-33 specific antibody. The sample numbers are given above each panel and the lanes corresponding to the normal (N), cut margins (CM) and tumor (T) samples are indicated along with position of the molecular weight standards used in kDa.

Figure 28 summarises the expression profile of the keratins as obtained by 1DE-western blotting. It is seen that K4, K13 and K14 are expressed in more normal/cut margin samples while K5, K16 and K17 are seen predominantly in tumors. It is to be noted that K14 was observed in more tumors samples by 1DE-mass spectrometry.

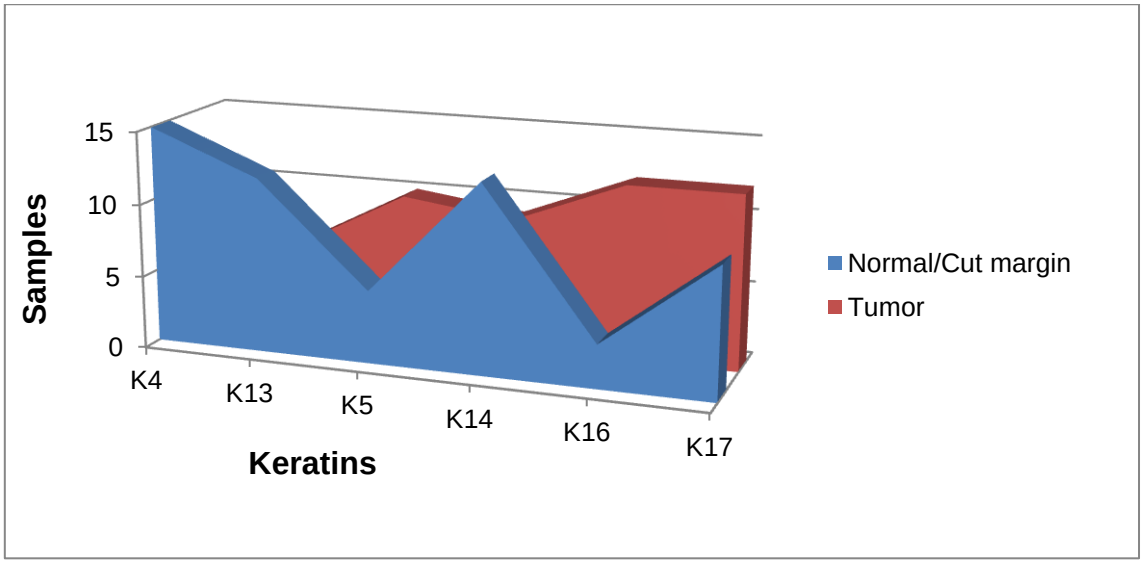


Figure 28. Graphical summary for the expression of keratins using 1DE-western blotting.

3.3. 2DE-Silver staining, western blotting, and PMF-MS for localization and identification of keratins.

To verify the results emerging from 1DE mass spectrometry and western blotting data, 2DE mass spectrometry was undertaken. Enriched keratin preparations from two sample sets i.e. 1737 and 1740 normal, cut margin and tumor were resolved by 2DE and the protein in the spots subjected to trypsin digestion followed by mass spectrometry. In parallel, 2DE gels were run for these samples for immunoblotting with antibodies to K4, K13, K14, K16 and K17, the keratins which are differentially expressed as per mass spectrometry data in section 3.1. **Figures 29(A-C) and (D-F)** show position of the acidic keratins K13, K14, K16 and K17 as determined by MS analysis.in 1737 and 1740 N, CM and T samples respectively. Detailed mass spectrometry data is given in **Appendix Table A3**. The isoelectric point and molecular weight of these keratins matched with reported literature. The position of K13 is clearly seen in the gels for samples 1737N and 1740N (Fig. 30A, 30D). In the gels for 1737T and 1740T shown in Fig. 30C and 30F, it is interesting to note that the band for K13 is absent in the tumor samples and keratin 14, 16 and 17 are clearly seen in tumor tissue profiles. In the cut margins there are two patterns seen. In sample 1737CM (Fig. 30B) the straight line of spots corresponding to K13 and below that the curved band of spots corresponding to K14, 16 and 17 are seen indicating that in the histologically normal cut margins there are molecular changes which are attributable to the transformation process. In sample 1740 CM (Fig. 30E), only the spots corresponding to K13 are seen.

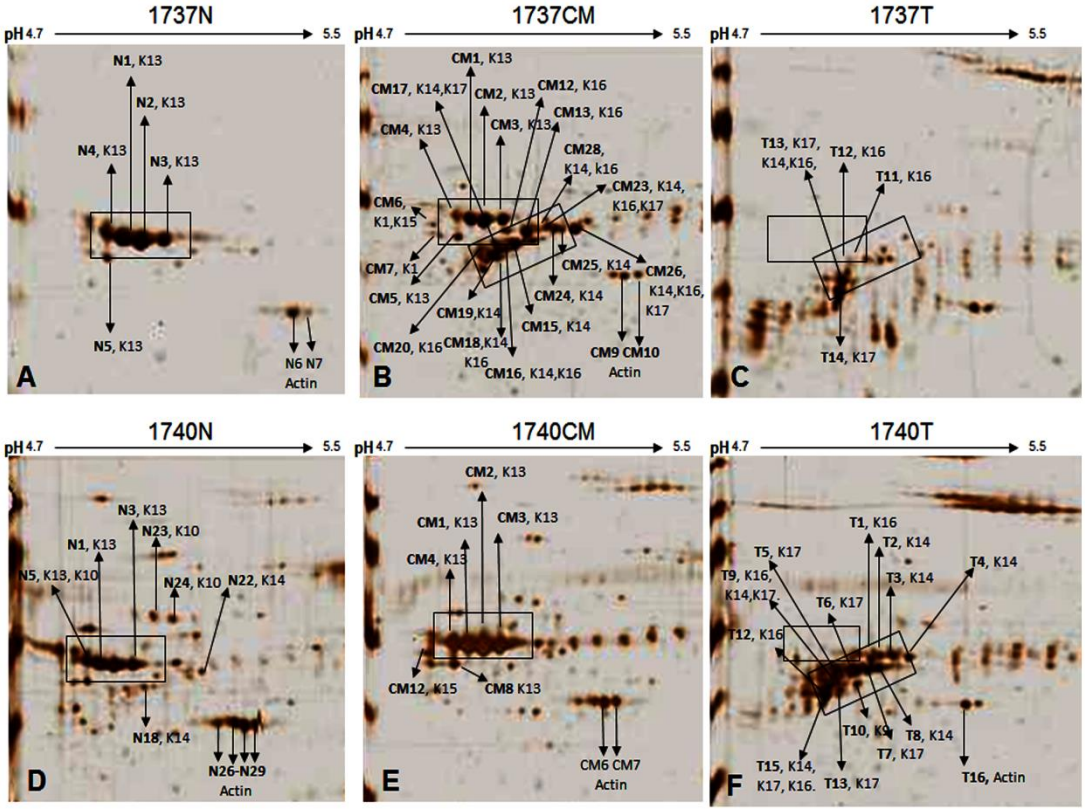


Figure 29. 2DE-Silver stained gel patterns for the keratins showing the position of the different keratins identified by mass spectrometry. A, B and C are the profiles for sample 1737 N, CM and T respectively. D, E and F show the profiles for sample1740 N, CM and T respectively. The pH range for IEF is shown above the gel profile. The spot numbers (Nx), (CMx) and (Tx) are followed by name of the keratin as identified by mass spectrometry. The details of the mass spectrometry data are given in Appendix Table 3**.**

The position of the keratins is further confirmed by 2DE western blotting. **Figures 30 to 35** show the silver gel patterns juxtaposed to the western blots for samples 1737 and 1740 N, CM and T probed with the antibodies to K13, K14, K16 and K17.

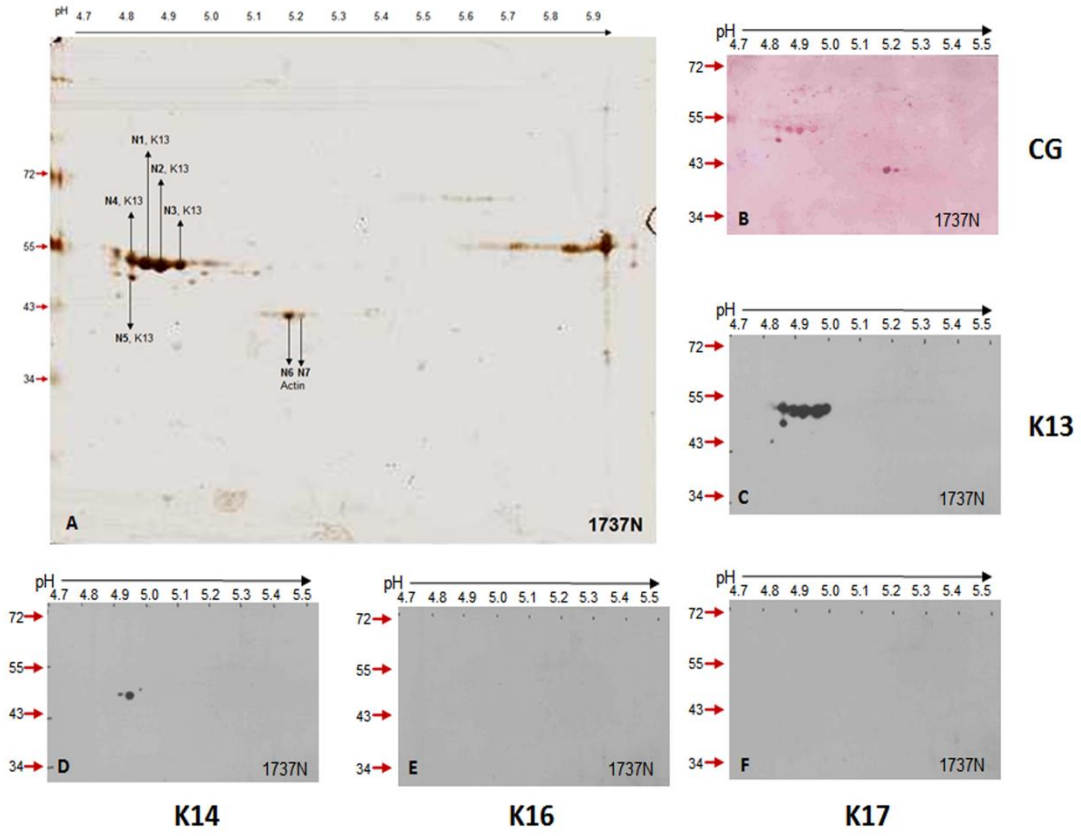


Figure 30. Validation of keratin identities obtained by 2DE-MS by Western Blotting using antibodies specific for K13, K14, K16 and K17. Figures show the profiles obtained for sample 1737N. (A) 2DE-silver stained gel profile and the location of the keratins. (B) Colloidal stained 2DE blot to show the position of the resolved proteins. (C) Autograph of the blot stained with antibody to K13, (D) for K14, (E) for K16, and (F) for K17. The pH range used for IEF is given above the gels and blots. On the silver gel profile, the spot number (Nx) and the identity of the keratin (Kx) as per mass spectrometry data in Appendix Table A3 is shown. The 2DE blot was cut beyond pH 5.5 to minimize antibody requirements.

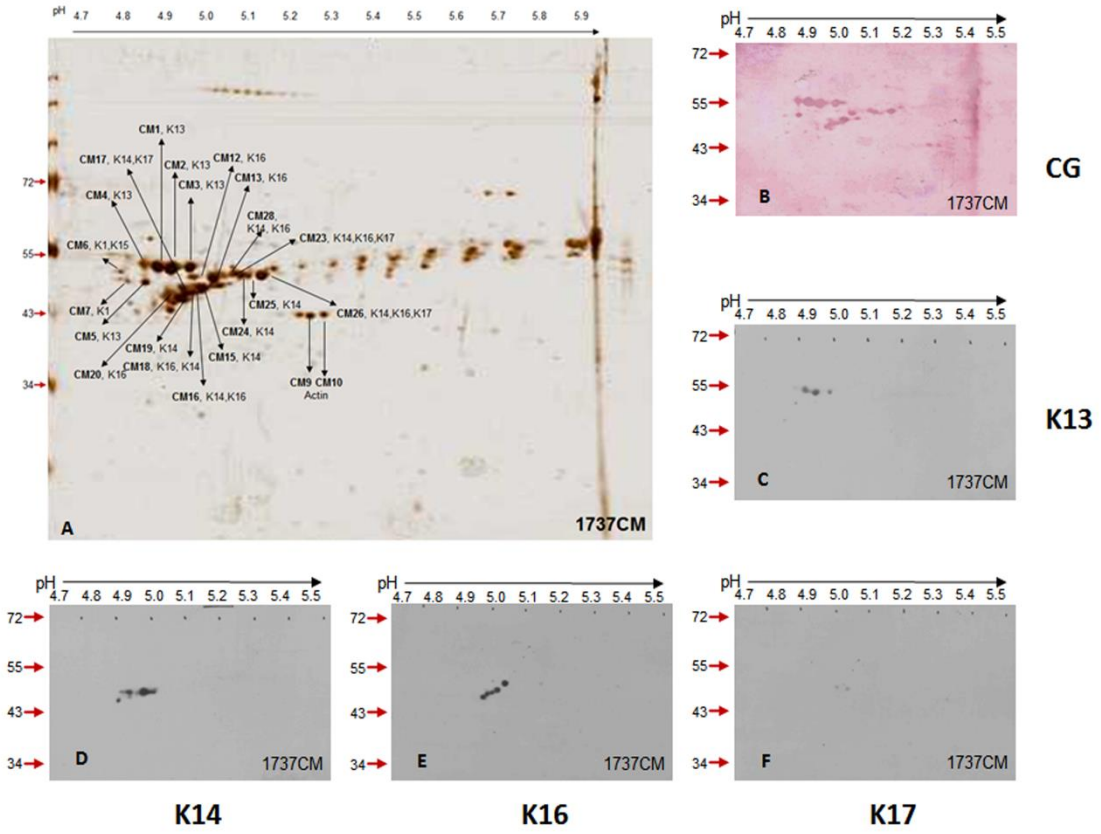


Figure 31. Validation of keratin identities obtained by 2DE-MS by Western Blotting using antibodies specific for K13, K14, K16 and K17. Figures show the profiles obtained for sample 1737 CM. (A) 2DE-silver stained gel profile and the location of the keratins. (B) Colloidal stained 2DE blot to show the position of the resolved proteins. (C) Autograph of the blot stained with antibody to K13, (D) for K14, (E) for K16, and (F) for K17. The pH range used for IEF is given above the gels and blots. On the silver gel profile, the spot number (CMx) and the identity of the keratin (Kx) as per mass spectrometry data in Appendix Table A3 is shown. The 2DE blot was cut beyond pH 5.5 to minimize antibody requirements.

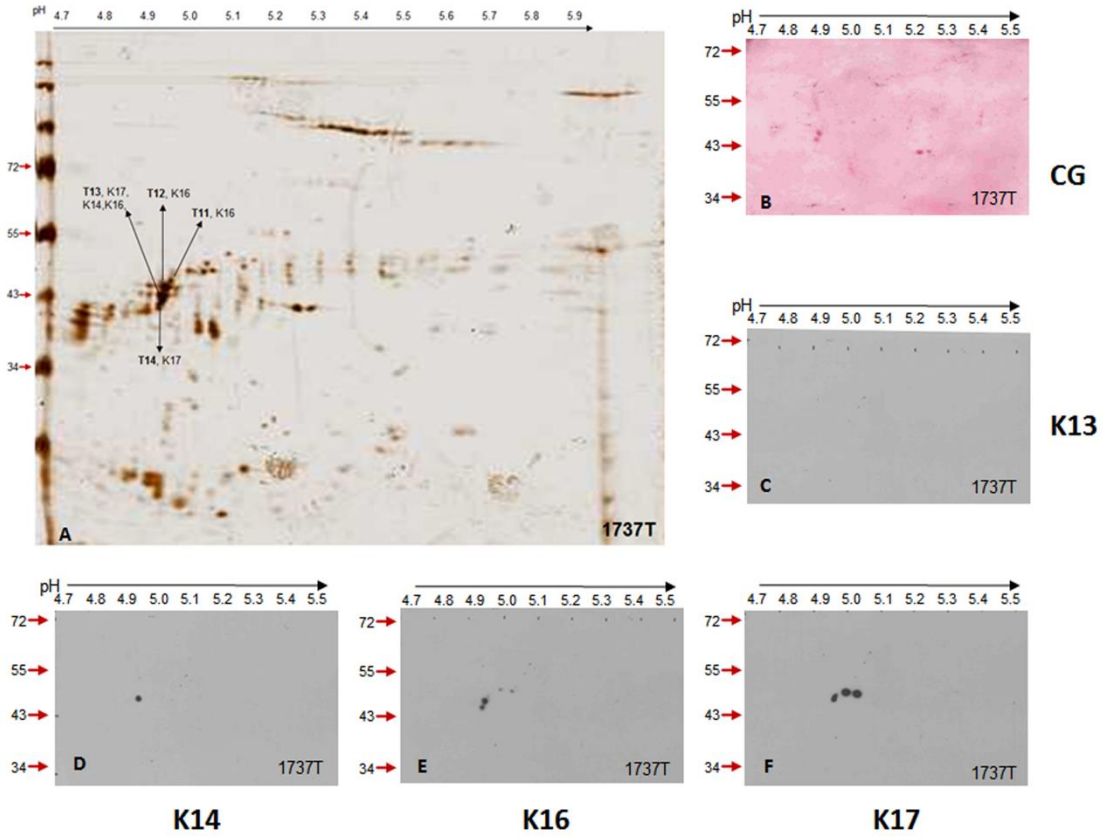


Figure 32. Validation of keratin identities obtained by 2DE-MS by Western Blotting using antibodies specific for K13, K14, K16 and K17. Figures show the profiles obtained for sample 1737 T. (A) 2DE-silver stained gel profile and the location of the keratins. (B) Colloidal stained 2DE blot to show the position of the resolved proteins. (C) Autograph of the blot stained with antibody to K13, (D) for K14, (E) for K16, and (F) for K17. The pH range used for IEF is given above the gels and blots. On the silver gel profile, the spot number (Tx) and the identity of the keratin (Kx) as per mass spectrometry data in Appendix Table A3 is shown. The 2DE blot was cut beyond pH 5.5 to minimize antibody requirements.

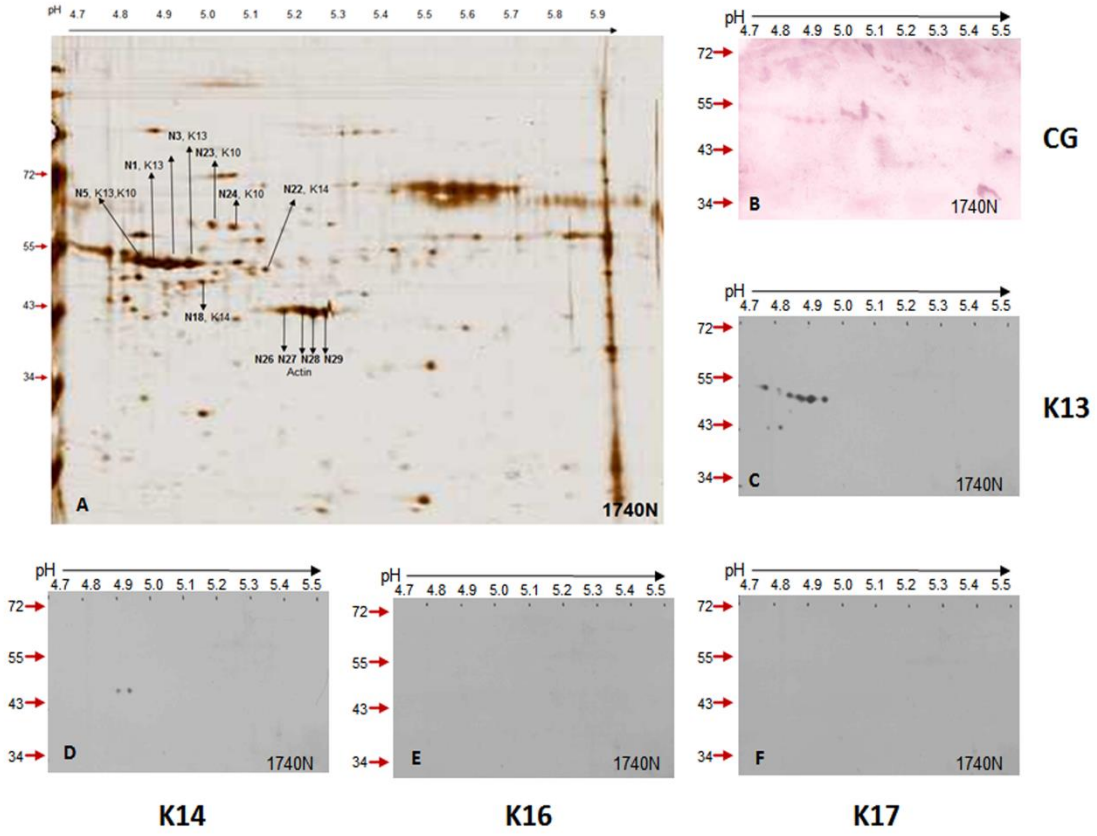


Figure 33. Validation of keratin identities obtained by 2DE-MS by Western Blotting using antibodies specific for K13, K14, K16 and K17. Figures show the profiles obtained for sample 1740 N. (A) 2DE-silver stained gel profile and the location of the keratins. (B) Colloidal stained 2DE blot to show the position of the resolved proteins. (C) Autograph of the blot stained with antibody to K13, (D) for K14, (E) for K16, and (F) for K17. The pH range used for IEF is given above the gels and blots. On the silver gel profile, the spot number (Nx) and the identity of the keratin (Kx) as per mass spectrometry data in Appendix Table A3 is shown. The 2DE blot was cut beyond pH 5.5 to minimize antibody requirements.

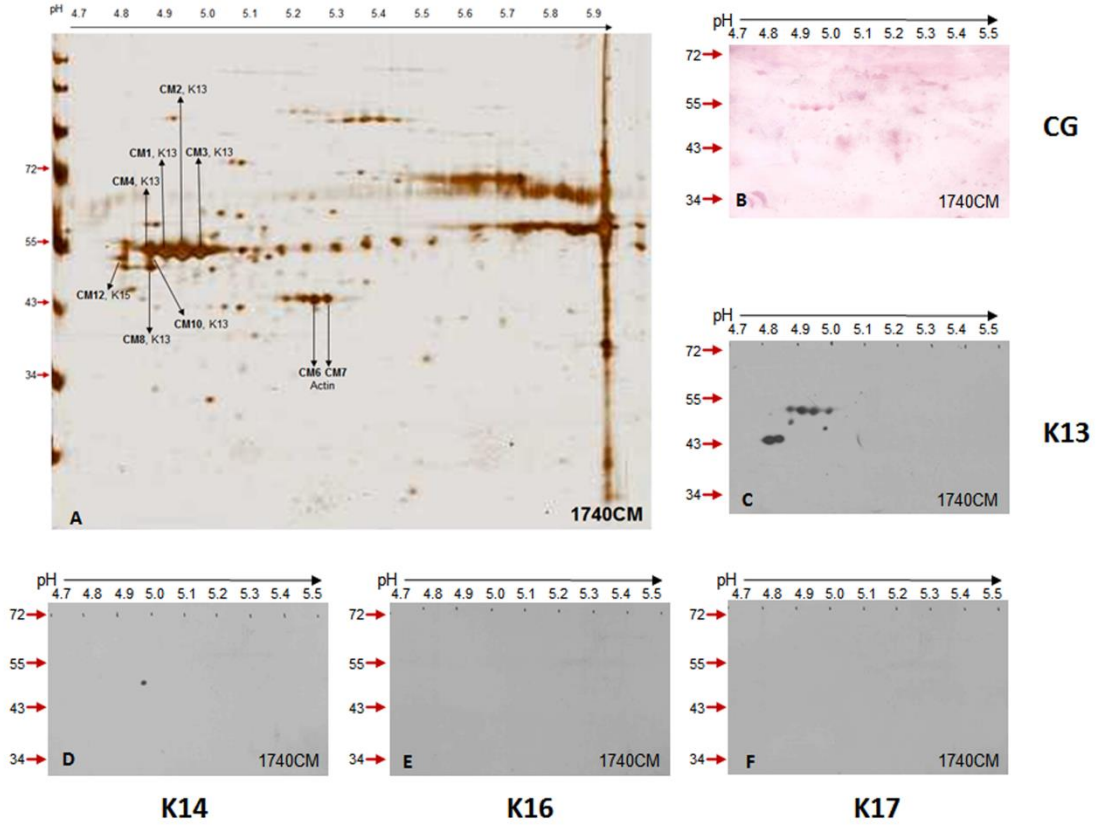


Figure 34. Validation of keratin identities obtained by 2DE-MS by Western Blotting using antibodies specific for K13, K14, K16 and K17. Figures show the profiles obtained for sample 1740 CM. (A) 2DE-silver stained gel profile and the location of the keratins. (B) Colloidal stained 2DE blot to show the position of the resolved proteins. (C) Autograph of the blot stained with antibody to K13, (D) for K14, (E) for K16, and (F) for K17. The pH range used for IEF is given above the gels and blots. On the silver gel profile, the spot number (CMx) and the identity of the keratin (Kx) as per mass spectrometry data in Appendix Table A3 is shown. The 2DE blot was cut beyond pH 5.5 to minimize antibody requirements.

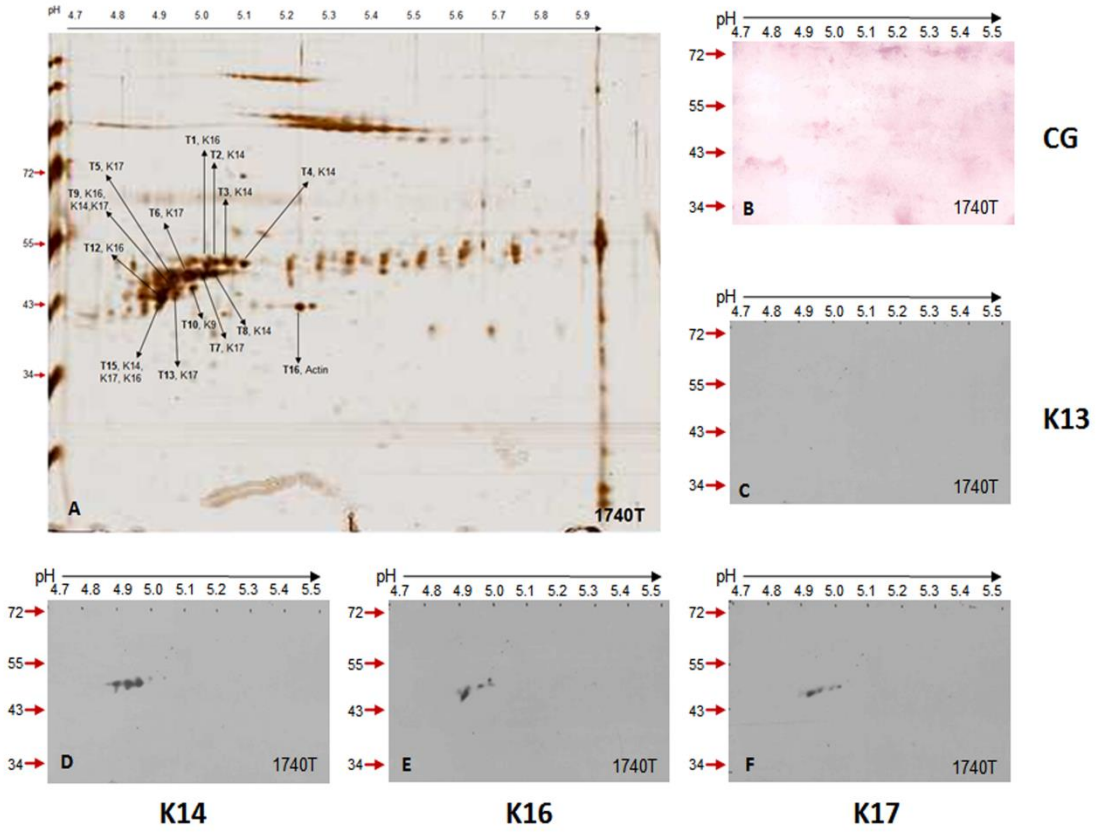


Figure 35. Validation of keratin identities obtained by 2DE-MS by Western Blotting using antibodies specific for K13, K14, K16 and K17. Figures show the profiles obtained for sample 1740 T. (A) 2DE-silver stained gel profile and the location of the keratins. (B) Colloidal stained 2DE blot to show the position of the resolved proteins. (C) Autograph of the blot stained with antibody to K13, (D) for K14, (E) for K16, and (F) for K17. The pH range used for IEF is given above the gels and blots. On the silver gel profile, the spot number (Tx) and the identity of the keratin (Kx) as per mass spectrometry data in Appendix Table A3 is shown. The 2DE blot was cut beyond pH 5.5 to minimize antibody requirements.

Figure 36 shows the position of Keratin 4 by 2DE-MS and WB. This basic keratin does not resolve well and a streak is obtained. Histologically, the cut margin tissues may appear similar to the normal tissues but their keratin signature clearly distinguishes if they have a keratin signature similar to that of the tumor tissue. The observed expression patterns can be of clinical significance so as to determine the surgical margins that are molecularly transformed and should be resected during surgery.

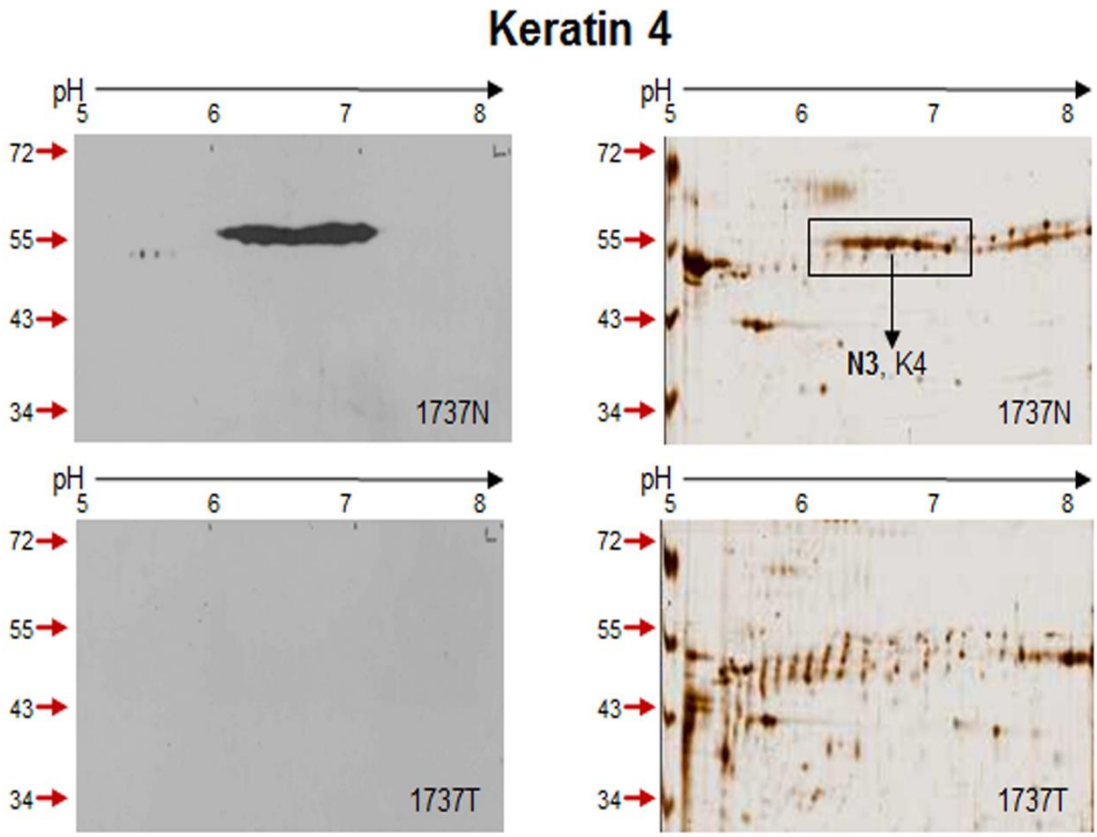


Figure 36. Profiles of enriched keratin preparations from a sample 1737N and 1737T resolved by 2DE on a 17cm IPG strip of pH range 5-8. Figure shows autographic profile of the blot stained with antibody to keratin 4 for normal and tumor samples and the position of K4 on silver stained gel as obtained by mass spectrometry. Mass spectrometry data is given in Appendix Table A3. The spot number N3 and identity of the keratin K4 is shown on the silver stained gel.

2DE-Western Blotting with K13 specific antibody-

Figure 37 shows the antibody reactivity to K13 in two more sample sets 1735 and 1736. The reported molecular weight of K13 is 49.9 kDa and pI is 4.91. Figure 38 shows that in the normal (except sample no. 1735N) and cut margin tissues the position of K13 is in the position seen above and K13 is entirely absent in tumor tissues. The striking pattern of 3 spots which are common in these samples are the isoforms of K13 (shown by mass spectrometry in **Appendix Table A3**. This observation is supported by the 1DE-MS and 1DE-western blotting experiments suggesting that K13 is downregulated in tumor samples.

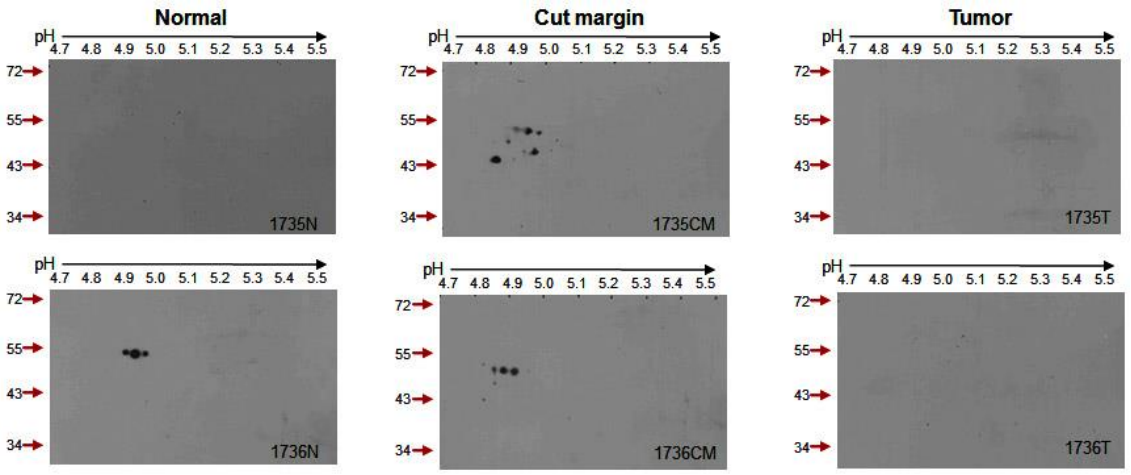


Figure 37. 2DE-western blotting profile for enriched keratin preparations using K13 specific antibody. The sample numbers and tissue type are given at the right corner in each panel. The position of the molecular weight standards and the pH are shown.

2DE-Western Blotting with K14 specific antibody-

Figure 38 shows the position of K14 in two more sample sets i.e. 1735 and 1736. The reported molecular weight of K14 is 51.9 kDa and pI is 5.09. The 2DE-western blotting result shows the position of K14 at ~50 kDa position with pI ranges between 4.9 to 5.0 which is the position of K14 on 2DE blots in tissue samples

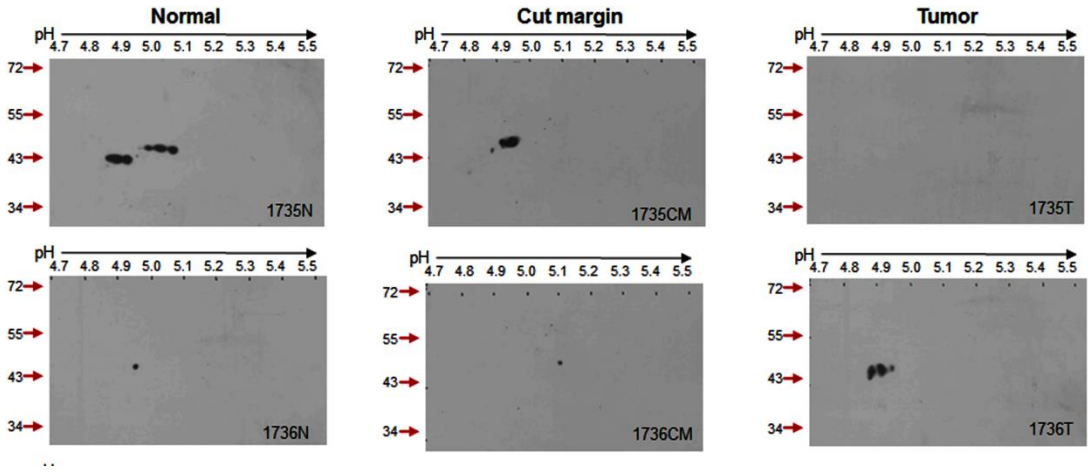


Figure 38. 2DE-western blotting profile for enriched keratin preparations using K14 specific antibody. The sample numbers and tissue type are given at the right corner in each panel. The position of the molecular weight standards and the pH are shown.

The 2DE-WB results suggest the specificity of the K14 specific rabbit monoclonal antibody **EP1612Y**.

2DE-Western Blotting with K16 specific antibody-

The mass spectrometry and 1DE-WB studies have shown that K16 was overexpressed in tumor samples**.** **Figure 39** shows the position of K16 in two more sample sets i.e. 1735 and 1736). The reported molecular weight of K16 is 51.6kDa with pI 4.99. The experimental result shows the position of K16 between 55 to 43 kDa with pI between 4.9 to 5 which is the position of K16 on 2DE-blot

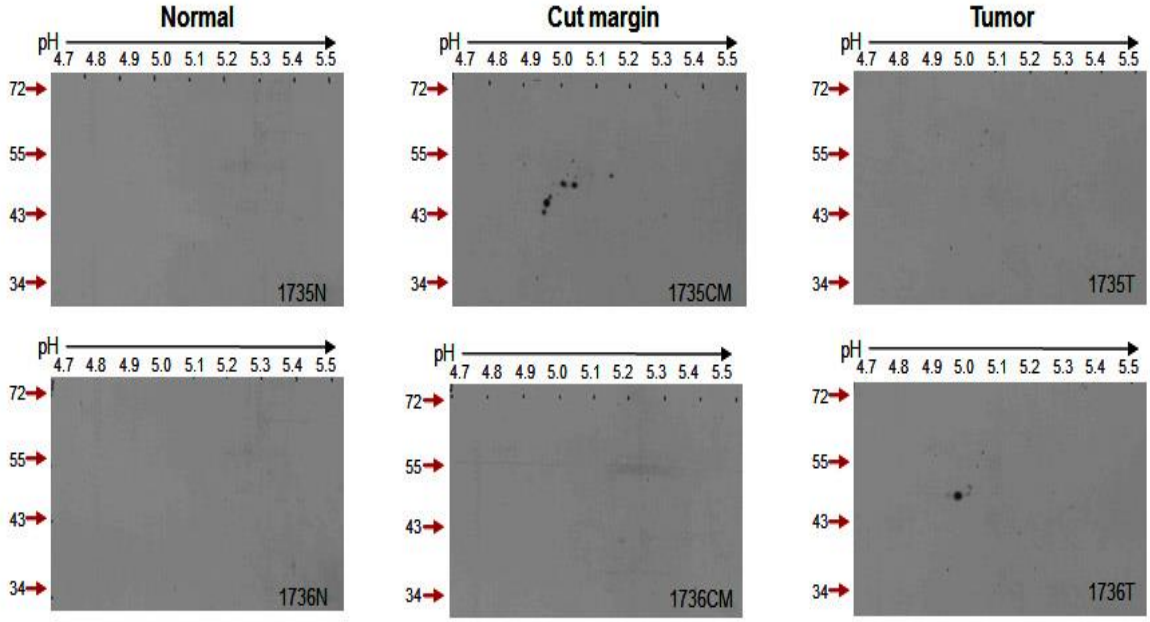


Figure 39. 2DE-western blotting profile for enriched keratin preparations using K16 specific antibody. The sample numbers and tissue type are given at the right corner in each panel. The position of the molecular weight standards and the pH are shown.

2DE-Western Blotting with K17 specific antibody-

The mass spectrometry and 1DE-WB studies have shown that K17 was overexpressed in tumor samples. **Figure 40** shows the position of K17 in two more sample sets i.e. 1735 and 1736. The reported molecular weight of K17 is 48.4 kDa and pI is 4.97. The result shows that K17 antibody stains at molecular weight between 55 to 43 kDa with pI range 4.9 to 5 which is the position of K17 on 2DE-blot.

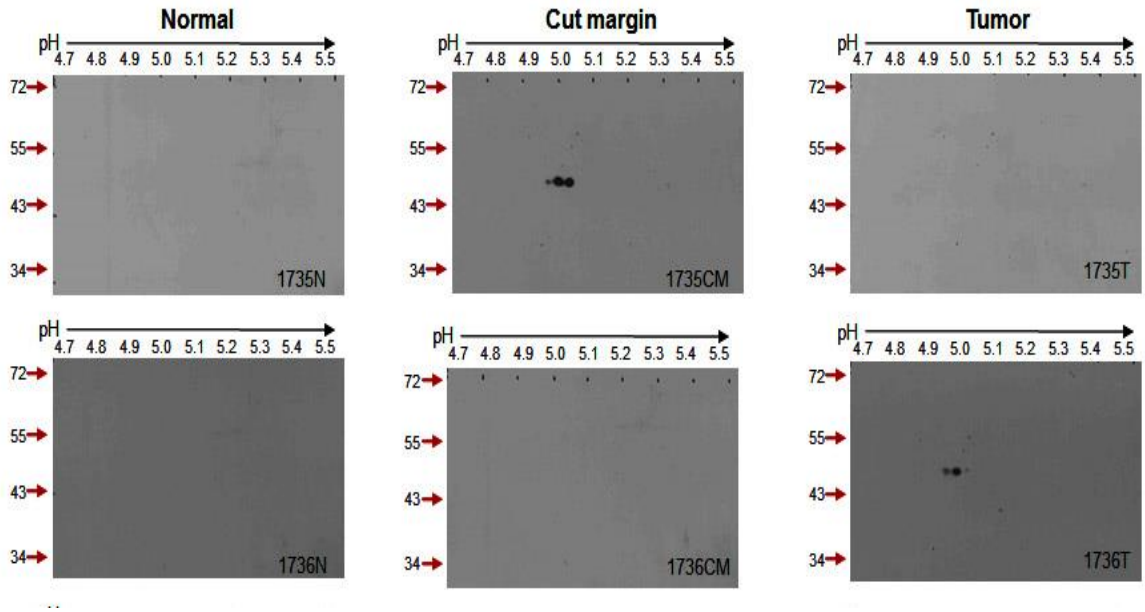


Figure 40. 2DE-western blotting profile for enriched keratin preparations using K17 specific antibody. The sample numbers and tissue type are given at the right corner in each panel. The position of the molecular weight standards and the pH are shown.

The results also suggest that K17 antibody does not show cross-reactivity at any other location on 2DE-blots.

2DE-Western Blotting with K18 specific antibody-

The molecular weight of K18 is ~48 kDa and pI is 5.4. Previous studies have reported the aberrant expression of K18 in oral cancer and the same has been observed in the previous section by 1DE western blotting. **Figure 41** shows the reactivity of K18 (CY90) antibody at ~49 kDa position with pI ~4.9 in sample 1735, 1736, 1737 and 1740 normal and 1736, 1737 and 1740 cut margin and another reactive spot at ~43 kDa position with pI between 5.1 to 5.4 (sample 1735 and 1740 normal, 1736 and 1740 cut margin) In 1736, 1737 and 1740 tumor samples, antibody show spots at ~43 kDa between pH 4.9 to 5.4.

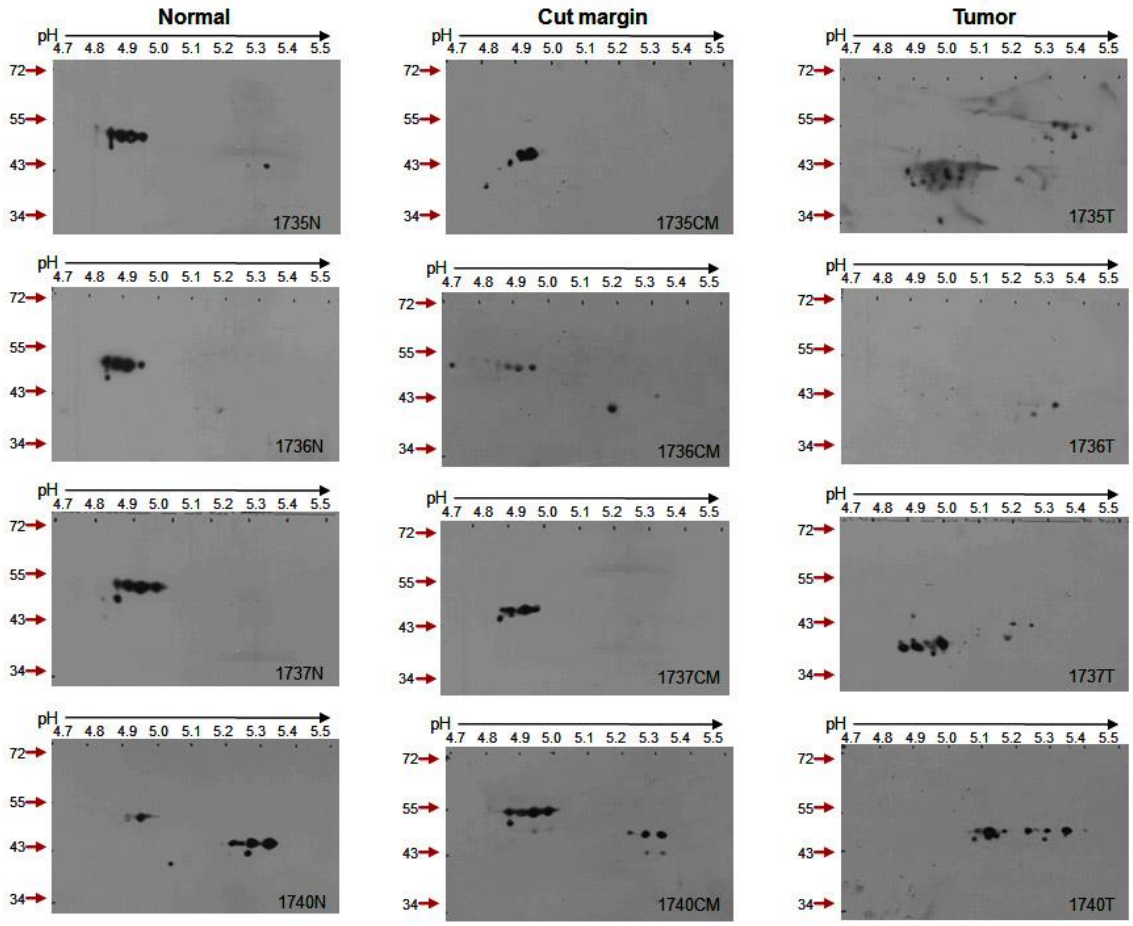


Figure 41. 2DE-western blotting profile for enriched keratin preparations using K18 (CY 90) specific antibody. The sample numbers and tissue type are given at the right corner in each panel. The position of the molecular weight standards in kDa and the pH are shown.

Comparison of 2DE-Western Blotting with K18 and K13 antibodies

It is observed from the western blotting results shown above that K13 and K18 have been identified by both the antibodies at ~48-49 kDa, suggesting the cross reactivity of K18 (CY90) antibody with K13 protein. **Figures 42 to 44** given below compare the K13 and K18(CY90) antibody staining side by side for samples 1736, 1737 and 1740 respectively and the colloidal stained blots are also shown in each figure. The colloidal gold stained blot for sample 1736N belongs to anti K13 stained blot whereas other blots were initially stained with anti K13 and then destained and reprobed with anti K18 to locate the exact position of these proteins on PVDF membrane. It is seen that an

antibody to K13 also recognises only the ~48KDa band and does not recognize the lower band.

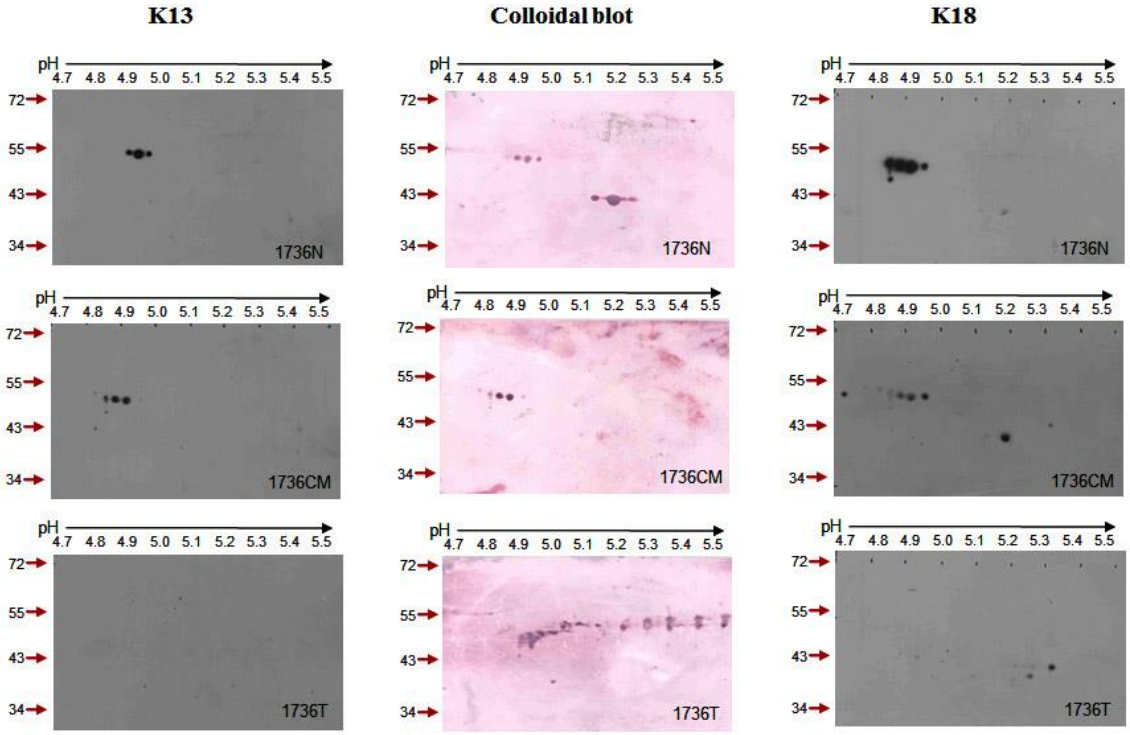


Figure 42. 2DE-western blotting profile for enriched keratin preparations using K18 (CY 90) and K13 specific antibody. The sample numbers and tissue type are given at the right corner in each panel. The position of the molecular weight standards in kDa and the pH are shown. The colloidal gold (CG) stained blot is also shown. The CG blot shown for 1736N is the one used to probe with K13. Another blot was used for K18. The other CG stained blots were used for both antibodies.

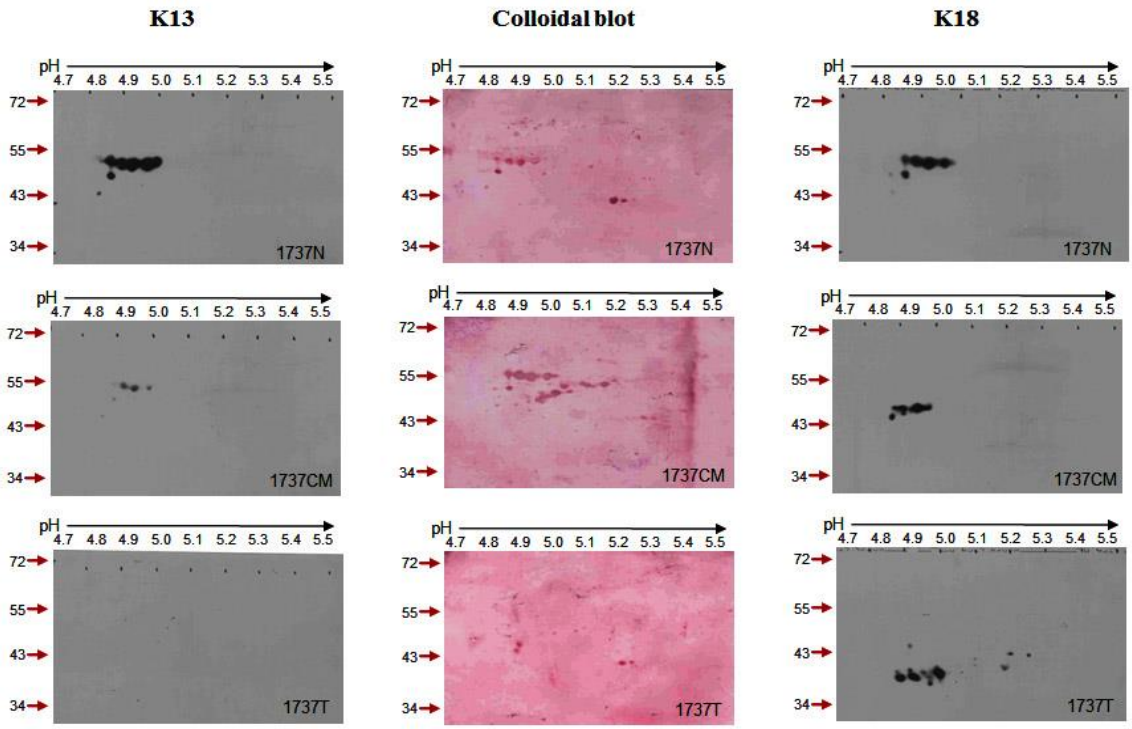


Figure 43. 2DE-western blotting profile for enriched keratin preparations using K18 (CY 90) and K13 specific antibody. The sample numbers and tissue type are given at the right corner in each panel . The position of the molecular weight standards in kDa and the pH are shown. The colloidal gold (CG) stained blot is also shown. For 1737 CM the antibody to K18 gave a signal which overlaps the curved set of spots below the K13 set of spots on the CG blot.

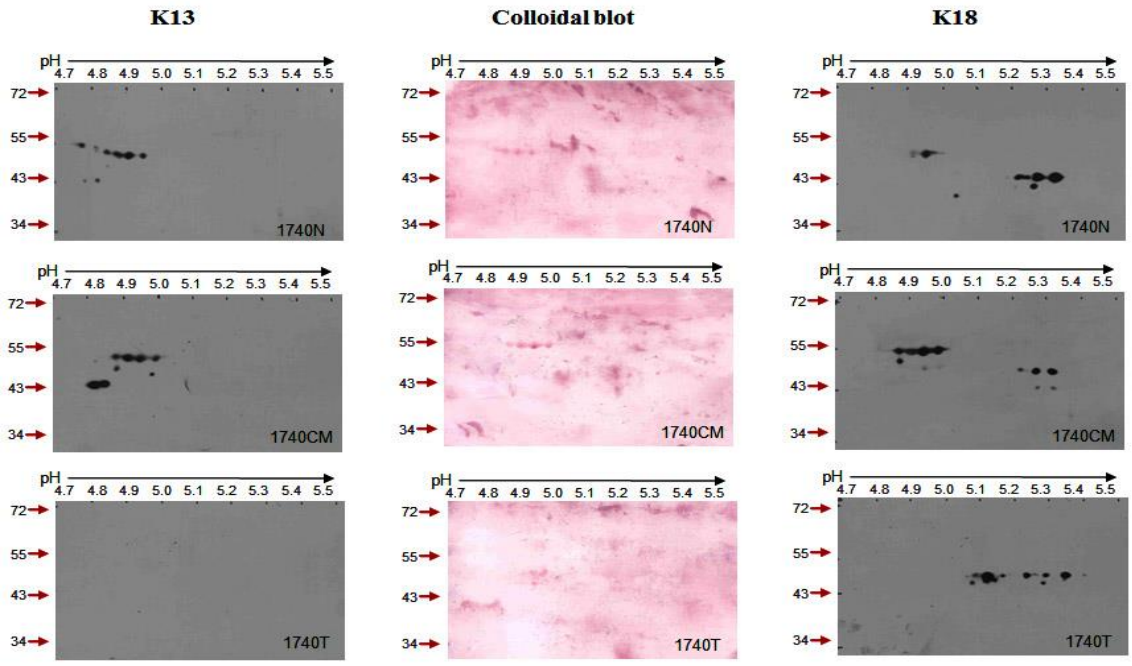


Figure 44. 2DE-western blotting profile for enriched keratin preparations using K18 (CY 90) and K13 specific antibody. The sample numbers and tissue type are given at the right corner in each panel . The position of the molecular weight standards in kDa and the pH are shown. The colloidal gold (CG) stained blot is also shown.

Evaluation of the reactivity of keratin 18 antibody from different sources

To determine whether the clone CY 90 from another source other than Sigma also detected K13, the experiment was repeated. The blots were probed with clone CY 90 from Abcam and LDK18 (an antibody to the C-terminal end of keratin 18). The results obtained are given in **Fig. 45**. The same signals were seen with the anti-keratin 18 antibody from Abcam as were seen with the Sigma antibody shown in Fig.42-44. Another antibody to keratin 18 i.e. LDK 18 does not detect any protein at 48 kDa or 45 kDa in the keratin preparations from 1736 N and T and 1740 CM and T samples.

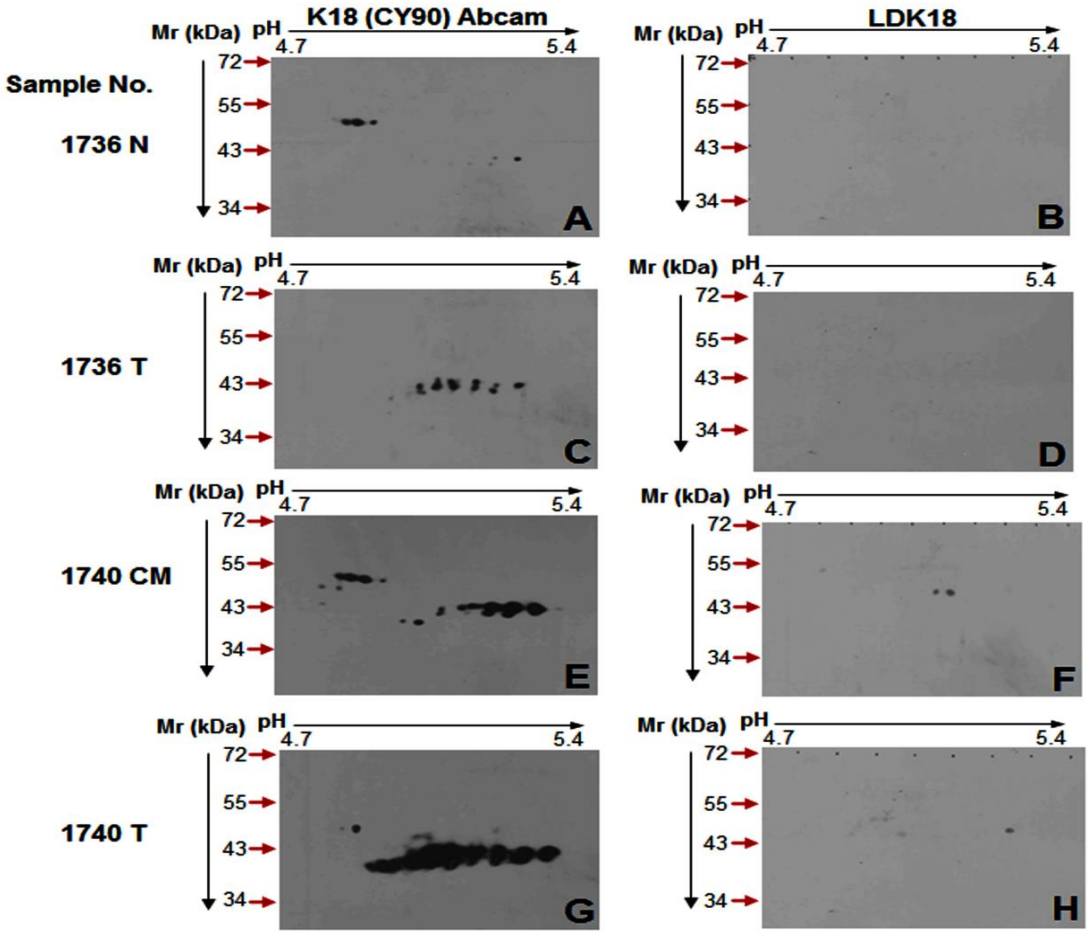


Figure 45. Autographic profiles of enriched keratin preparation from 1736 normal (N) and tumor (T) and 1740 cut margin (CM) and tumor (T) samples resolved by 2DE on a 17 cm IPG strip of pH range 4.7 to 5.9 and blots probed with antibodies to K18 (CY-90 from Abcam) and LDK 18 from Abcam. Autographs A, C, E and G are for antibody to K18, CY 90 from Abcam and autographs B, D, F and H are for antibody to K 18, LDK18 from Abcam. The 2DE blots were cut beyond pH 5.4 to minimise antibody requirements.

Confirmation of the identity of the Keratin 13 by 2DE-LC-MS/MS

To confirm if keratin 18 was entirely absent, enriched keratin preparation from another histologically normal epithelium of 1574 CM was resolved by 2DE. **Fig. 46** shows the 2DE profile of enriched keratins from the sample 1574 CM. The silver stained spots in the boxed area were subjected to trypsin digestion and the eluted peptides resolved by liquid chromatography and analysed by mass spectrometry. Only keratin 13 along with keratin 15 was detected and K18 identity was not obtained **(Appendix Table A4).**

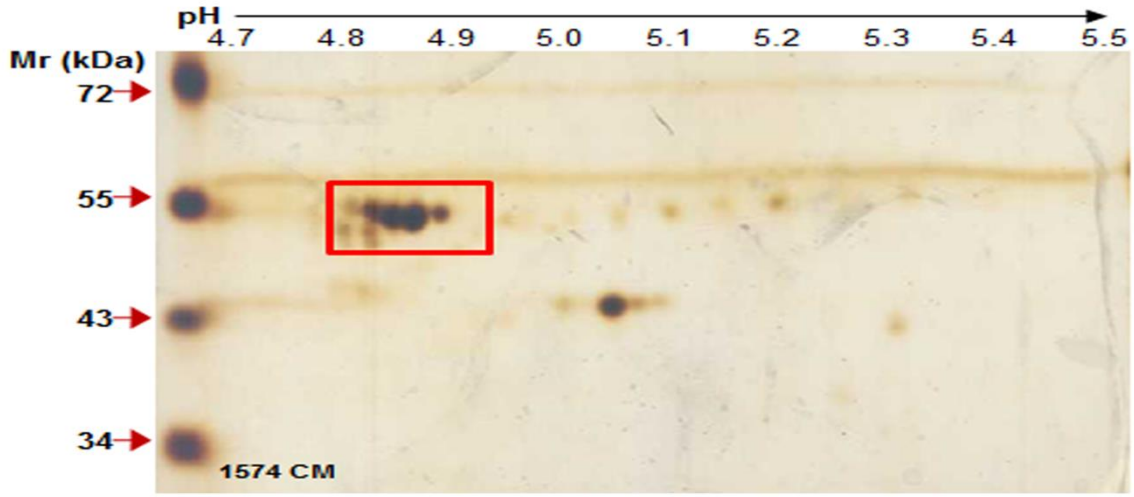


Figure 46. Silver stained gel profile of enriched keratin preparation (30 µg) from histologically normal cut margin tissue sample (1574 CM) resolved by 2DE on a 17 cm IPG strip of pH range 4.7 to 5.9. Boxed area shows the spots taken for LC-MS/MS analysis.

Evaluation of the reactivity of clone CY90 with breast cancer cells and breast cancer tissue which express keratin 18

Breast cancer cells MDA-MB-468 are reported to express keratin 18 [217]. Cell lysates were resolved by 2DE and the proteins transferred to PVDF membranes. The blots were probed with anti-keratin 18 clone CY 90 from Sigma and Abcam, LDK 18 antibody, K13 antibody and actin specific antibody. The data is given in Fig. 47A to 47G which show the autographic profiles obtained for each of the antibodies. Sigma

and Abcam anti K18 antibodies (CY90) detected a band overlapping the position of actin (Fig. 47- A and B) while LDK 18 antibody reacted more specifically to the spots above actin (Fig. 47 C). No signal was seen in the position of K13 and this was confirmed as shown by the autographic profile obtained with K13 antibody (Fig. 47-D). Actin specific antibody reacted with actin (Fig. 47-E).

MDA-MB-468 lysate (50 µg protein) was **spiked** with 30 µg of enriched keratin preparation from sample 1740 CM. The blotted proteins were probed with anti K13 which detected a band in the upper left hand corner Fig. 47-F. The blot was stripped and reprobed with Sigma clone CY 90. The profile in Fig. 47-G shows that the clone CY90 from Sigma has detected the additional upper band at the position of K13 even in the presence of K18.

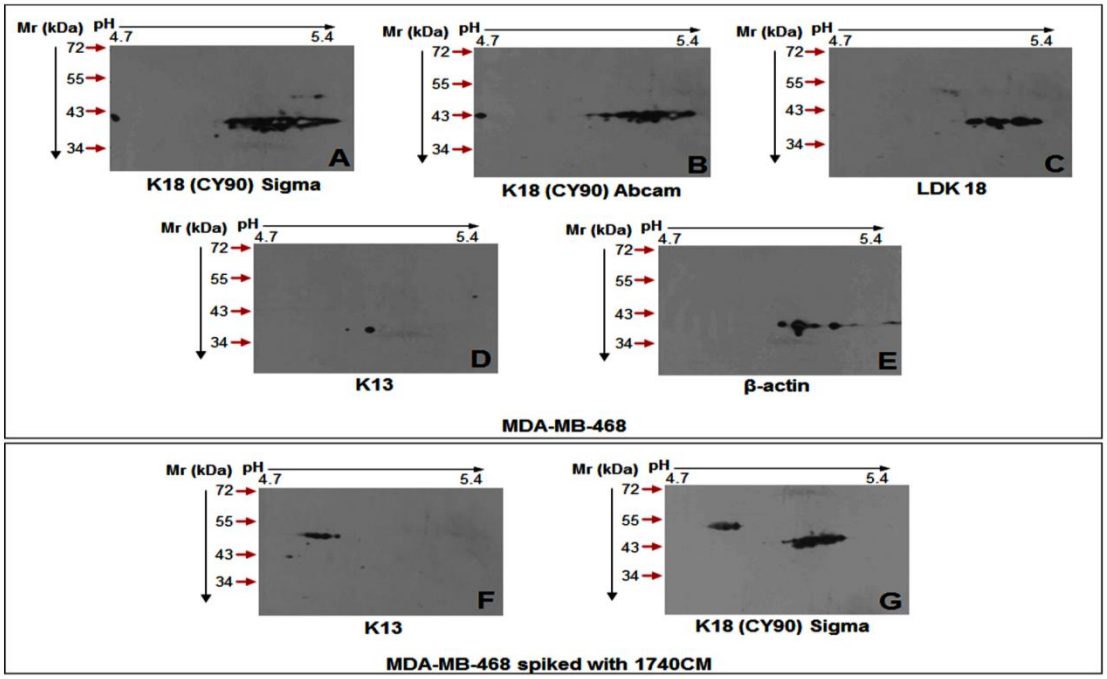


Figure 47. Autographic profiles of total cell lysate proteins (50 µg) from MDA-MB-468 cells resolved by 2DE on a 17 cm IPG strip of pH range 4.7 to 5.9 and blots probed with antibodies to K18 (CY-90 from Sigma and Abcam), LDK 18, antibodies to K13 and β actin. Autographs A, B, C are for antibody to K18,(CY 90) from Sigma and Abcam and LDK respectively. Autograph D is for keratin 13 and E for anti β actin. The MDA-MB-468 cell lysate (50 µg protein) was mixed with 30 µg of enriched keratins from 1740 CM and the same was resolved by 2DE and blotted as above. Autograph F shows the profile of the blot probed with anti-keratin 13. The same blot was stripped and reprobed with anti K18 CY90 clone from Sigma. The 2DE blots were cut beyond pH 5.4 to minimise antibody requirements.

Figure 48- A to D show a close up of the autographs in Fig. 47 with the corresponding colloidal stained profiles. It is seen in Fig. 46A and 46B that the autographic signals obtained with antibody CY90 overlaps with actin and the spots immediately above it, The LDK 18 signal in C is also very close to actin with focus on the upper spots. Autographic signal for actin in Fig. 46-D is directly on the intensely stained protein and there is no signal for K18 in the autograph.

Figure 48- E shows the silver stained profile of 400 µg of the MDA-MB-468 cell lysate resolved on 2DE and stained with silver. The spots which were subjected to in-gel digestion followed by mass spectrometry of the peptides generated are numbered. The MS results given in Appendix **Table A5** showed the identity for actin in spot numbers 6 to 15, while K18 was detected in spot numbers 1, 3, 4 and 5.

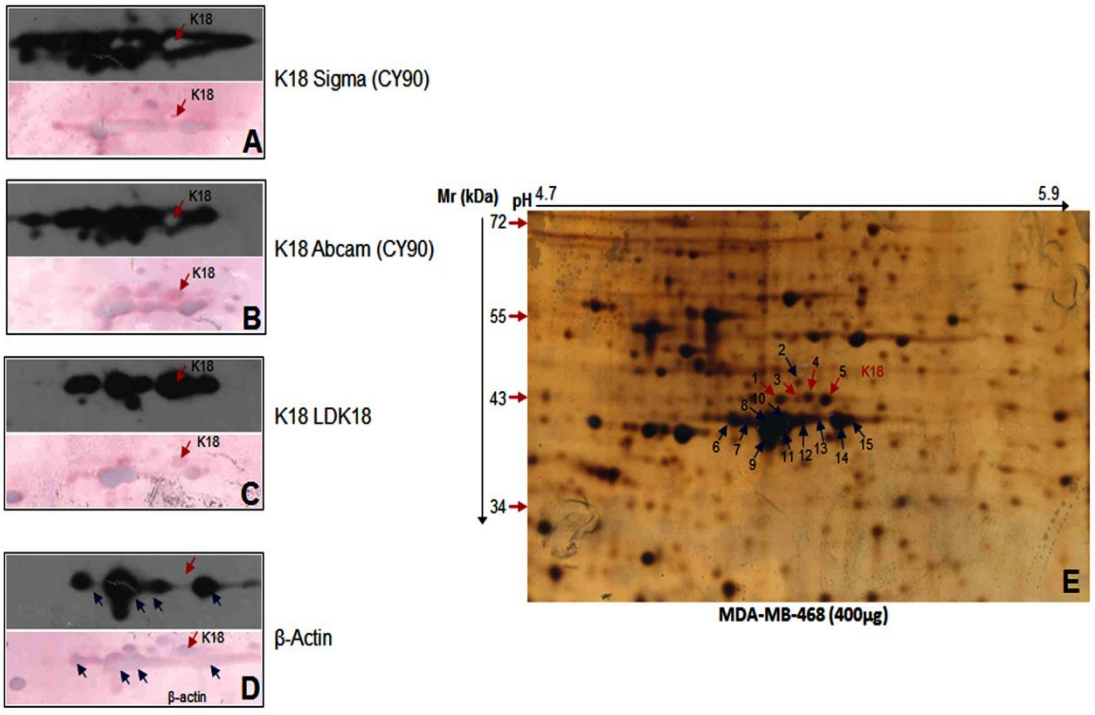


Figure 48. A-D.- A close up of the autographs shown in Fig. 45 with the corresponding colloidal stained profile. E.-The silver stained profile of 400 µg of the MDA-MB-468 cell lysate resolved on 2DE and stained with silver. The spots which were subjected to in-gel digestion followed by mass spectrometry of the peptides generated are numbered. Spots 6 to 15 are actin and spots 1, 3, 4, 5 are K18. Details given in Table A5 in Appendix.

As another example **breast cancer tissue** which is known to express K18 [218, 219] was processed for keratin enrichment, 2DE, western blotting and mass spectrometry as described ahead.

Figure 49 shows the profile of enriched keratin preparations (30 µg) resolved by 2DE and the transferred proteins probed with CY 90 from Sigma (Fig. 49-A) and Abcam (Fig. -49B). It is seen that the antibodies detect a band to the left of actin. A third blot was probed with anti K18, LDK 18. Fig. 49-C shows that the antibody detects a spot on actin and one slightly above actin. Another blot was probed for K13 (Fig. 49-D) and the same blot probed with antibody to actin after stripping (Fig 49-E). There is no signal for K13 in Fig.49-D, while the position for actin is clearly seen in Fig. 49-E.

Fifty microgram of enriched keratins from the breast cancer tissue was also **spiked** with 30 µg of enriched keratins from oral tissue sample 1740 CM and the same resolved by 2DE. One blot was probed with anti K13, Fig. 49-F. The same blot was stripped and reprobed with anti K18 clone CY90 from Sigma Fig. 49-G. Fig. 49-F shows that anti K13 detects the upper band which we know is K13 by mass spectrometry. Anti K18 (CY90) from Sigma detects the upper band (K13) and shows a signal which spans from the left of the intense colloidal band for actin and across it encompassing K18 as seen in Fig. 49-A. CY90 therefore detects both K13 and K18 when they are present.

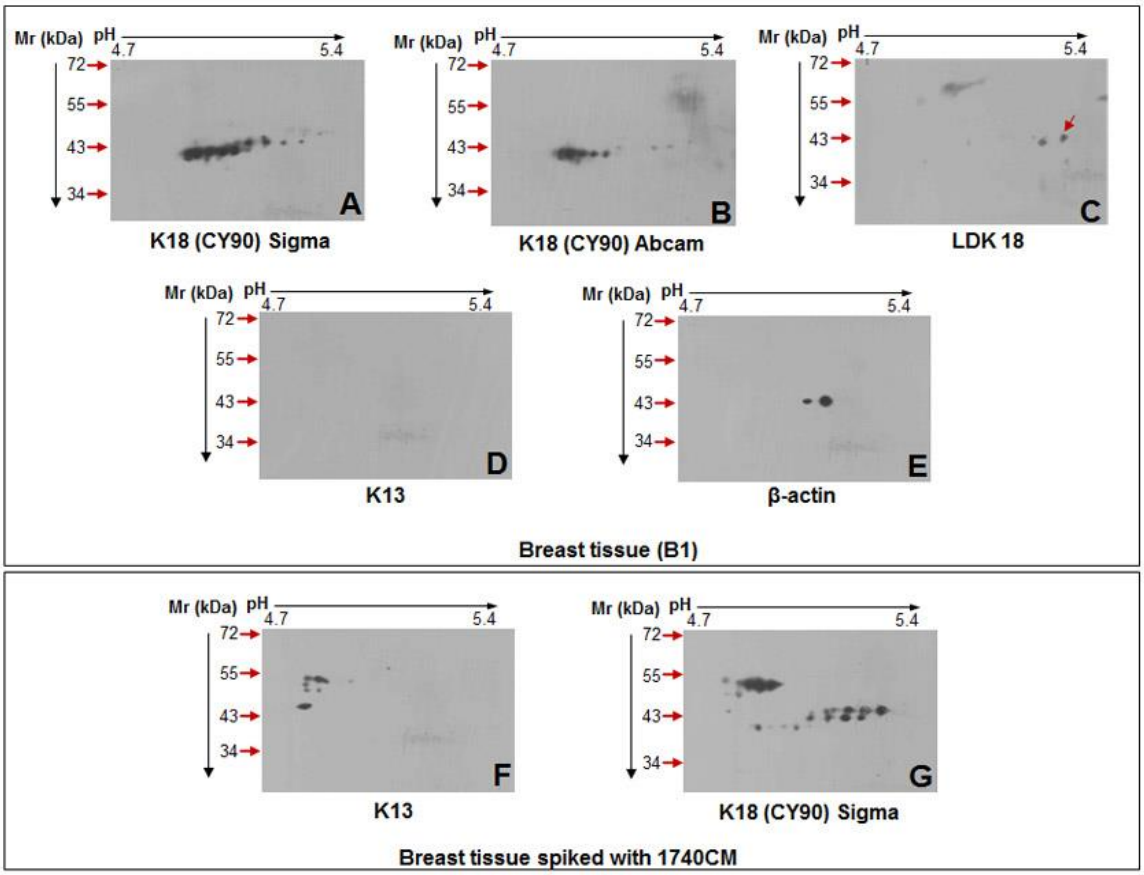


Figure 49. Profile of enriched keratin preparations of breast cancer tissue (30 µg) resolved by 2DE and the transferred proteins probed with CY 90 from Sigma, (Fig.49-A) and Abcam (Fig. 49-B). It is seen that the antibodies detect a set of strong bands to the left of actin. Fig. 49-C shows an autograph of the blot stained with LDK 18. Two clear spots are seen. The spot marked with an arrow is K18. Fig. 49-D shows the autograph of a blot probed with anti K13, wherein no signal is seen. The same blot was probed with anti β actin after stripping (Fig 49-E) and two clear spots are seen for actin. Fig. 49-F shows the autograph of the blot for which 30 µg of enriched keratin preparations from breast tissue B1 and 30 µg from sample 1740 CM were resolved and the blot probed with anti K13. The blot was probed with anti K18, CY90 from Sigma after restriping blot in Fig. 49-F. The spot on the upper left and a set of lower spots near and to the left of actin are clearly seen (Fig. 49-G).

Figures 50 A to D shows a close up of the autographs in Fig. 49 with the corresponding colloidal stained profile. It is seen that the autographic signals obtained with antibody CY90 are to the left of the intense actin protein band and there are few faint signals above actin shown by an arrow. The LDK 18 signal shows two spots, one on actin (a) and one above (K18). In the blot probed with anti actin, the signal is directly on the intensely stained protein.

Fig. 50-E shows a silver stained profile of enriched keratins (400 µg) from the breast cancer tissue and 400 µg keratins from oral tissue 1740 T (Fig. 50-F). It is seen in Fig. 50-E that there are clearly defined protein spots above actin very similar to those seen in MDA-MB-468 in Fig. 48-E. These spots are not seen in the oral tissue gel in Fig. 50-F. The spots corresponding to the position of K18 and actin from these silver stained gels were processed for PMF and the identities obtained by mass spectrometry are shown in Appendix Table A5. It is seen that the breast tissue contains K18 in spot 4.

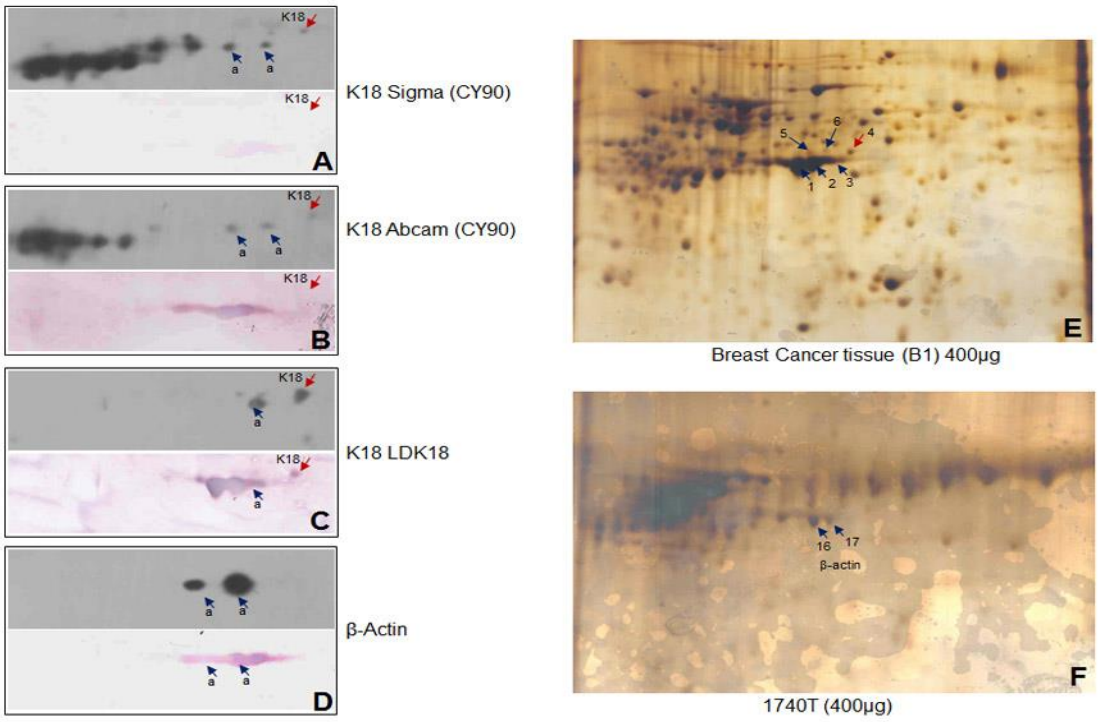


Figure 50-A to D show a close up of the autographs in Fig.49 with the corresponding colloidal stained profiles of the blots. It is seen that the autographic signals obtained with antibody CY90 are to the left of colloidal stained actin band with few spots immediately above it. With LDK 18 there is a single spot on actin and another above it shown by arrow. In the blot probed with anti actin, the signal is directly on the intensely stained protein. Fig 50-E shows a silver stained profile of enriched keratins (400 µg) from the breast cancer tissue B1 and Fig 50-F, the profile of 400 µg keratins from oral tissue 1740 T. It is seen in Fig 50-E, that there are clearly defined protein spots above actin very similar to those seen in MDA-MB-468 in Fig. 48-E. These spots are not seen in the oral tissue gel in Fig 50-F. The spots with numbers were processed for PMF and the identities obtained by mass spectrometry. Data is given in Appendix Table A5. Spots 1, 2 and 3 gave an identity for actin, spot 4 was K18. No identity could be obtained for spots 5 and 6.

These experimental evidences have confirmed the cross reactivity of K18 (CY90) antibody with K13 protein in the tissues from cancer of gingivo buccal complex.

Comparison of O-linked Glycosylation pattern with K13 antibody staining pattern on 2DE blots

There is a very early report of glycosylation of K13 in the human keratinocytes cultured from juvenile foreskin on irradiated 3T3 feeder layers and A431 cells derived from epidermoid carcinomas of the vulva [161]. To see the status in tissue samples, western blots from enriched keratins resolved by 2DE were probed with antibody to O-GlcNAc. It is seen that this antibody recognizes the same series of spots identified as K13 in the gels shown in **Figure 51** suggesting that K13 is glycosylated in tissues from the oral buccal mucosa. The colloidal stained blot shows the exact location of the spots reactive for the K13 and O-GlcNAc antibodies and it also supports the above finding.

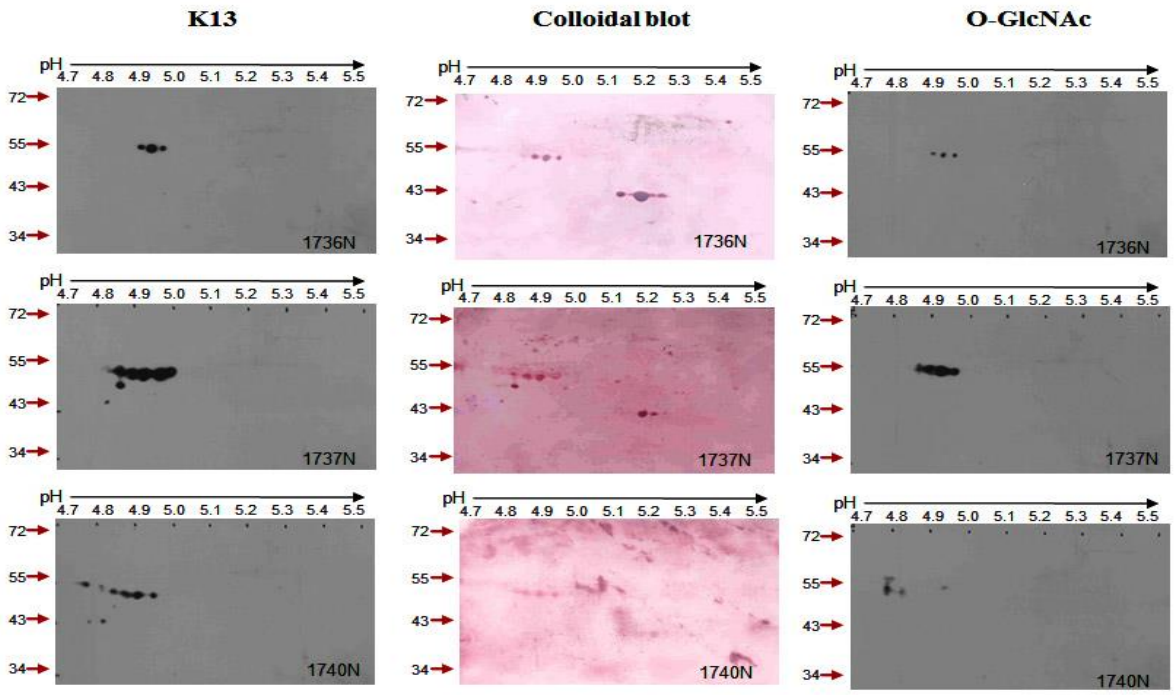


Figure 51. 2DE-western blotting profile for enriched keratin preparations using K13 and O-GlcNAc specific antibody and comparison of spots with colloidal stained blot. The sample numbers and tissue type are given at the right corner in each panel. The position of the molecular weight standards in kDa and the pH are shown.

This study reports for the first time the O-linked glycosylation of K13 in the gingivo buccal complex tissues.

3.4. 2DE-Silver staining pattern of keratins from normal, cut margin and tumor tissue.

The 2DE silver stained profiles seen in Section 3.3 above indicated that there are distinct patterns for keratin expression in normal, cut margin and tumor tissue samples. To confirm the emerging patterns, all the tissue samples were resolved by 2DE and compared with the histological patterns on the corresponding H&E stained sections. **Figures 52 to 57** show the pattern for comparison of silver staining with H&E histology of the tissue with normal, cut margin and tumor sets for sample 1606, 1735, 1736, 1737, 1740 and 1751 and with normal and tumor pairs for sample 1579, 1580, 1657, 1686, 1687, 1725, 1726, 1731 and 1741.

Figure 52 shows the Keratin 13 signature spots (boxed in red) below 55 kDa (pI 4.7-4.9) position in both 1606N and 1735N silver stained gels. The sample 173 5 N in addition has a curved streak below K13. The 1DE-MS data in Figs. 6 and 8 show mainly K13 in 1606N while in 1735N, K13 and K16 are seen and this presentation is supported by the silver pattern. The histology of the tissue is given alongside with the H&E images. Silver staining pattern for 1606CM is more or less similar to the normal but the silver staining pattern for 1735CM shows streak of spots (boxed in blue) below K13. The histology of the sample shows it as hyperplasia with mild dysplasia. K13 signature pattern (boxed in red) is lost in the 1606T silver stained gel and the streak of spots (comprising of K14, K16, K17) between 55 kDa to 43 kDa are more prominent (boxed in blue). These keratins have also been seen by 1DE-MS and are shown in Fig. 6. Sample 1735T is degraded and therefore no such pattern could be obtained.

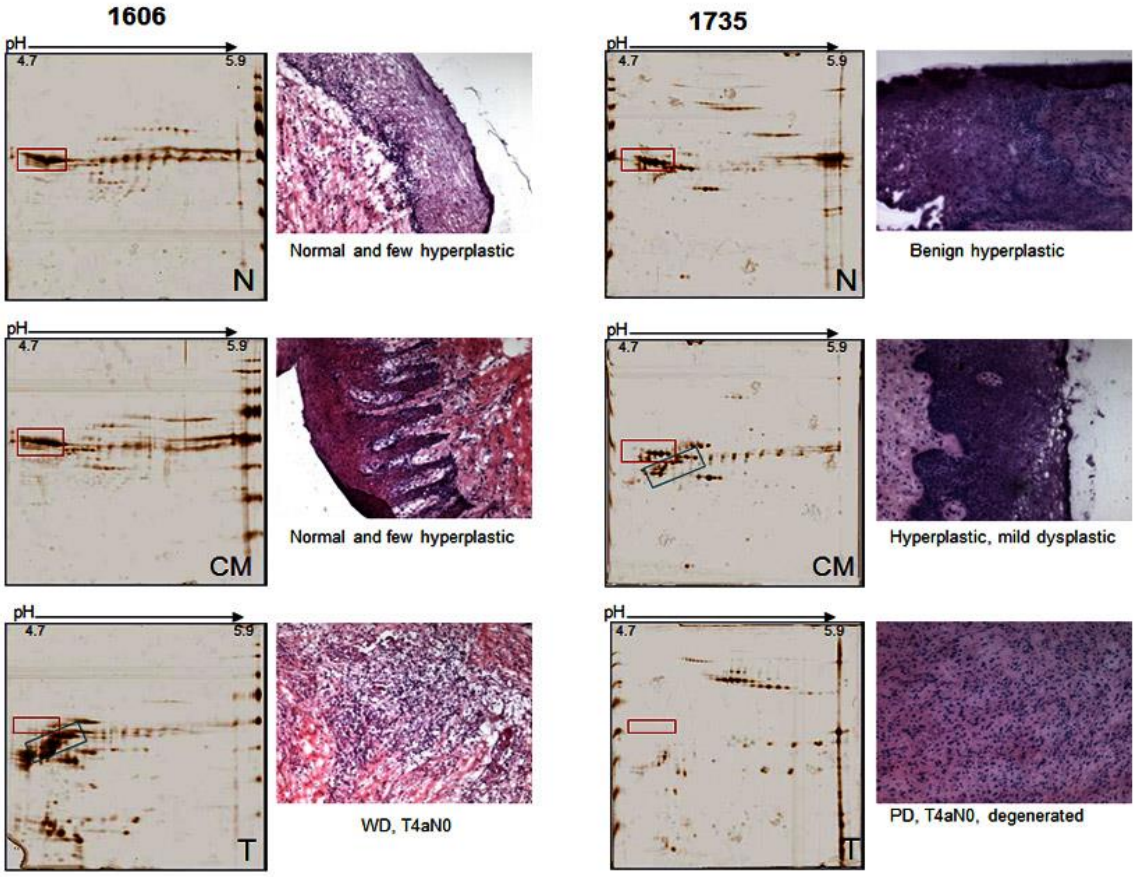


Figure 52. Comparison of the 2DE-silver stained pattern with the H&E stained sections for samples 1606 (N, CM and T) and 1735 (N, CM and T). The pH range for IEF is indicated above each gel profile. The histological status of the tissue is given below each H&E stained section. WD; Well differentiated, PD; Poorly differentiated.

Figure 53 shows the Keratin 13 signature spot (boxed in red) below 55 kDa (pI 4.7-4.9) positions in both 1736 and 1737 normal (N) silver stained gels. The tissue histology from H&E sections shows the status as benign hyperplastic. Sample 1736CM silver stained gel shows K13 signature pattern (boxed in red) and faint streak below and H&E histology for the samples shows hyperplasia with mild dyaplasia. Sample 1737CM shows both K13 signature pattern (boxed in red) and a streak below and H&E histology status shows as a benign hyperplastic. *Thus, silver staining pattern for 1737CM may be more predictive of the status of the tissue than H&E histology*. Both 1736 and 1737

tumor tissue silver stained gels do not show the K13 signature spots (boxed in red) but show the more prominent lower streak (boxed in blue).

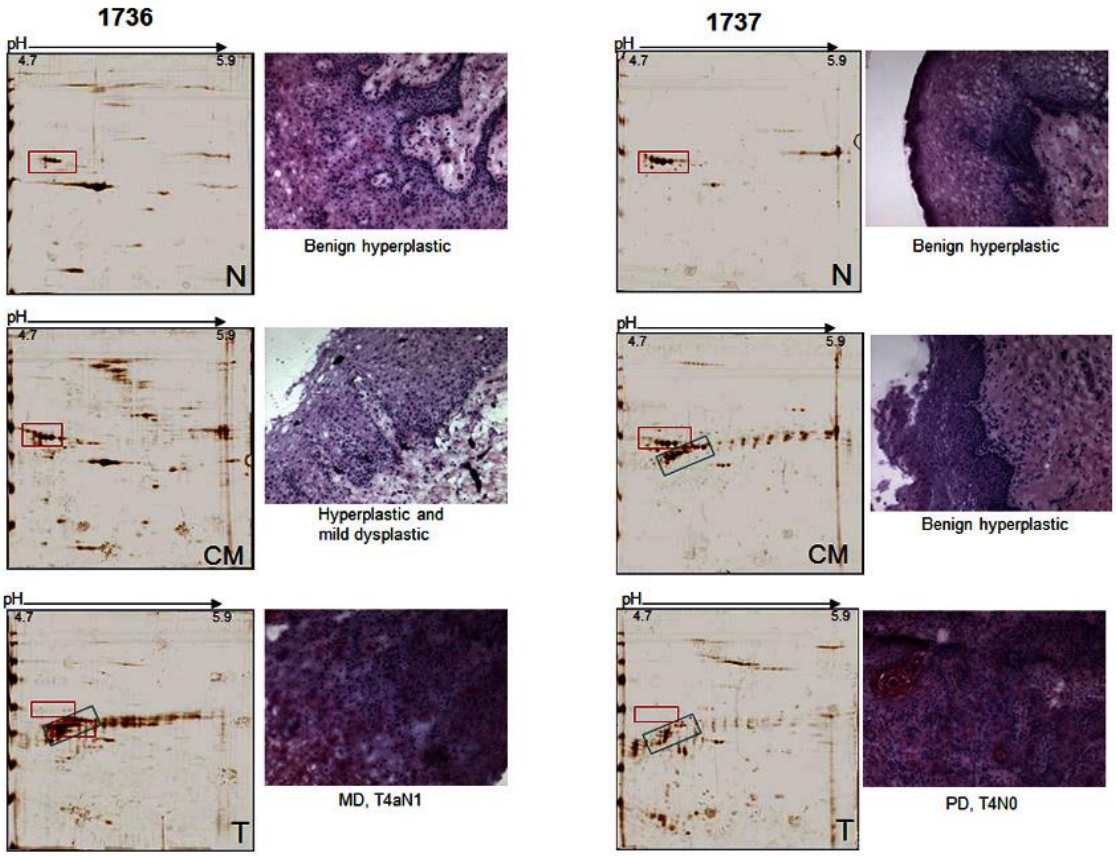


Figure 53. Comparison of the 2DE-silver stained pattern with the H&E stained sections for samples 1736 (N, CM and T) and 1737 (N, CM and T). The pH range for IEF is indicated above each gel profile. The histological status of the tissue is given below each H&E stained section. MD; Moderately differentiated, PD; Poorly differentiated.

Figure 54 shows the keratin 13 signature spots (boxed in red) below 55 kDa (pI 4.7-4.9) in both 1740 and 1751 normal silver stained gels. The tissue histology from H&E sections shows the status of 1740N as normal plus benign hyperplastic and for 1751N as benign. Sample 1740CM shows K13 signature spots (boxed in red) and no lower streak while 1751CM shows both the signatures *suggesting although the histology status is benign hyperplastic but the silver staining pattern shows the precursor for transformed phenotype.* Both the tumor silver stained gels showed the typical lower streak (boxed in

blue) which is the signature for transformation.

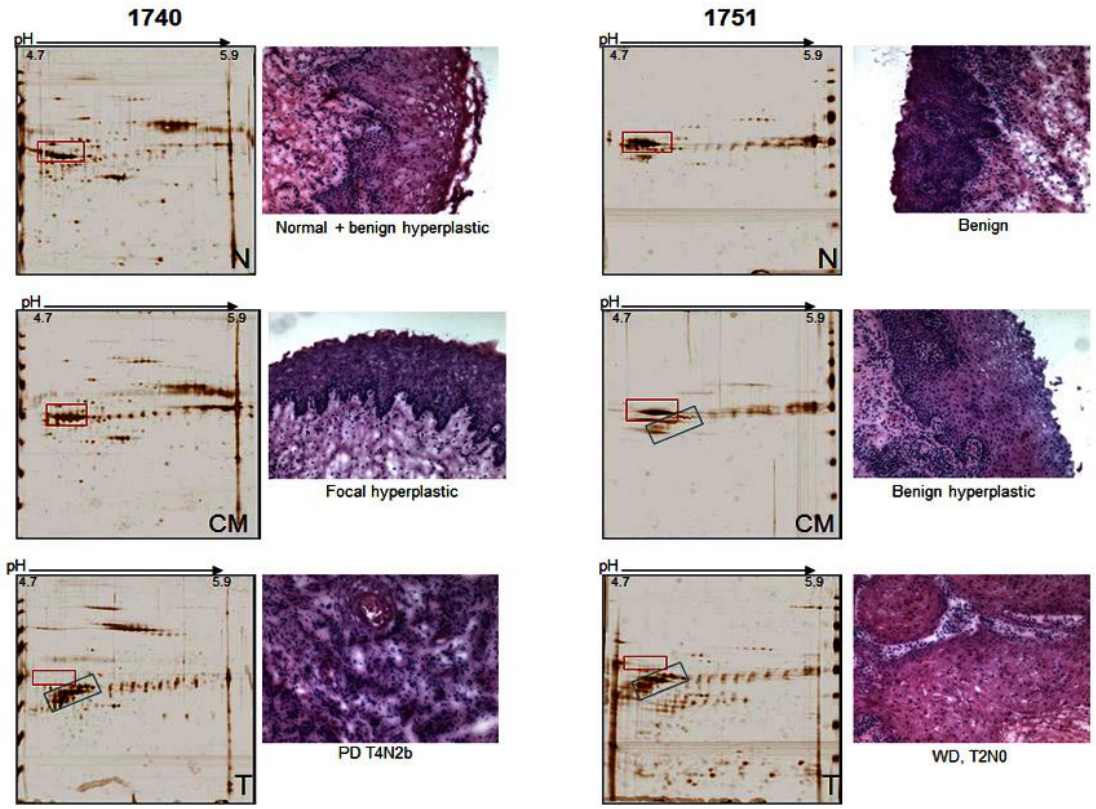


Figure 54. Comparison of the 2DE-silver stained pattern with the H&E stained sections for samples 1740 (N, CM and T) and 1751 (N, CM and T). The pH range for IEF is indicated above each gel profile. The histological status of the tissue is given below each H&E stained section. WD; Well differentiated, PD; Poorly differentiated.

The same pattern of expression as discussed for above mentioned six samples is applicable for normal and tumor samples 1579, 1580, 1657, 1686, 1687, 1725, 1726, 1731 and 1741 given in **Fig. 55 to 57**.

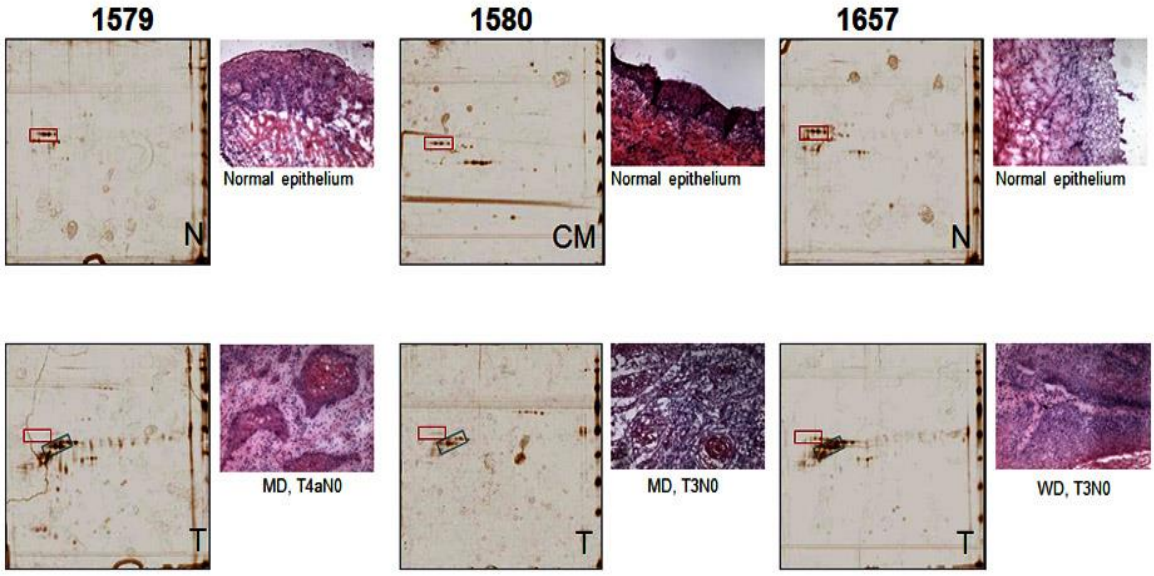


Figure 55. Comparison of the 2DE-silver staining pattern for enriched keratins with the H&E stained profile of the tissue sections for sample 1579 (N, and T), 1580 (CM and T) and 1657 (N and T). The pH range used for IEF is shown above the gel profile and the magnification is indicated on the H&E stained sections. WD; Well differentiated, MD; Moderately differentiated.

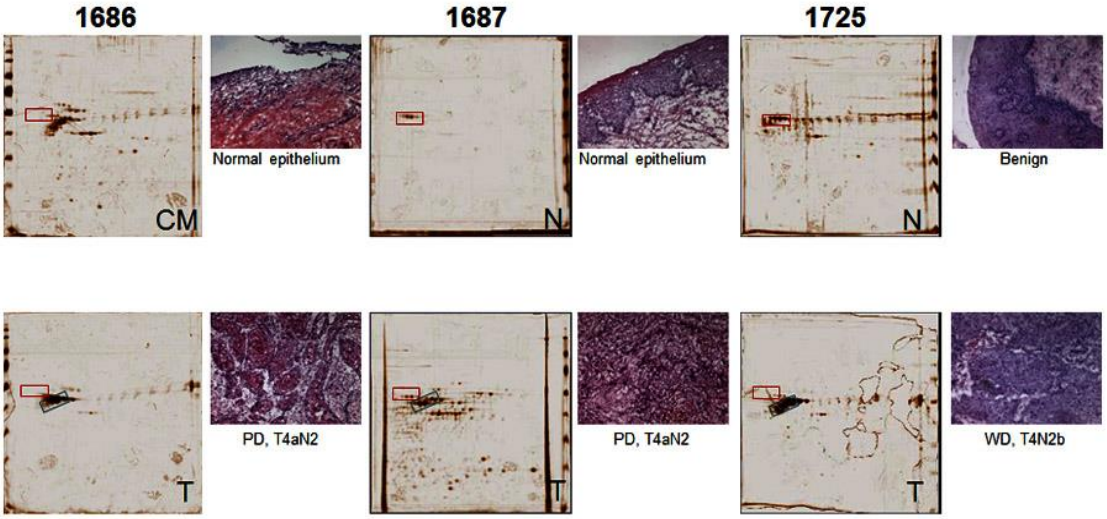


Figure 56. Comparison of the 2DE-silver staining pattern for enriched keratins with the H&E stained profile of the tissue sections for sample 1726 (N, and T), 1731 (N and T) and 1741 (N and T). The pH range used for IEF is shown above the gel profile and the magnification is indicated on the H&E stained sections. WD; Well differentiated, MD; Moderately differentiated.

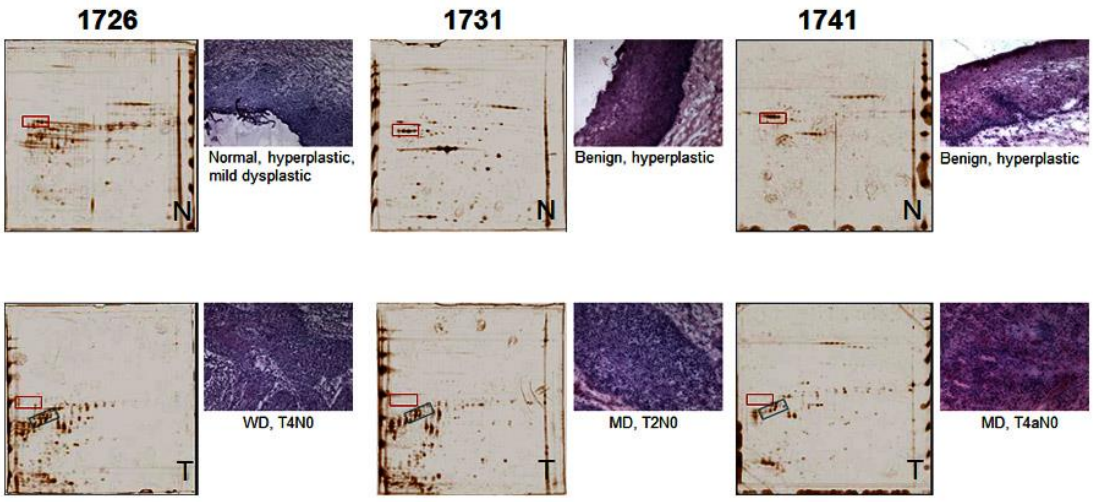


Figure 57. Comparison of the 2DE-silver staining pattern for enriched keratins with the H&E stained profile of the tissue sections for sample 1726 (N, and T), 1731 (N and T) and 1741 (N and T). The pH range used for IEF is shown above the gel profile and the magnification is indicated on the H&E stained sections. WD; Well differentiated, MD; Moderately differentiated.

It is apparent from all the above 2DE silver stained gel patterns that these could be used to advantage to detect early transformation precursor signatures which may have otherwise been missed by mere analysis of histology sections.

3.5 Confirmation of keratin expression by Immunohistochemistry.

The evaluation of keratin expression by 2DE, mass spectroscopy and western blotting has short listed keratin 4, 13, 16, 17 as differentially expressed keratins. Their utility in the clinics would be possible if the immunohistochemistry pattern on tissue sections matches with the information obtained. Tissue section from each of the samples were therefore analysed by IHC.

Figure 58 shows the sections from normal, cut margin and tumor tissues for the sample 1657 stained with antibodies to K4, K13, K14, K16 and K17. The pattern for these

keratins in samples, 1687, 1736, 1737, 1740, 1741 and 1751 are shown in Figures 59 to 64. It is seen that K4 and K13 are mainly seen in the normal or cut margin tissue.

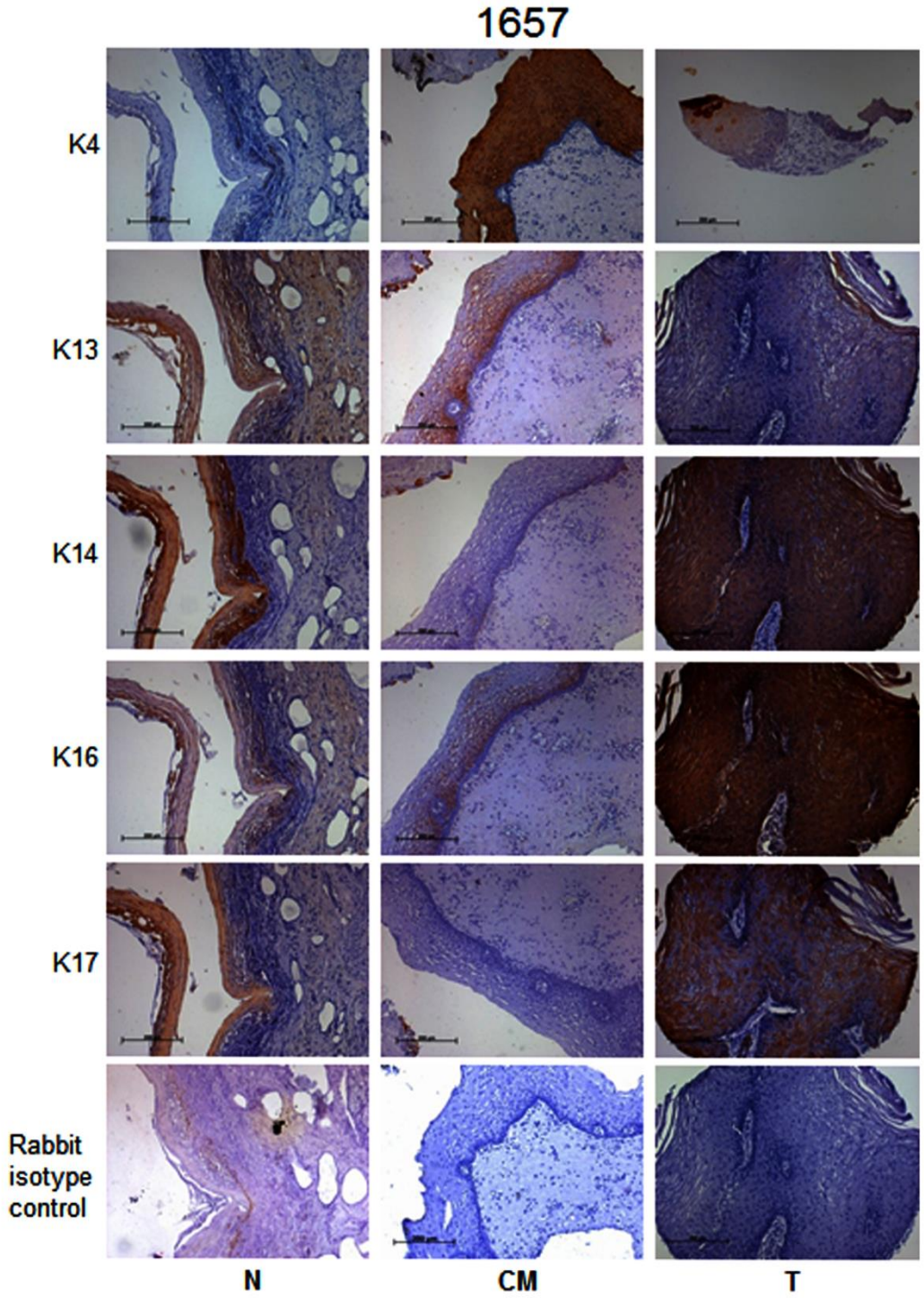


Figure 58. Profiles of keratin expression on tissue sections from sample 1657 immunostained with antibodies to keratin K4, K13, K14, K16 and K17. Sections from normal (N), cut margin (CM) and tumor (T) tissues are shown. Rabbit isotype control antibody was used as a negative control. The magnification used is indicated on the panels.

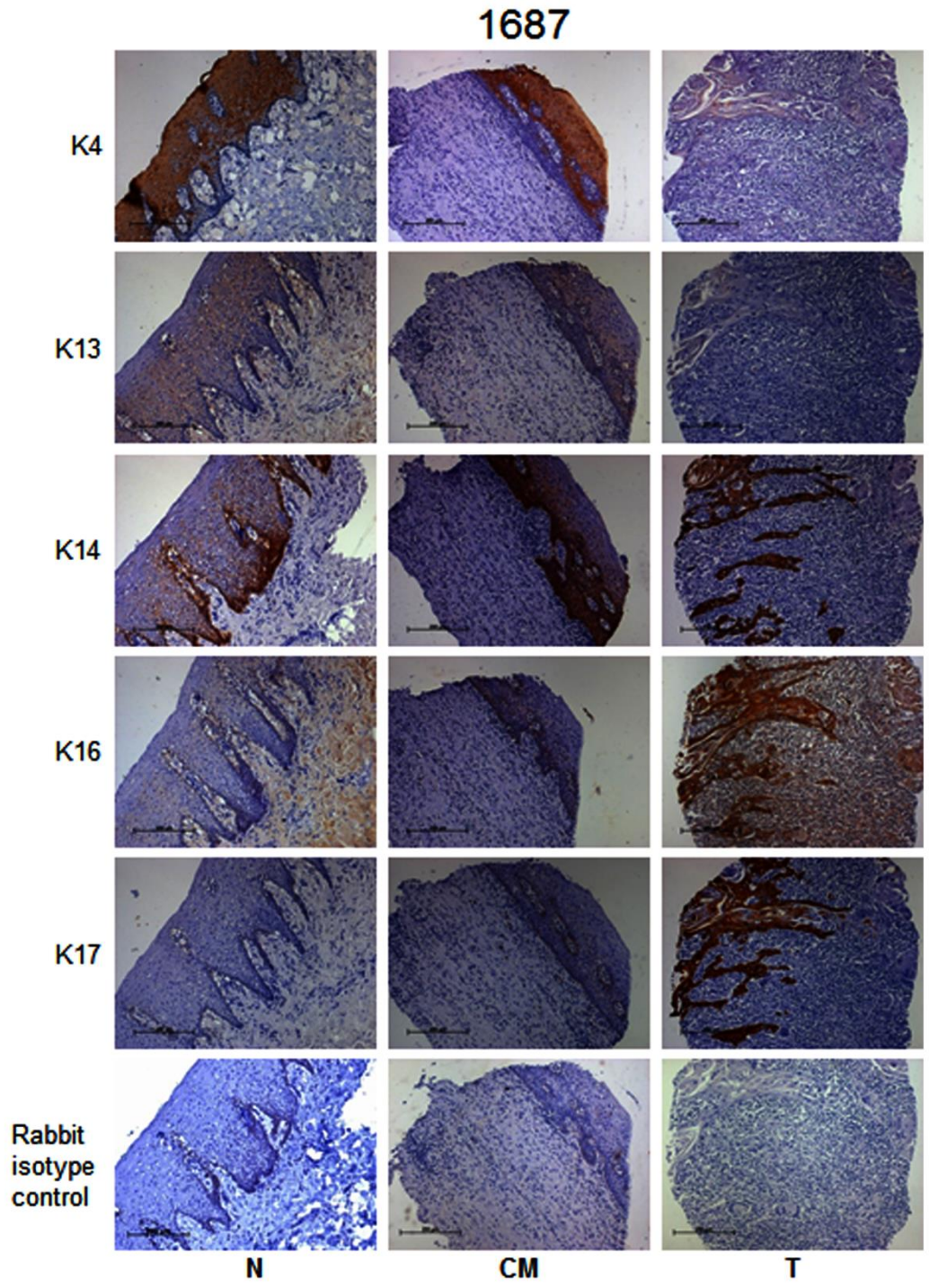


Figure 59. Profiles of keratin expression on tissue sections from sample 1687 immunostained with antibodies to keratin K4, K13, K14, K16 and K17. Sections from normal (N), cut margin (CM) and tumor (T) tissues are shown. Rabbit isotype control antibody was used as a negative control. The magnification used is indicated on the panels.

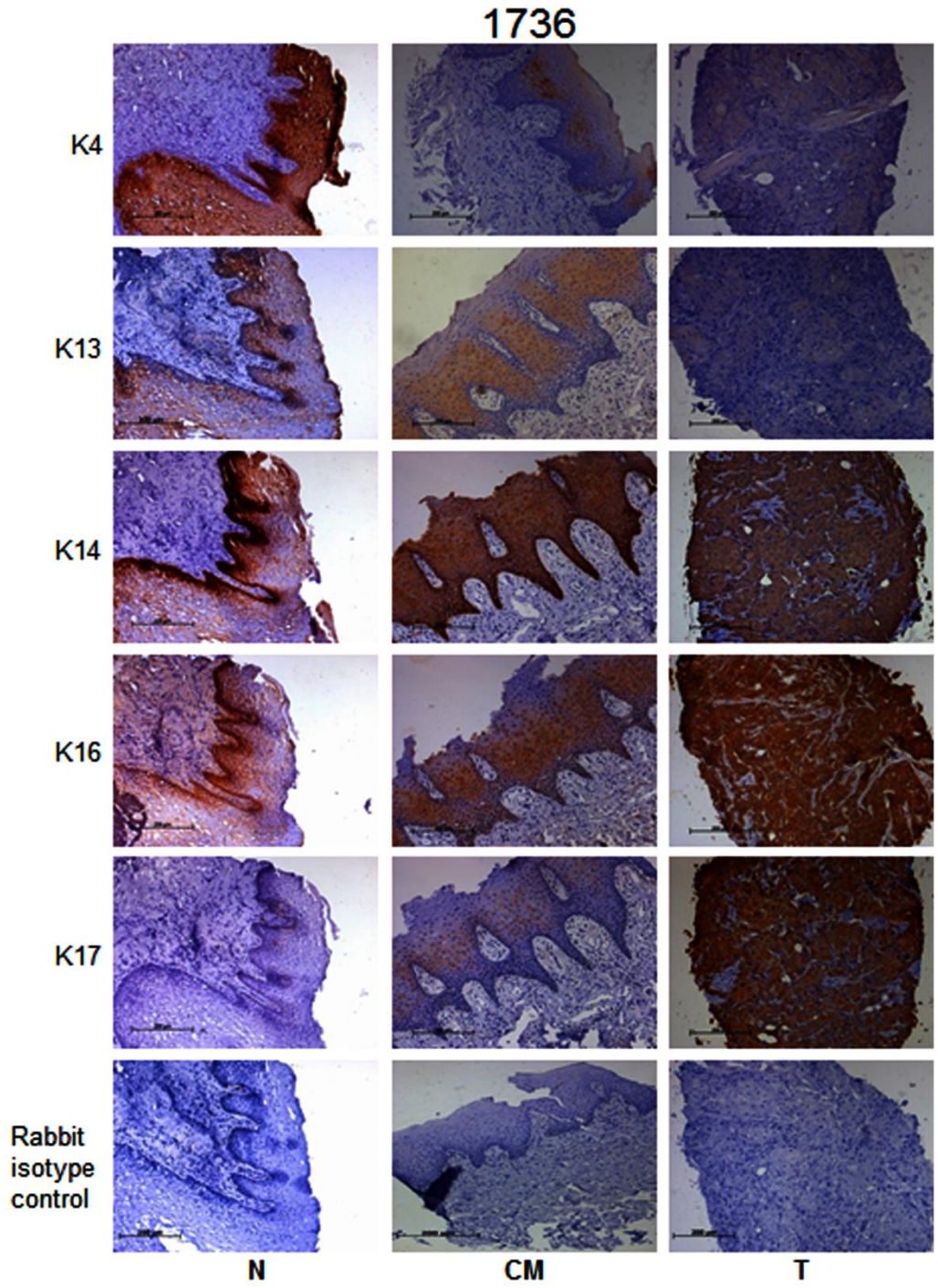


Figure 60. Profiles of keratin expression on tissue sections from sample 1736 immunostained with antibodies to keratin K4, K13, K14, K16 and K17. Sections from normal (N), cut margin (CM) and tumor (T) tissues are shown. Rabbit isotype control antibody was used as a negative control. The magnification used is indicated on the panels.

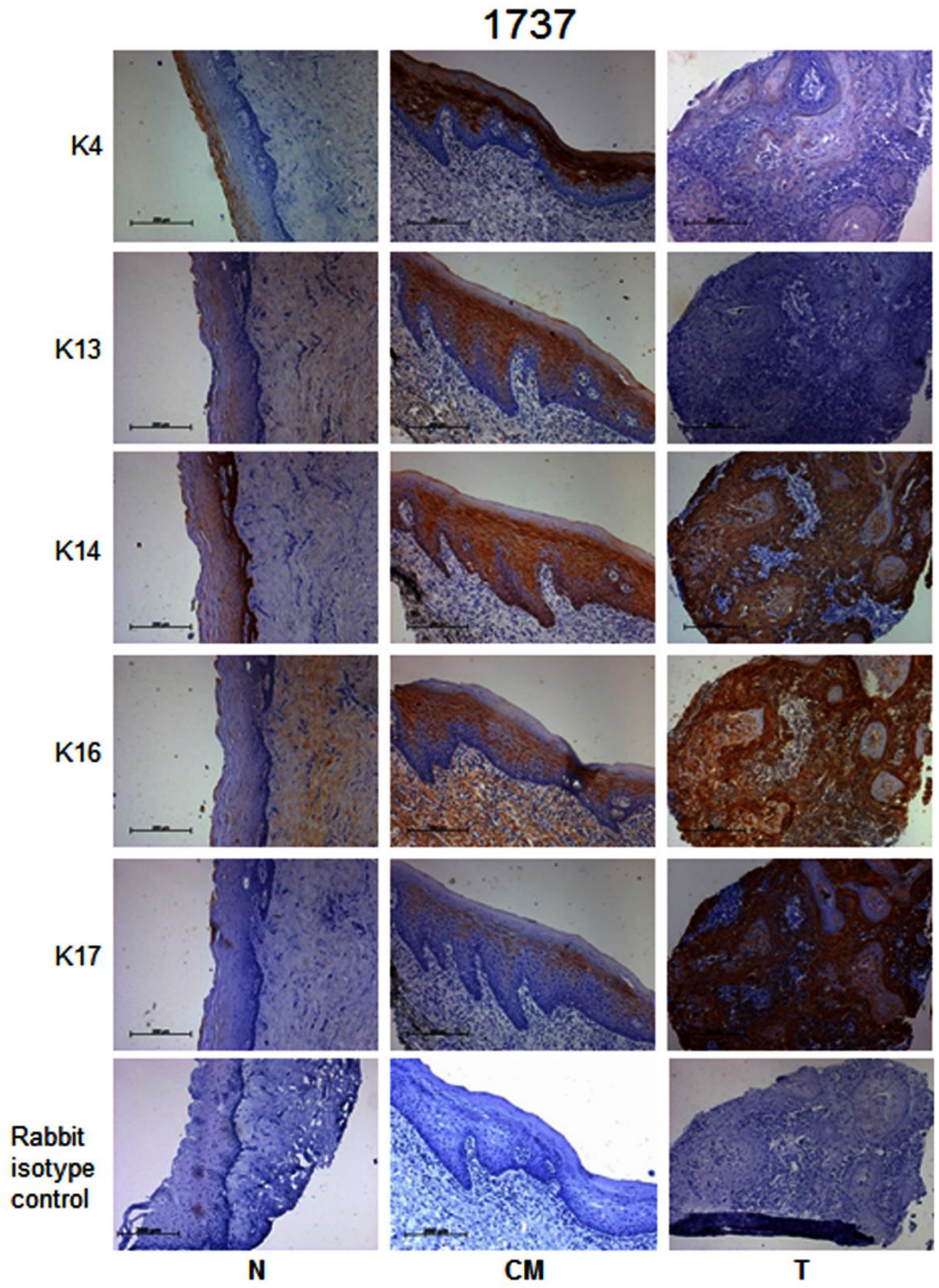


Figure 61. Profiles of keratin expression on tissue sections from sample 1737 immunostained with antibodies to keratin K4, K13, K14, K16 and K17. Sections from normal (N), cut margin (CM) and tumor (T) tissues are shown. Rabbit isotype control antibody was used as a negative control. The magnification used is indicated on the panels.

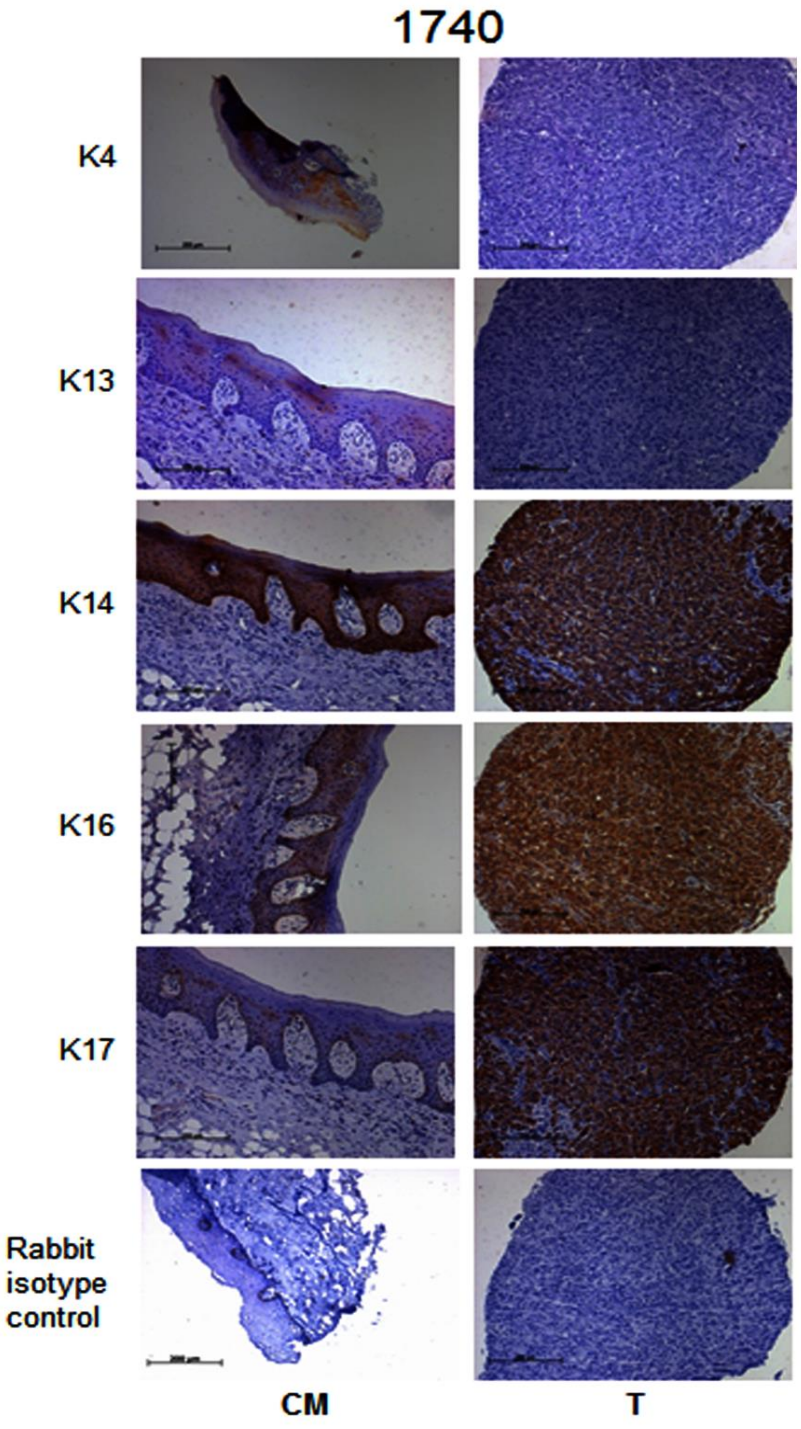


Figure 62. Profiles of keratin expression on tissue sections from sample 1740 immunostained with antibodies to keratin K4, K13, K14, K16 and K17. Sections from normal (N), and tumor (T) tissues are shown. Rabbit isotype control antibody was used as a negative control. The magnification used is indicated on the panels.

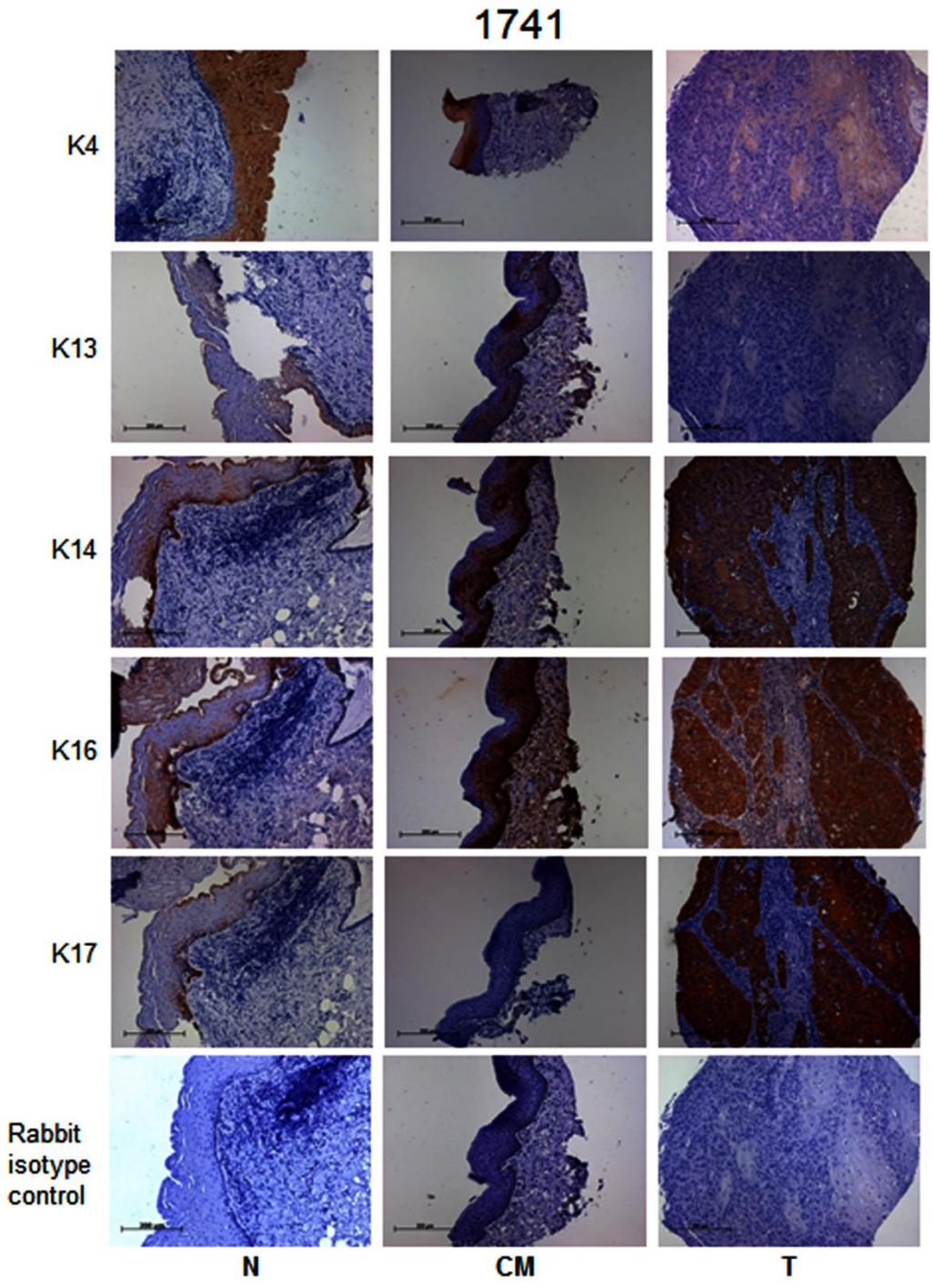


Figure 63. Profiles of keratin expression on tissue sections from sample 1741 immunostained with antibodies to keratin K4, K13, K14, K16 and K17. Sections from normal (N), cut margin (CM) and tumor (T) tissues are shown. Rabbit isotype control antibody was used as a negative control. The magnification used is indicated on the panels.

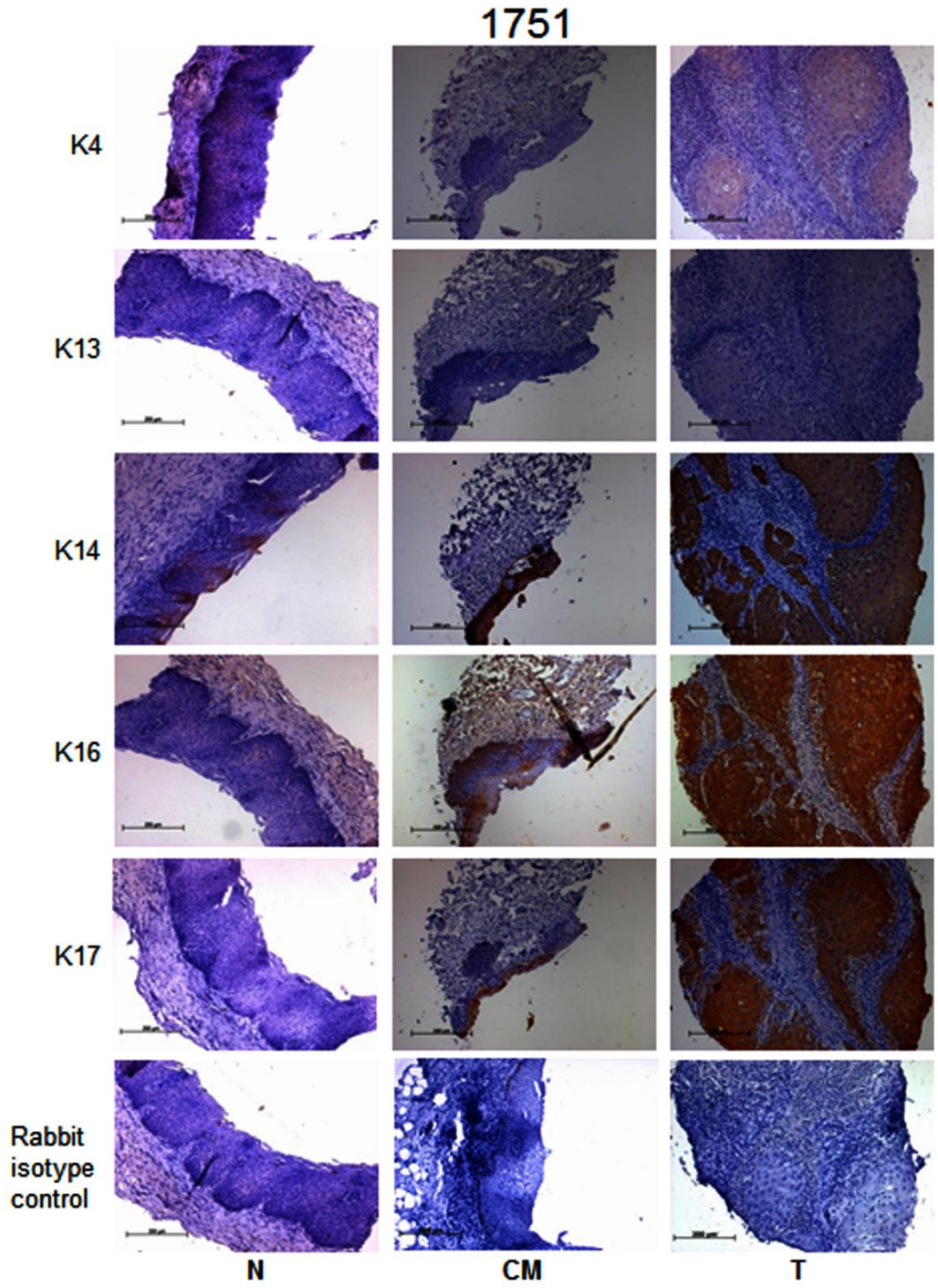


Figure 64. Profiles of keratin expression on tissue sections from sample 1751 immunostained with antibodies to keratin K4, K13, K14, K16 and K17. Sections from normal (N), cut margin (CM) and tumor (T) tissues are shown. Rabbit isotype control antibody was used as a negative control. The magnification used is indicated on the panels.

3.6 Comparison of the expression pattern obtained by mass spectrometry, silver staining, western blotting and immunohistochemistry.

Each technique has its pros and cons and it is always advisable to verify any finding with one or more techniques. Towards this end, in this section, the keratin profile obtained above by the different techniques has been collated. The data using different techniques is juxtaposed. All the samples could not be analysed by all the techniques. The 1DE and 2DE mass spectrometry information is from the details given in sections 3.1 and 3.3, the WB data from section 3.4 and IHC from section 3.5. Figs. 65 to 68 show the compilation for the comparison of expression pattern by various techniques. It is apparent that the presence of a keratin is supported by atleast three of the four techniques *i.e.* silver staining, mass spectrometry, 2DE western blotting and immunohistochemistry.

Figure 65 shows the composite for comparison of expression pattern obtained by MS, silver staining, WB and IHC for sample 1736. The presence of K13 is seen on the silver stained gel, by 2DE-MS, 2DE-WB and by IHC for 1736N. Good 1DE mass spectrometry data could not be obtained for this sample even with 5 µg of enriched sample. K16 was seen only by IHC, suggesting that it may be a nonspecific interaction.

The **cut margin** sample shows K13 positivity by all the methods. The string of three spots which has been shown to be K13 by 2DE-MS., IHC and 2DE-WB all support the presence of K13. K14 was identified by IHC and 2DE-WB. The faintly stained set of spots curve below the position of K13 on the silver stained gel indicates the possibility of presence of K14. The amount (3µg) used may not contain enough K14 detectable by 1DE-WB and MS, but even the more amount (5µg) also did not give the identity for K14). K16 and K17 were identified only by IHC. The amount of protein loaded (3µg

for 1DE and 30µg for 2DE) may not be sufficient to detect the K16 and K17 in this sample. The tumor sample shows presence of K14, K16 and K17 by silver staining, IHC, and 2DE-WB but not by 1DE-MS.

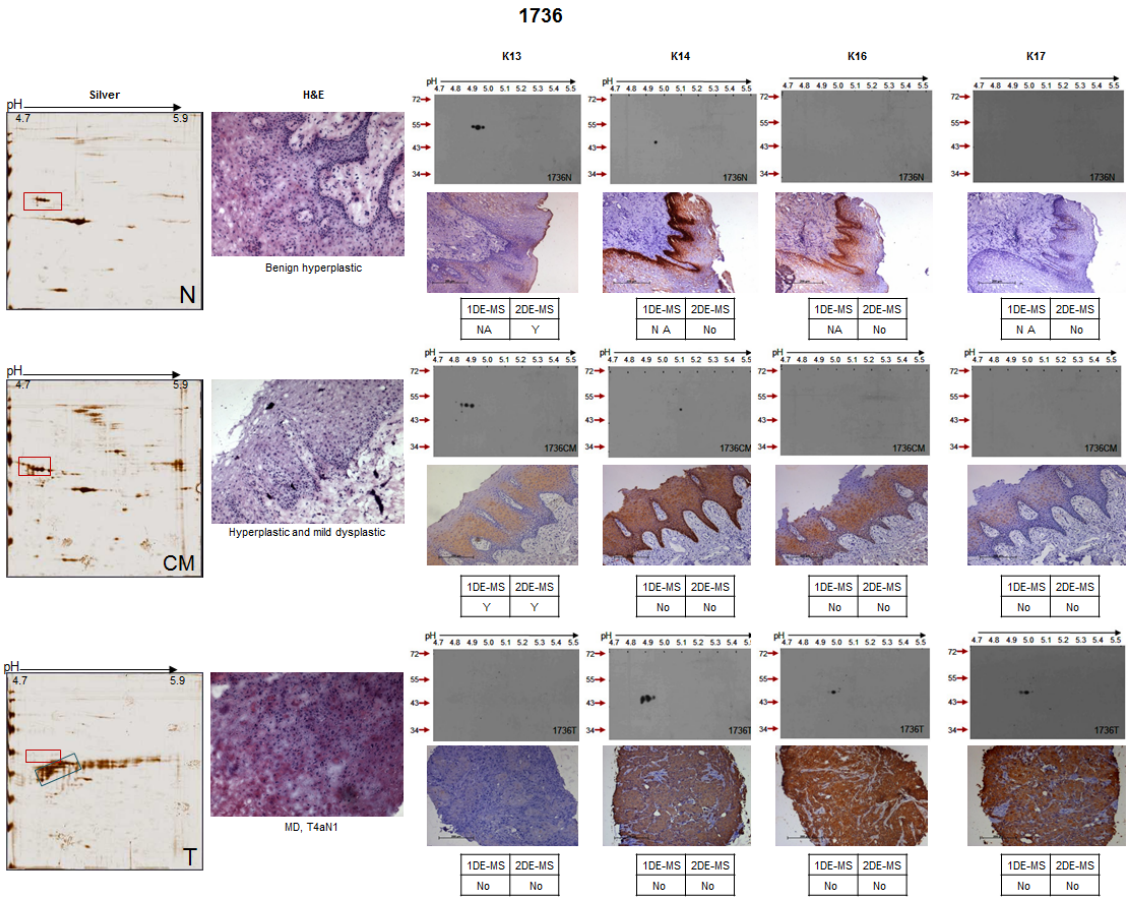


Figure 65. Comparison of the expression pattern obtained by 1DE/2DE- mass spectrometry, 2DE-silver staining, 2DE western blotting and immunohistochemistry staining for Sample No. 1736.

Fig. 66 shows the pattern for sample 1737. In the **normal** sample K13 is clearly seen as a string of spots on the silver stained gel. Its presence is confirmed by 2DE WB and IHC. K14 is seen faintly by 2DE WB and IHC but not supported by mass spectrometry. K16 and K17 are absent as shown by all the techniques. This pattern of expression is as expected for normal epithelium. In the **cut margin** sample, presence of K13, K14, K16 and K17 is shown by silver stained pattern, 2DE-MS, 2DE-WB and IHC. In the **tumor**

sample, K13 is absent, K14, 16, 17 are present as seen from silver staining pattern, 2DE WB, IHC and 2DE-MS.

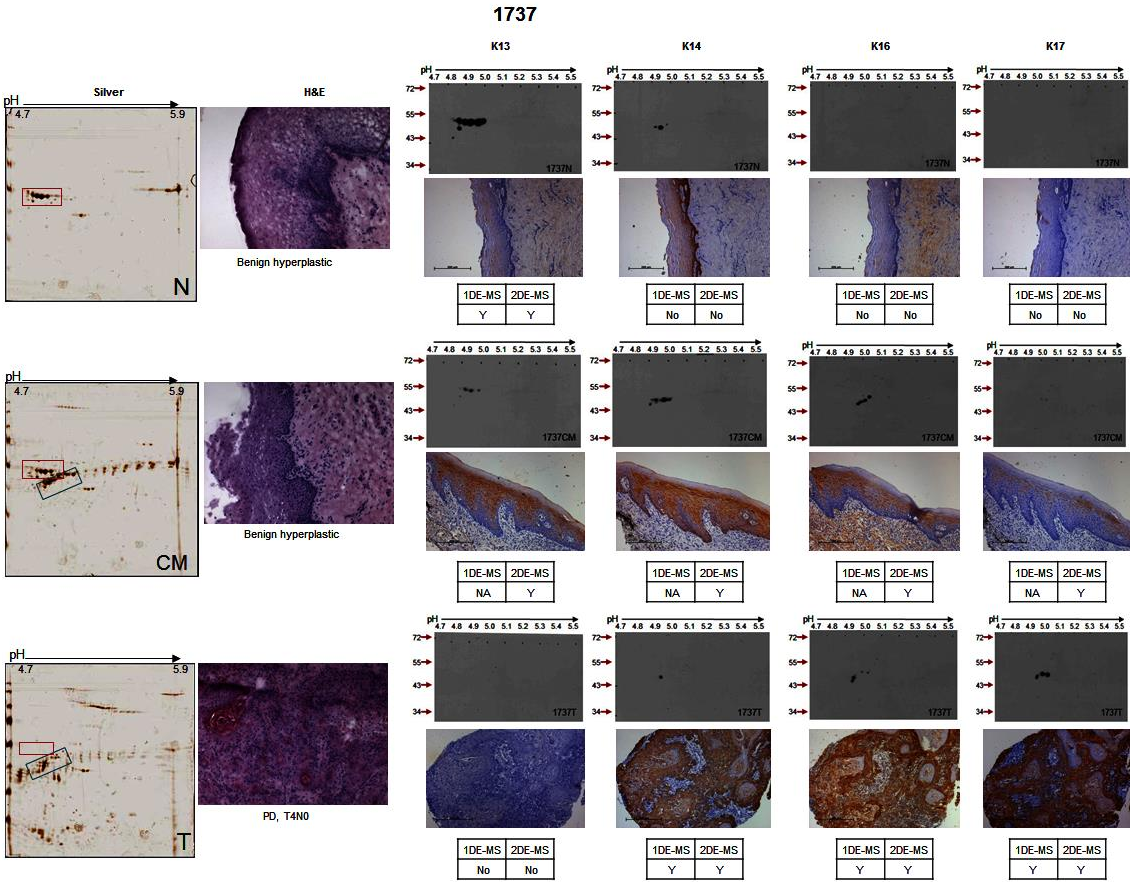


Figure 66. Comparison of the expression pattern obtained by 1DE/2DE- mass spectrometry, 2DE-silver staining, 2DE western blotting and immunohistochemistry staining for Sample No. 1737.

Fig. 67 shows the profile for sample 1740. In the normal sample, K13 is present, as supported by silver staining, 2DE WB, IHC, 1DE and 2DE-MS. K14 is seen by 2DE WB and 2DE-MS and maybe present in the faint curved set of spots below position of K13 on the silver stained gel. K16 and K17 were not identified by 1DE-MS, 2DE-WB, and 2DE-MS. Tissue was not available for IHC.

In the cut margin sample, K13 is present, as supported by silver staining, 2DE WB, IHC, and 2DE-MS. K14 is seen by IHC and faintly on the silver stained gel pattern and by 2DE-WB. K16 is seen faintly by IHC and maybe present in the faint curved set of

spots below position of K13 on the silver stained gel. K17 is absent by all the techniques. In the tumor **s**ample, K13 is absent and K14, K16, K17 are present as shown by all the techniques.

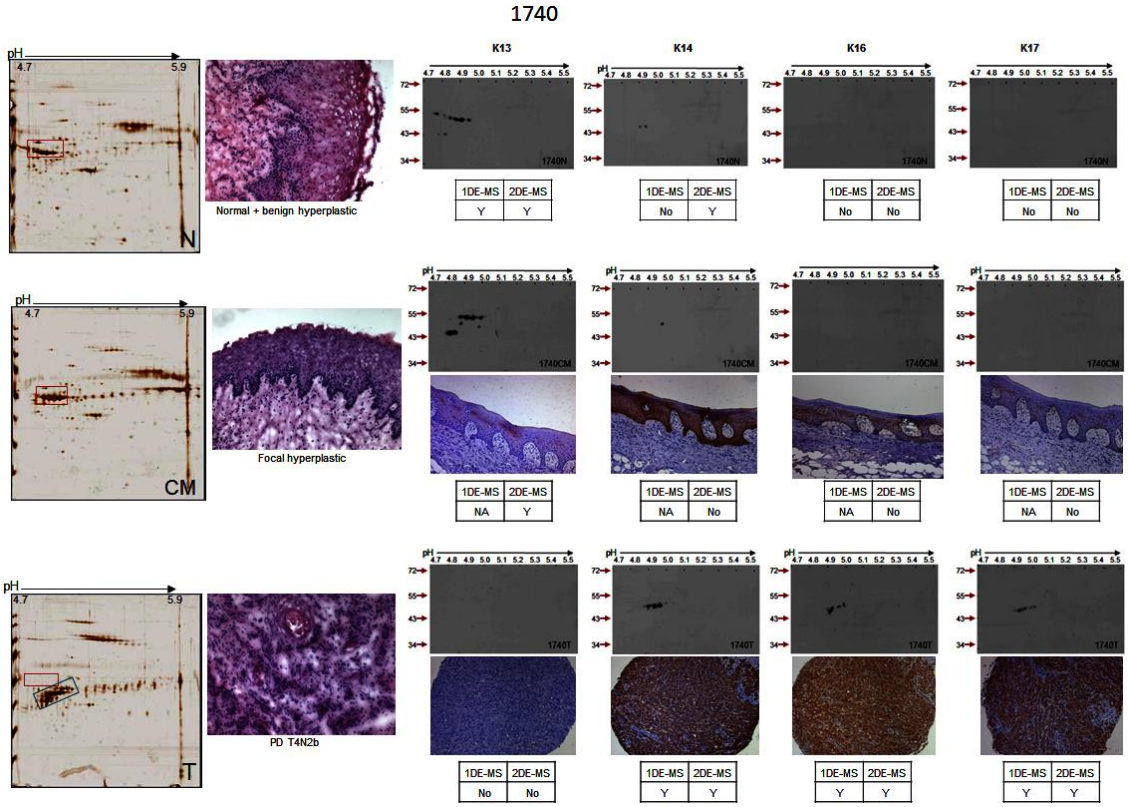


Figure 67. Comparison of the expression pattern obtained by 1DE/2DE- mass spectrometry, 2DE-silver staining, 2DE western blotting and immunohistochemistry staining for Sample No. 1740. Tissue was not available for IHC for 1740 N.

Fig. 68 shows the pattern for sample 1751. In the normal sample, K13 is present as shown by silver stained pattern of the 2DE gel, by 2DE-WB and by mass spectrometry. K14 is faintly seen by 2DE-WB and IHC and there is silver stained curved set of spots on the gel below K13 in which K14 may be present. K16 and K17 are absent by all the techniques. In the cut margin sample, presence of K13 is shown by the silver stained gel pattern, 2DE-WB. IDE-MS and direct 2DE-MS data is not available for this sample. K16 and K17 are faintly seen by IHC and they could be present in the curved set of spots below K13 on the 2DE gel. No signal was seen for K 16 on 2DE-WB

although it is seen for K17. In the tumor sample, K13 is absent as supported by silver stained pattern on the gel, 2DE-WB, IHC and mass spectrometry. K14 and K17 are seen by the silver staining pattern, 2DE-WB and IHC. K16 is supported only by IHC.

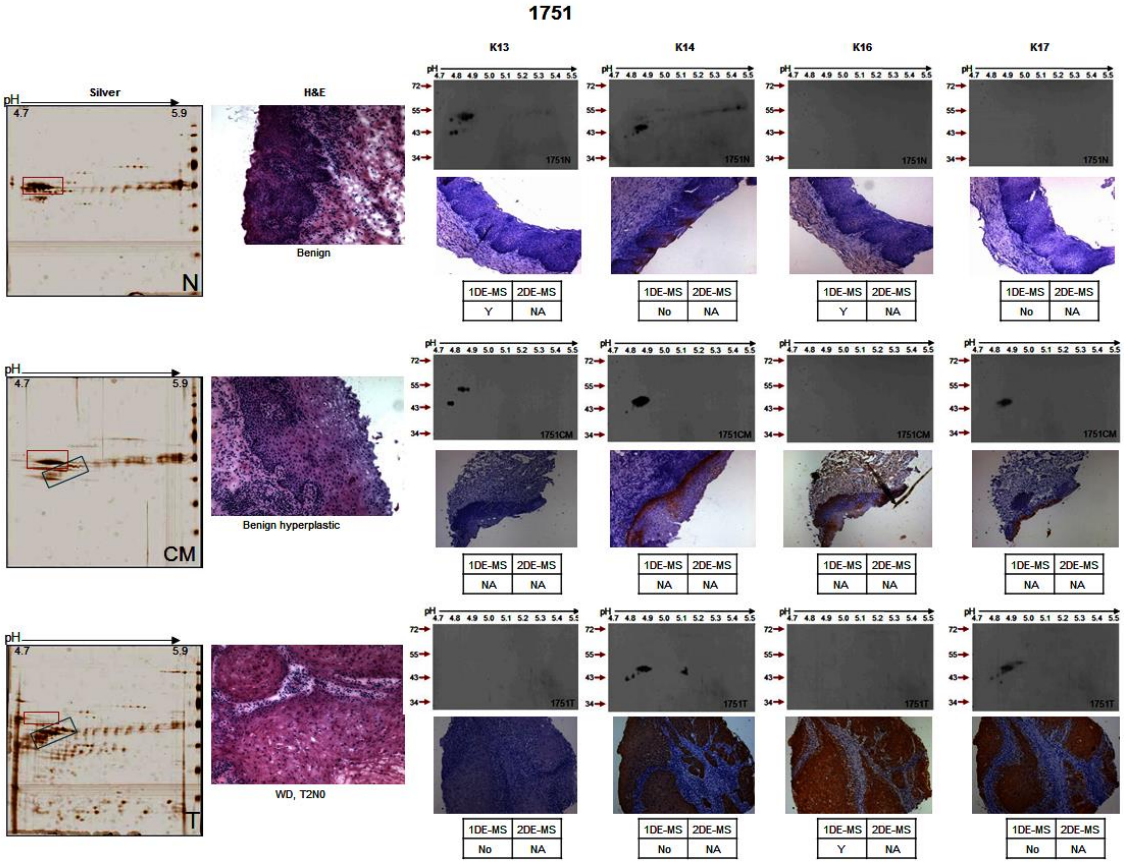


Figure 68. Comparison of the expression pattern obtained by 1DE/2DE- mass spectrometry, 2DE-silver staining, 2DE western blotting and immunohistochemistry staining for Sample No. 1751.

Comparison of information for other samples is provided in **Appendix Figures A2** (Fig. A 2.1-2.8)**.**

Chapter 4

DISCUSSION

Chapter 4. DISCUSSION

Keratins have been used as biomarkers for diagnosis and prognosis in various cancers [18]. Histological status of the tissue is often used by the pathologist to define the normal area from the tumor during surgery. In most studies of Head & Neck cancer the expression of a few specific keratins has been evaluated by IHC [20-25, 141-143, 205-214]. Aberrant expression of the simple epithelial K8/18 has been reported in the literature for the stratified non-keratinized epithelium of oral cancer [22, 27, 29, 143]. In our earlier investigations we have reported the need of mass spectrometry to confirm antibody based findings. This is with reference to the use of an antibody (clone CY90) to K18 which has shown to cross react with K13 irrespective of presence or absence of K18. These observations necessitate caution in accepting the reported presence of K18 in head & neck cancers with this antibody. In the present work, the expression of keratins in a subsite of the oral cavity, the gingivo buccal complex has been investigated by mass spectrometry, 2DE, western blotting, silver staining and immunohistochemistry. This study reports the presence and glycosylation of K13 in normal epithelium, absence of K4, K13 and increased expression of K14, K16 and K17 in the cancer of gingivo buccal complex. The mixed expression pattern seen in cut margins shows the continuum of expression from normal to transformed state and validates the observations. K8/18 were not identified by mass spectrometry in the samples evaluated in this study.

K4/13 are reported to be expressed in the suprabasal layer of stratified squamous epithelium [204]. Literature reports show that in some squamous cell carcinomas they are lost and in some they are focally and variably expressed such as in head and neck carcinoma and in transitional cell carcinoma where there is pronounced expression in poorly differentiated tumors [135, 220, 221]. Mouse K4 plays key a role to maintain

epithelial cell integrity [222]. Point mutations in K4/13 have been associated with the autosomal dominant disorder white sponge nevus [119, 120]. In this study K4/13 are identified in normal epithelium by mass spectrometry which is seen in the suprabasal layer of the normal epithelium by IHC and has been shown to be downregulated in the tumor suggesting loss of or disintegration of suprabasal layer during the tumorogenesis. The suprabasal layer may control and protect the proliferating basal layer from uncontrolled expansion. The loss of K4 and K13 may therefore help the tumor cells to facilitate the uncontrolled expansion which in turn help to adapt and survive in the body system. Although, their role is not very clear in the human tissues, they may have a key role in maintaining the tissue architecture.

K5/14 are strongly expressed in undifferentiated basal cell layers of stratified epithelium containing stem cells and down regulated in the differentiating suprabasal levels [136, 221]. They bundle up as tonofilaments and are attached to desmosomes and hemidesmosomes. Most squamous cell carcinomas strongly express these keratins while little, focal or no staining is seen in adenocarcinomas [14, 128, 135, 223, 224]. K14 have previously been reported to be over expressed in oral cancer shown by immunohistochemistry [22] and by mass spectrometry in tongue cancers [30, 31]. The increased expression of K5/14 could contribute to the disorganised arrangement by facilitating the dividing capacity of the epithelial cells in transformed tissues.

K6/16 are constitutive keratins of stratified epithelia and are often expressed as a pair. They are expressed in mucosal tissue where it is in a state of high proliferation [13, 128, 225]. There are three very homologous isoforms of K6, K6a, K6b, and K6c which are encoded by separate genes. K6 is expressed in the suprabasal layers of non-keratinizing squamous epithelium. They are involved in mechanical scaffolding and migration and re-epithelialization. K6/16 are strongly expressed in squamous cell carcinoma of

several sites [128, 135]. Previous reports have suggested the over expression of K16 in tongue cancer by immunohistochemistry and in-situ hybridization [226] and by mass spectrometry [30]. Keratin 16 is regarded as proliferation marker and the over expression of K16 may aid the tumor cells in proliferation of its progeny to invade the surrounding area. The increased expression of K6/16 could contribute to the invasiveness of the cancers.

K17 is absent in non-keratinizing stratified squamous epithelia and reported to be expressed in squamous cell carcinomas. K17 has been reported to be over expressed in oral submucous fibrosis by immunohistochemistry [23] and in tongue cancer by immunohistochemistry [211] and by mass spectrometry [30]. Toyoshima *et.al.* [38] have reported the over expression of K17 in oral squamous cell carcinoma by DNA microarray but have not mentioned the subsites of the oral site. This study confirms the overexpression of K17 by mass spectrometry, western blotting and by IHC.

K8/18 are components of simple epithelia. They are involved in structural and mechanical properties of the cell as well as they have a role in cell cycle and apoptosis. They are reported to be aberrantly expressed in squamous cell carcinomas of various tissues including that of the head and neck [128, 227]. *This study did not identify K8/18 by mass spectrometry in either normal or GBC cancer tissues.* The 2DE western blotting and mass spectrometry results have shown the cross reactivity of K18 (CY90) antibody.

In summary the keratin signature obtained by mass spectrometry and validated by 2DE, western blotting and IHC provides markers to distinguish the normal tissue from the non-transformed area. The early disappearance of K4 and K13 and appearance of K14, K16, and K17 as seen in the cut margins indicate that the histologically normal tissue

has already acquired molecular characteristics of the tumor. As cut margins are used to identify limits of surgical resection, evaluation of the keratin profile could be used to advantage by the clinician to monitor and/or reduce recurrence in patients. Studies to understand the mechanisms regulating the expression of these keratins will provide clues to the transformation process. The combination of K14, 16 and K17 could be tested as markers for identification of micro-metastasis in the cervical lymph nodes.

Chapter 5

SUMMARY AND CONCLUSION

Chapter 5. SUMMARY AND CONCLUSION

Summary:-

- 1DE-mass spectrometry of enriched keratin preparations revealed the presence of K1, K2, K4, K5, K6, K10, K13, K14, K15, K16, K17 of which K4 and K13 were most often seen only in normal and or cut margin samples and K14, K16 and K17 were seen in tumor tissues. Keratins 8 and 18 were not seen in any of the samples in this study.
- 1DE immunoblotting alternatively confirmed the mass spectrometry identity of keratins 1, 4, 5, 6, 10, 13, 14, 16 and 17. 2DE followed by silver staining and mass spectrometry of the spots confirmed the presence of the keratins identified by 1DE-mass spectrometry.
- 2DE immunoblotting evaluation confirmed the antibody based keratin identity with respect to molecular weight and isoelectric point of the keratins.
- Silver staining of the 2DE gels for enriched keratins showed distinct pattern for normal, cut margin and tumor tissue keratins.
- Immunohistochemistry of the tissue sections confirmed the keratin expression pattern. Specific observations are the downregulation of K4 and K13 and over expression of K14, 16 and 17 in tumor tissue.

Conclusion:-

- Samples in which the cut margin tissue do not show the expression of K4 and K13 can be considered as partially transformed as indicated by this molecular change which is not apparent by histology. *The patient with this presentation could be*

prone for recurrence of the disease and this observation may have clinical relevance.

- The keratin pattern seen on the silver stained gel was reflective of the histologically normal and transformed state of the tissue.
- Presence of the normal plus tumor pattern in the cut margin shows early molecular changes not apparent by histology.
- This study has identified the differentially expressed keratins in normal and tumor tissue and confirmed their presence by mass spectrometry, western blotting, pI and molecular weight and localization on the tumor sections, thereby *providing markers for use in the clinic.*
- Follow-up of the patient history and its clinical correlation with above findings can be instrumental for avoiding recurrence in the cancer patients.
- The combination of K14, 16 and K17 could be tested as markers for identification of micro-metastasis in the cervical lymph nodes.

Potential utility of the findings in the clinic:

This study has identified K4, K13, K14, K16 and K17 which can distinguish between normal and tumor tissues and has potential as biomarkers in cancer of gingivo buccal complex.

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APPENDIX

APPENDIX

Table A1. Samples used for profiling of keratins.

Sr. No.	Sample No.	Age	Sex	Differentiation Status	pTNM	Site
1.	1579	60	F	MD	T4aN0	BM+GBS+Alv
2.	1580	45	F	MD	T3N0	BM+GBS
3.	1606	67	M	WD	T4aN0	Alv+GBS
4.	1657	70	M	WD	T3N0	BM
5.	1686	45	M	PD	T4aN2	BM+GBS
6.	1687	39	M	PD	T4aN2	BM+GBS
7.	1725	61	M	WD	T4N2b	Alv+RMT
8.	1726	50	F	WD	T4N0	BM+GBS+RMT
9.	1731	41	M	MD	T2N0	BM
10.	1735	45	M	PD	T4aN0	BM+RMT
11.	1736	36	M	MD	T4aN1	GBS recurrent
12.	1737	48	M	PD	T4aN0	GBS+RMT
13.	1740	43	M	PD	T4N2b	BM+lower lip
14.	1741	57	M	MD	T4aN0	BM+GBS
15.	1751	70	M	WD	T2N0	BM

Table A2. 1DE-mass spectrometry analysis.

1DE-Mass spectrometry analysis for sample 1579N (Fig. 6, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerance (Da)
N5	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	76	66170	26.7	36	100ppm	95	15	K.YEELQITAGR.H R.FLEQQNQVLQTK.W K.QISNLQQSISDAEQR.G R.FSSCGGGGGSFGAGGGFGSR.S R.GGGGGGYGSGGSSYGSGGGSYGSGGGGGGGR.G	4.9e-05 0.0014 0.00027 2.9e-05 9.1e-18	60 45 51 56 177	289	13	5 (5)	0.7
N6	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	138	66170	31.5	41	100ppm	85	23	K.IEISELNR.V K.YEDEINKR.T K.YEELQITAGR.H K.NMQDMVEDYR.N R.FLEQQNQVLQTK.W R.GGGGGGYGSGGSSYGSGGGSYGSGGGGGGGR.G	0.19 0.0071 3.3e-05 0.058 0.0035 1.4e-10	25 38 62 26 41 110	167	12	6 (4)	0.7
	Keratin, type II cytoskeletal 5	K2C5_HUMAN	P13647	121	62568	25.7	34	100ppm	85	22	K.FASFIDK.V R.GRLDSELR.N K.YEDEINKR.T K.AQYEEIANR.S K.LALDVEIATYR.K R.VSLAGACGVGGYGSR.S	0.031 2.1 0.0071 0.03 5.6e-05 1.5	31 15 38 32 59 14	73	9	6 (4)	0.7
	Keratin, type I cytoskeletal 10	K1C10_HUMAN	P13645	109	59020	20.6	30	100ppm	85	19	R.LENEIQTYR.S R.QSVEADINGLR.R R.SQYEQLAEQNRK.D	0.34 6 0.00019	21 9 54	54	5	3 (1)	0.7
	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	105	60293	24.3	33	100ppm	85	19	K.FASFIDK.V R.GRLDSELR.G K.YEDEINKR.T K.AQYEEIAQR.S K.YEELQVTAGR.H K.LALDVEIATYR.K	0.031 2.1 0.0071 0.1 0.058 5.6e-05	31 15 38 27 29 59	71	9	6 (4)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	105	60273	24.3	33	100ppm	85	19	K.FASFIDK.V R.GRLDSELR.G K.YEDEINKR.T K.AQYEEIAQR.S K.YEELQVTAGR.H K.LALDVEIATYR.K	0.031 2.1 0.0071 0.1 0.058 5.6e-05	31 15 38 27 29 59	71	9	6 (4)	0.7
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	97	60315	25.8	32	100ppm	85	18	K.FASFIDK.V R.GRLDSELR.N K.YEDEINKR.T K.AQYEEIAQR.S K.YEELQITAGR.H K.LALDVEIATYR.K	0.031 2.1 0.0071 0.1 3.3e-05 5.6e-05	31 15 38 27 62 59	103	9	6 (4)	0.7
	Keratin, type II cytoskeletal 2 epidermal	K22E_HUMAN	P35908	74	65678	22.8	26	100ppm	85	15	K.FASFIDK.V K.IEISELNR.V K.YEDEINKR.T K.AQYEEIAQR.S K.LALDVEIATYR.K R.FLEQQNQVLQTK.W	0.031 0.19 0.0071 0.1 5.6e-05 0.0035	31 25 38 27 59 41	82	8	6 (4)	0.7
N7	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	91	66170	32	37	100ppm	75	15	K.YEELQITAGR.H R.FLEQQNQVLQTK.W R.SGGGFSSGSAGIINYQR.R R.FSSCGGGGGSFGAGGGFGSR.S R.GGGGGGYGSGGSSYGSGGGSYGSGGGGGGGR.G	0.0028 0.0077 5.8e-06 2.2e-08 1.3e-16	42 37 68 91 172	306	13	5 (5)	0.7
	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	63	60293	19.1	29	100ppm	75	13	K.FASFIDK.V K.AQYEEIAQR.S K.YEELQVTAGR.H R.QNLEPLFEQYINNLR.R	0.0033 0.92 0.22 7.1e-05	41 18 23 57	69	7	4 (2)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	63	60273	19.1	29	100ppm	75	13	K.FASFIDK.V K.AQYEEIAQR.S K.YEELQVTAGR.H R.QNLEPLFEQYINNLR.R	0.0033 0.92 0.22 7.1e-05	41 18 23 57	69	7	4 (2)	0.7

	Keratin, type II cytoskeletal 2 epidermal	K22E_HUMAN	P35908	58	65678	26.7	26	100ppm	75	11	K.FASFIDK.V K.AQYEEIAQR.S R.FLEQQNQVLQTK.W	0.0033 0.92 0.0077	41 18 37	53	4	3 (2)	0.7
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	56	60315	19.2	27	100ppm	75	12	K.FASFIDK.V K.AQYEEIAQR.S K.YEELQITAGR.H R.QNLEPLFEQYINNLR.R	0.0033 0.92 0.0028 7.1e-05	41 18 42 57	83	7	4 (3)	0.7
N8	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	120	60293	34.9	37	100ppm	91	20	K.FASFIDK.V K.AQYEEIAQR.S R.QNLEPLFEQYINNLR.R	0.00032 11 0.0005	51 7 48	71	5	3 (2)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	110	60273	34.9	34	100ppm	91	19	K.FASFIDK.V K.AQYEEIAQR.S R.QNLEPLFEQYINNLR.R	0.00032 11 0.0005	51 7 48	71	5	3 (2)	0.7
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	112	60315	32.7	35	100ppm	91	19	K.FASFIDK.V K.AQYEEIAQR.S R.QNLEPLFEQYINNLR.R	0.00032 11 0.0005	51 7 48	71	5	3 (2)	0.7
	Keratin, type II cytoskeletal 5	K2C5_HUMAN	P13647	80	62568	21.3	26	100ppm	91	17	K.FASFIDK.V K.AQYEEIANR.S K.YEELQQTAGR.H R.QNLEPLFEQYINNLR.R	0.00032 0.021 0.088 0.0005	51 34 27 48	78	6	4 (3)	0.7
N9	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	85	60293	23.5	29	100ppm	69	14	K.FASFIDK.V R.QNLEPLFEQYINNLR.R	3.3 2.4e-08	10 91	91	3	2(1)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	76	60273	23.5	27	100ppm	69	13	K.FASFIDK.V R.QNLEPLFEQYINNLR.R	3.3 2.4e-08	10 91	91	3	2(1)	0.7
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	76	60315	23.1	27	100ppm	69	13	K.FASFIDK.V R.QNLEPLFEQYINNLR.R	3.3 2.4e-08	10 91	91	3	2(1)	0.7
	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	116	49900	30.1	37	100ppm	69	16	R.LKYENELALR.Q R.LQSSSASYGGGFGGGSCQLGGGR.G	0.035 2.8e-07	32 77	82	7	2(2)	0.7
N10	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	122	51872	43.2	41	100ppm	75	17	K.IRDWYQR.Q K.NHEEEMNALR.G K.TRLEQEIATYR.R R.GQVGGDVNVEMDAAPGVDLSR.I	0.086 7.4e-05 0.00011 6.5e-09	28 57 56 96	158	10	4 (3)	0.7
	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	105	49900	30	34	100ppm	75	17	K.IRDWHLK.Q R.LKYENELALR.Q K.TRLEQEIATYR.S	3 0.00073 0.00011	12 48 56	76	6	3 (2)	0.7
	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	56	48361	34.6	24	100ppm	75	12							

1DE-Mass spectrometry analysis for sample 1579T (Fig. 6, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
T2	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	80	60293	26.8	27	100ppm	64	13	K.FASFIDK.V K.LALDVEIATYR.K R.QNLEPLFEQYINNLR.R	0.0063 0.025 4.8e-05	38 33 59	72	5	3(3)	0.7
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	71	60315	27.1	27	100ppm	64	12	K.FASFIDK.V K.LALDVEIATYR.K R.QNLEPLFEQYINNLR.R	0.0063 0.025 4.8e-05	38 33 59	72	5	3(3)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	71	60273	26.2	27	100ppm	64	12	K.FASFIDK.V K.LALDVEIATYR.K R.QNLEPLFEQYINNLR.R	0.0063 0.025 4.8e-05	38 33 59	72	5	3(3)	0.7
T3	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	70	60315	26.9	30	100ppm	73	13	K.AQYEEIAQR.S K.YEELQITAGR.H R.QNLEPLFEQYINNLR.R	0.36 0.46 0.00053	21 20 48	48	6	3(1)	0.7
T4	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	71	60315	29.6	30	100ppm	83	14	K.FASFIDK.V K.AQYEEIAQR.S K.YEELQITAGR.H R.ISIGGGSCAISGGYGSR.A R.QNLEPLFEQYINNLR.R	0.0043 0.017 0.022 0.7 1.1e-09	40 35 33 17 105	126	10	5(4)	0.7
	Keratin, type II cytoskeletal 5	K2C5_HUMAN	P13647	61	62568	23.7	25	100ppm	83	14							
T5	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	96	60315	33.4	27	100ppm	59	12	K.FASFIDK.V K.YEELQITAGR.H K.LALDVEIATYR.K R.QNLEPLFEQYINNLR.R	0.021 6.4 0.041 9.5e-05	33 9 30 56	61	7	4(3)	0.7
T6	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	94	51578	38.8	34	100ppm	97	16	K.IRDWYQR.Q K.NHEEEMLALR.G K.TRLEQEIATYR.R K.IIAATIENAQPILQIDNAR.L R.GQTGGDVNVEMDAAPGVDLSR.I	0.14 7.2 0.51 1.4e-09 2.2	26 7 19 102 11	102	14	5(1)	0.7
	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	77	60293	18.6	33	100ppm	97	15	K.FASFIDK.V K.AQYEEIAQR.S R.QNLEPLFEQYINNLR.R	0.013 2.7 0.00028	35 13 51	57	5	3(2)	0.7
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	70	60315	17.3	31	100ppm	97	14	K.FASFIDK.V K.AQYEEIAQR.S R.QNLEPLFEQYINNLR.R	0.013 2.7 0.00028	35 13 51	57	5	3(2)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	69	60273	18.2	30	100ppm	97	14	K.FASFIDK.V K.AQYEEIAQR.S R.QNLEPLFEQYINNLR.R	0.013 2.7 0.00028	35 13 51	57	5	3(2)	0.7
T7	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	136	51578	49.5	41	100ppm	86	19	R.VLDELTLAR.T K.IRDWYQR.Q K.NHEEEMLALR.G K.TRLEQEIATYR.R K.IIAATIENAQPILQIDNAR.L	3 0.081 0.0082 0.00013 1.3e-10	12 28 37 55 113	153	11	5(3)	0.7
	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	91	51872	47	43	100ppm	86	20	R.VLDELTLAR.A K.IRDWYQR.Q K.NHEEEMNALR.G K.TRLEQEIATYR.R R.ILNEMRDQYEK.M	3 0.081 1.2e-07 0.00013 9.7	12 28 85 55 6	112	10	5(2)	0.7
	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	140	48361	54.8	40	100ppm	86	19	R.VLDELTLAR.A K.IRDWYQR.Q K.NHEEEMNALR.G K.TRLEQEIATYR.R R.ILNEMRDQYEK.M	3 0.081 1.2e-07 0.00013 9.7	12 28 85 55 6	112	11	5(2)	0.7

T8	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	168	51578	56.7	53	100ppm	89	24	R.LEQEIATYR.R K.NHEEEMLALR.G K.TRLEQEIATYR.R K.IIAATIENAQPILQIDNAR.L	7.4 0.13 0.028 4.1e-11	9 25 32 118	122	8	4(2)	0.7
	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	92	48361	44.6	36	100ppm	89	16	R.DYSQYYR.T R.LEQEIATYR.R K.NHEEEMNALR.G K.TRLEQEIATYR.R	2.1 7.4 2.8e-06 0.028	13 9 72 32	75	6	4(2)	0.7
T9	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	81	48361	36.4	34	100ppm	100	16	R.DYSQYYR.T K.IRDWYQR.Q K.NHEEEMNALR.G K.TRLEQEIATYR.R R.LLEGEDAHLTQYK.K	0.73 0.1 4.9e-06 0.015 3.6	18 27 70 34 11	75	11	5(2)	0.7
	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	65	51578	31.7	26	100ppm	100	15	K.IRDWYQR.Q K.NHEEEMLALR.G K.TRLEQEIATYR.R K.IIAATIENAQPILQIDNAR.L	0.1 0.028 0.015 2.3e-05	27 32 34 59	72	9	4(3)	0.7
	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	63	66170	17.1	32	100ppm	100	14	K.YEELQITAGR.H K.NMQDMVEDYR.N K.WELLQQVDTSTR.T R.FSSCGGGGGSFGAGGGFGSR.S	0.0073 1.3 0.11 0.09	38 15 26 26	38	8	4(1)	0.7

1DE-Mass spectrometry analysis for sample 1580CM (Fig. 6, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
CM4	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	115	60293	38.2	27	100ppm	53	11	K.FASFIDK.V K.YEELQVTAGR.H K.QCANLQAAIADAEQR.G R.QNLEPLFEQYINNLR.R	2.8e-05 1.3 0.43 2.9e-05	61 15 19 60	93	8	4(2)	0.7
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	87	60315	36.7	27	100ppm	53	11	K.FASFIDK.V K.YEELQITAGR.H K.QCANLQAAIADAEQR.G R.QNLEPLFEQYINNLR.R	2.8e-05 5.9 0.43 2.9e-05	61 9 19 60	93	8	4(2)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	86	60273	36.1	25	100ppm	53	10							
CM5	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	69	66170	29.4	24	100ppm	58	10	K.YEELQITAGR.H K.SLNNQFASFIDK.V R.FLEQQNQVLQTK.W K.QISNLQQSISDAEQR.G R.FSSCGGGGGSFGAGGGFGSR.S	0.0028 0.013 0.0083 0.14 0.0006	42 35 37 24 48	81	10	5(4)	0.7

1DE-Mass spectrometry analysis for sample 1580T (Fig. 6, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
T3	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	132	66170	52.3	34	100ppm	42	12	K.YEELQITAGR.H K.NMQDMVEDYR.N R.FLEQQNQVLQTK.W R.SGGGFSSGSAGIINYQR.R K.QISNLQQSISDAEQR.G R.FSSCGGGGGSFGAGGGFGSR.S R.GGGGGGGYGSGGSSYGSGGGSYGSGGGGGGGR.G	0.003 1.3 0.35 0.014 0.52 0.001 2.5e-18	42 9 21 34 18 42 185	227	17	7(4)	0.7
T4	Keratin, type I cytoskeletal 10	K1C10_HUMAN	P13645	94	59020	24	33	100ppm	72	15	R.LENEIQTYR.S K.IRLENEIQTYR.S K.GSLGGGFSSGGFSGGSFSR.G R.AETECQNTEYQQLLDIK.I R.GSSGGGCFGGSSGGYGGLGGFGGGSFR.G	2.7 0.0018 0.003 1.3e-05 7.5e-13	12 44 39 63 132	199	12	5(4)	0.7
	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	72	66170	21.7	31	100ppm	72	13	K.YEELQITAGR.H K.SLNNQFASFIDK.V K.WELLQQVDTSTR.T R.SGGGFSSGSAGIINYQR.R R.FSSCGGGGGSFGAGGGFGSR.S R.GGGGGGYGSGGSSYGSGGGSYGSGGGGGGGR.G	0.00076 6.2 0.12 0.00073 1.1e-06 2.7e-09	48 9 25 46 72 94	186	15	6(4)	0.7
	Keratin, type II cytoskeletal 5	K2C5_HUMAN	P13647	78	62568	30	25	100ppm	72	15	K.FASFIDK.V K.AQYEEIANR.S K.YEELQQTAGR.H R.QNLEPLFEQYINNLR.R	0.033 0.81 0.00049 1.3e-09	31 17 49 104	127	6	4(3)	0.7
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	72	60315	26.2	28	100ppm	72	13	K.FASFIDK.V K.AQYEEIAQR.S K.YEELQITAGR.H R.QNLEPLFEQYINNLR.R	0.033 0.48 0.00076 1.3e-09	31 20 48 104	126	7	4(3)	0.7
	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	79	60293	26.5	29	100ppm	72	14	K.FASFIDK.V K.AQYEEIAQR.S K.YEELQVTAGR.H R.QNLEPLFEQYINNLR.R	0.033 0.48 0.18 1.3e-09	31 20 24 104	107	7	4(2)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	79	60273	26.5	29	100ppm	72	14	K.FASFIDK.V K.AQYEEIAQR.S K.YEELQVTAGR.H R.QNLEPLFEQYINNLR.R	0.033 0.48 0.18 1.3e-09	31 20 24 104	107	7	4(2)	0.7
T5	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	199	60293	69	43	100ppm	76	27	K.FASFIDK.V K.AQYEEIAQR.S K.YEELQVTAGR.H K.LALDVEIATYR.K K.ADTLTDEINFLR.A R.GSGGLGGACGGAGFGSR.S R.SRAEAESWYQTK.Y R.ISIGGGSCAISGGYGSR.A K.QCANLQAAIADAEQR.G R.QNLEPLFEQYINNLR.R R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	0.031 0.005 0.00033 0.57 6.4e-09 0.0041 0.13 8.1e-10 8.8e-10 4e-11 5.3e-15	33 42 54 22 101 39 26 108 108 122 157	524	26	11(9)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	176	60273	65.1	41	100ppm	76	25	K.FASFIDK.V K.AQYEEIAQR.S K.YEELQVTAGR.H K.LALDVEIATYR.K K.ADTLTDEINFLR.A R.GSGGLGGACGGAGFGSR.S R.SRAEAESWYQTK.Y R.ISIGGGSCAISGGYGSR.A K.QCASLQAAIADAEQR.G R.QNLEPLFEQYINNLR.R R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	0.031 0.005 0.00033 0.57 6.4e-09 0.0041 0.13 8.1e-10 2.3 4e-11 5.3e-15	33 42 54 22 101 39 26 108 14 122 157	446	26	11(8)	0.7
	Keratin, type II cytoskeletal 5	K2C5_HUMAN	P13647	94	62568	39.9	28	100ppm	76	18	K.FASFIDK.V K.AQYEEIANR.S + Methyl (C-term) K.LALDVEIATYR.K K.QCANLQNAIADAEQR.G R.QNLEPLFEQYINNLR.R	0.031 0.099 0.57 0.083 4e-11	33 29 22 27 122	125	9	5(2)	0.7

T6	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	113	60293	49.9	33	100ppm	74	17	K.FASFIDK.V K.YEELQVTAGR.H R.ISIGGGSCAISGGYGSR.A K.QCANLQAAIADAEQR.G R.QNLEPLFEQYINNLR.R R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	0.018 2.7e-05 0.00036 0.00016 2.4e-09 9.3e-06	33 62 49 53 102 62	226	16	6(6)	0.7
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	99	60315	45.5	31	100ppm	74	16	K.FASFIDK.V K.YEELQITAGR.H R.ISIGGGSCAISGGYGSR.A K.QCANLQAAIADAEQR.G R.QNLEPLFEQYINNLR.R R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	0.018 2.3 0.00036 0.00016 2.4e-09 9.3e-06	33 13 49 53 102 62	191	16	6(5)	0.7
T7	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	163	51872	48.8	51	100ppm	68	21	K.IRDWYQR.Q K.DAEEWFFTK.T K.NHEEEMNALR.G K.TRLEQEIATYR.R K.ILTATVDNANVLLQIDNAR.L R.GQVGGDVNVEMDAAPGVDLSR.I R.ADLEMQIESLKEELAYLK.K R.LLEGEDAHLSSSQFSSGSQSSR.D R.YCMQLAQIQEMIGSVEEQLAQLR.C	0.014 0.52 0.0004 0.14 1e-11 4.3e-05 0.19 0.1 2.8e-06	35 17 48 25 124 58 22 23 69	226	29	9(5)	0.7
	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	89	51578	42.4	31	100ppm	68	15	K.IRDWYQR.Q K.TRLEQEIATYR.R K.IIAATIENAQPILQIDNAR.L R.TDLEMQIEGLKEELAYLR.K R.LLEGEDAHLSSQQASGQSYSSR.E R.YCMQLSQIQGLIGSVEEQLAQLR.C	0.014 0.14 6.9e-11 1.3e-06 0.016 3.3	35 25 116 73 31 8	174	21	6(4)	0.7
T8	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	172	51872	57.9	45	100ppm	72	22	R.LAADDFR.T K.DYSPYFK.T K.IRDWYQR.Q R.LEQEIATYR.R K.NHEEEMNALR.G K.TRLEQEIATYR.R K.ILTATVDNANVLLQIDNAR.L R.GQVGGDVNVEMDAAPGVDLSR.I R.LLEGEDAHLSSSQFSSGSQSSR.D	0.45 0.12 0.035 0.12 9.5e-07 0.00089 2.1e-05 3.5e-14 2.1e-15	20 24 31 26 74 47 61 149 160	391	22	9(6)	0.7
	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	112	51578	56.1	37	100ppm	72	18	R.LAADDFR.T K.DYSPYFK.T K.IRDWYQR.Q R.LEQEIATYR.R K.NHEEEMLALR.G R.TKYEHELALR.Q K.TRLEQEIATYR.R K.IIAATIENAQPILQIDNAR.L R.LLEGEDAHLSSQQASGQSYSSR.E	0.45 0.12 0.035 0.12 0.0004 1.8 0.00089 4.7e-10 6.9e-11	20 24 31 26 49 14 47 108 115	244	19	9(5)	0.7
	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	159	48361	61.2	44	100ppm	72	20	R.LAADDFR.T R.DYSQYYR.T K.IRDWYQR.Q R.LEQEIATYR.R R.TKFETEQALR.L K.NHEEEMNALR.G R.LSVEADINGLRR.V K.TRLEQEIATYR.R R.LLEGEDAHLTQYK.K R.GQVGGEINVEMDAAPGVDLSR.I	0.45 0.0074 0.035 0.12 0.31 9.5e-07 0.9 0.00089 2.4e-06 2.4e-11	20 34 31 26 22 74 17 47 72 121	243	22	10(6)	0.7
T9	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	125	51578	52.3	38	100ppm	63	17	K.IRDWYQR.Q K.NHEEEMLALR.G K.TRLEQEIATYR.R K.IIAATIENAQPILQIDNAR.L R.LLEGEDAHLSSQQASGQSYSSR.E	0.041 16 0.78 1.7e-06 1	31 2 18 73 13	75	14	5(2)	0.7
	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	86	51872	44.5	32	100ppm	63	14	K.IRDWYQR.Q K.DAEEWFFTK.T K.NHEEEMNALR.G K.TRLEQEIATYR.R K.ILTATVDNANVLLQIDNAR.L R.GQVGGDVNVEMDAAPGVDLSR.I R.LLEGEDAHLSSSQFSSGSQSSR.D	0.041 0.87 0.0049 0.78 0.075 0.0038 1.1	31 14 37 18 26 38 13	51	20	7(3)	0.7
	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	94	48361	47.1	31	100ppm	63	14	K.IRDWYQR.Q K.NHEEEMNALR.G K.TRLEQEIATYR.R R.GQVGGEINVEMDAAPGVDLSR.I	0.041 0.0049 0.78 0.013	31 37 18 33	46	11	4(3)	0.7

1DE-Mass spectrometry analysis for sample 1606N (Fig. 6, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
N2	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	63	66170	36.3	20	100ppm	38	9							
N5	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	93	60293	47.5	32	100ppm	67	15	K.AQYEEIAQR.S K.YEELQVTAGR.H K.ADTLTDEINFLR.A R.ISIGGGSCAISGGYGSR.A K.QCANLQAAIADAEQR.G R.QNLEPLFEQYINNLR.R	0.0021 0.0003 0.0029 4.6 2.4 0.0085	44 52 42 9 12 36	88	13	6(4)	0.7
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	106	60315	45.1	33	100ppm	67	15							
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	94	60273	46.3	32	100ppm	67	15							
	Keratin, type II cytoskeletal 4	K2C4_HUMAN	P19013	103	57649	37.8	28	100ppm	67	16	K.LLEGEEYR.M R.AQYEEIAQR.S	0.25 0.0021	23 44	44	3	2(1)	0.7
N6	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	93	49900	25.5	30	100ppm	59	13	R.VILEIDNAR.L R.LKYENELALR.Q K.MIGFPSSAGSVSPR.S R.LQSSSASYGGGFGGGSCQLGGGR.G	1.9 0.0006 1.7 7.2e-06	13 47 14 66	86	12	4(2)	0.7
	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	100	60293	35.5	30	100ppm	59	14	K.AQYEEIAQR.S K.YEELQVTAGR.H K.ADTLTDEINFLR.A R.ISIGGGSCAISGGYGSR.A K.QCANLQAAIADAEQR.G R.QNLEPLFEQYINNLR.R R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	0.32 0.4 0.03 0.35 0.14 0.0017 0.009	22 21 31 20 24 42 33	53	18	7(3)	0.7
	Keratin, type II cytoskeletal 4	K2C4_HUMAN	P19013	76	57649	15.9	23	100ppm	59	12	K.LLEGEEYR.M R.AQYEEIAQR.S R.NLDLDSIIAEVR.A	0.38 0.32 0.044	21 22 29	29	5	3(1)	0.7
N7	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	185	49900	46.6	50	100ppm	60	20	K.IRDWHLK.Q R.VILEIDNAR.L R.LEQEIATYR.S R.LKYENELALR.Q K.TRLEQEIATYR.S K.MIGFPSSAGSVSPR.S R.LQSSSASYGGGFGGGSCQLGGGR.G	0.17 0.068 15 0.0013 0.025 0.09 3.6e-18	23 28 5 45 32 27 189	211	16	7(3)	0.7

1DE-Mass spectrometry analysis for sample 1606CM (Fig. 6, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
CM4	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	59	60293	31.6	21	100ppm	51	9	R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.NLDLDSIIAEVKAQYEEIAQR.S	4.5 0.058 2.4	8 25 10	25	6	3(0)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	59	60273	31.6	21	100ppm	51	9	R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.NLDLDSIIAEVKAQYEEIAQR.S	4.5 0.058 2.4	8 25 10	25	6	3(0)	0.7
CM5	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	75	60293	27.1	26	100ppm	42	10	K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.NLDLDSIIAEVKAQYEEIAQR.S R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	4.9 4.5 0.12 0.69 0.095	9 9 24 16 22	24	13	5(0)	0.7
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	60	60315	27.1	20	100ppm	42	9	K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.NLDLDSIIAEVKAQYEEIAQR.S R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	4.9 4.5 0.12 0.69 0.095	9 9 24 16 22	24	13	5(0)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	75	60273	27.1	26	100ppm	42	10	K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.NLDLDSIIAEVKAQYEEIAQR.S R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	4.9 4.5 0.12 0.69 0.095	9 9 24 16 22	24	13	5(0)	0.7
CM7	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	122	49900	49.5	41	100ppm	69	13	R.VLAEMREQYEAMAER.N R.LQSSSASYGGGFGGGSCQLGGGR.G K.ILTATIENNRVILEIDNAR.L K.NHEEEMKEFSNQVVGQVNVEMDATPGIDLTR.V	0.024 0.015 1.8 0.0013	34 36 11 45	59	19	4(3)	0.7
CM8	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	62	66170	28.9	26	100ppm	80	12	K.SLNNQFASFIDKVR.F R.THNLEPYFESFINNLR.R R.THNLEPYFESFINNLRR.R	0.024 0.000 43 8.9	31 49 5	54	4	3(2)	0.7
CM9	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	64	66170	39	23	100ppm	61	11	K.SLNNQFASFIDKVR.F R.FSSCGGGGGSFGAGGGFGSR.S R.THNLEPYFESFINNLR.R	0.043 0.048 0.0014	29 25 43	49	7	3(3)	0.7

1DE-Mass spectrometry analysis for sample 1606T (Fig. 6, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
T2	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	117	66170	59.7	32	100ppm	42	12	K.WELLQQVDTSTR.T K.SLNNQFASFIDKVR.F R.SGGGFSSGSAGIINYQR.R R.FSSCGGGGGSFGAGGGFGSR.S R.GGGGGGYGSGGSSYGSGGGSYGSGGGGGGGR.G R.GSYGSGGSSYGSGGGSYGSGGGGGGHGSYGSGSSSGGYR.G	0.59 0.99 6.8 0.0064 7e-05 0.29	17 15 4 27 42 13	53	20	6(2)	0.7
T5	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	118	60293	42.9	36	100ppm	78	18	K.AQYEEIAQR.S K.YEELQVTAGR.H K.ADTLTDEINFLR.A R.ISIGGGSCAISGGYGSR.A R.QNLEPLFEQYINNLR.R R.AEAESWYQTKYEELQVTAGR.H R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	0.013 1.4 1.7e-06 0.022 6.4 1.1 0.0022	35 14 73 30 7 12 37	96	17	7(4)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	118	60273	42.9	36	100ppm	78	18	K.AQYEEIAQR.S K.YEELQVTAGR.H K.ADTLTDEINFLR.A R.ISIGGGSCAISGGYGSR.A R.QNLEPLFEQYINNLR.R R.AEAESWYQTKYEELQVTAGR.H R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	0.013 1.4 1.7e-06 0.022 6.4 1.1 0.0022	35 14 73 30 7 12 37	96	17	7(4)	0.7
	Keratin, type I cytoskeletal 10	K1C10_HUMAN	P13645	87	59020	21.8	27	100ppm	78	15							
T6	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	104	60293	39.4	33	100ppm	59	15	K.FASFIDKVR.F R.QNLEPLFEQYINNLR.R R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	6.7 0.54 0.00027	8 18 47	47	9	3(1)	0.7
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	77	60315	37	28	100ppm	59	13	K.FASFIDKVR.F R.QNLEPLFEQYINNLR.R R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	6.7 0.54 0.00027	8 18 47	47	9	3(1)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	104	60273	39.4	30	100ppm	59	14	K.FASFIDKVR.F R.QNLEPLFEQYINNLR.R R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	6.7 0.54 0.00027	8 18 47	47	9	3(1)	0.7
T7	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	105	51578	39.3	40	100ppm	74	19	K.IRDWYQR.Q K.TRLEQEIATYR.R R.ILNEMRDQYEQMAEK.N K.IIAATIENAQPILQIDNAR.L R.LLEGEDAHLSSQQASGQSYSSR.E	0.035 5.5 0.054 4.6e-06 3.9	31 9 26 68 6	73	15	5(2)	0.7
	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	105	48361	36.8	36	100ppm	74	16	R.DYSQYYR.T K.IRDWYQR.Q K.TRLEQEIATYR.R R.ILNEMRDQYEK.M R.GQVGGEINVEMDAAPGVDLSR.I	0.047 0.035 5.5 0.0068 0.006	24 31 9 37 36	49	13	5(4)	0.7
	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	121	51872	40.1	47	100ppm	74	19	K.IRDWYQR.Q K.TRLEQEIATYR.R R.ILNEMRDQYEK.M R.QRPAEIKDYSPYFK.T R.LLEGEDAHLSSSQFSSGSQSSR.D	0.035 5.5 0.0068 0.02 0.55	31 9 37 32 15	44	13	5(3)	0.7
	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	68	60293	15.6	21	100ppm	74	12	K.FASFIDKVR.F K.ADTLTDEINFLR.A	0.033 0.61	32 18	32	3	2(1)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	68	60273	15.6	21	100ppm	74	12	K.FASFIDKVR.F K.ADTLTDEINFLR.A	0.033 0.61	32 18	32	3	2(1)	0.7
T8	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	87	51578	42.8	34	100ppm	56	13	K.IRDWYQR.Q K.IIAATIENAQPILQIDNAR.L R.TDLEMQIEGLKEELAYLR.K	0.042 0.00055 0.4	31 47 18	50	9	3(2)	0.7
	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	111	48361	40.9	36	100ppm	56	14	R.DYSQYYR.T K.IRDWYQR.Q R.GQVGGEINVEMDAAPGVDLSR.I	0.84 0.042 0.14	13 31 23	31	8	3(1)	0.7
	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	72	51872	36.2	31	100ppm	56	11	K.IRDWYQR.Q K.ILTATVDNANVLLQIDNAR.L	0.042 0.18	31 22	31	5	2(1)	0.7

T9	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	60	51872	41.9	36	100ppm	75	16	R.VLDELTLAR.A K.IRDWYQR.Q K.DAEEWFFTK.T K.NHEEEMNALR.G K.TRLEQEIATYR.R K.ILTATVDNANVLLQIDNAR.L R.GQVGGDVNVEMDAAPGVDLSR.I R.LLEGEDAHLSSSQFSSGSQSSR.D	12 0.081 0.77 0.025 2.2 0.018 0.00011 2.5	6 28 16 30 13 32 54 10	62	22	8(3)	0.7
	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	117	48361	48.9	40	100ppm	75	16	R.DYSQYYR.T R.VLDELTLAR.A K.IRDWYQR.Q K.NHEEEMNALR.G K.TRLEQEIATYR.R R.GQVGGEINVEMDAAPGVDLSR.I	0.23 12 0.081 0.025 2.2 0.00035	20 6 28 30 13 49	53	15	6(2)	0.7

1DE-Mass spectrometry analysis for sample 1657N (Fig. 7, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
N4	Keratin, type II cytoskeletal 2 epidermal	K22E_HUMAN	P35908	60	65678	27.7	23	100ppm	53	11							
N5	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	80	60273	40.1	26	100ppm	42	11	R.QNLEPLFEQYINNLR.R R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	0.0021 0.00011	42 52	68	7	2(2)	0.7
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	92	60315	37.9	29	100ppm	42	12	R.QNLEPLFEQYINNLR.R R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	0.0021 0.00011	42 52	68	7	2(2)	0.7
	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	92	60293	40.1	29	100ppm	42	12	R.QNLEPLFEQYINNLR.R R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	0.0021 0.00011	42 52	68	7	2(2)	0.7
	Keratin, type I cytoskeletal 10	K1C10_HUMAN	P13645	66	59020	10.3	23	100ppm	42	9	K.NQILNLTTDNANILLQIDNAR.L	0.014	32	32	3	1(1)	0.7
N6	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	79	60293	45.3	21	100ppm	37	10	K.AQYEEIAQR.S K.YEELQVTAGR.H K.ADTLTDEINFLR.A R.ISIGGGSCAISGGYGSR.A R.QNLEPLFEQYINNLR.R	0.048 1.5 0.067 2 0.0024	31 15 28 13 41	43	11	5(2)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	67	60273	45.3	21	100ppm	37	9	K.AQYEEIAQR.S K.YEELQVTAGR.H K.ADTLTDEINFLR.A R.ISIGGGSCAISGGYGSR.A R.QNLEPLFEQYINNLR.R	0.048 1.5 0.067 2 0.0024	31 15 28 13 41	43	11	5(2)	0.7
N7	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	81	60293	31.4	28	100ppm	53	12	K.AQYEEIAQR.S K.ADTLTDEINFLR.A K.QCANLQAAIADAEQR.G R.QNLEPLFEQYINNLR.R	4.8 0.038 0.41 0.006	11 30 19 38	40	9	4(2)	0.7

	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	81	60315	30.3	28	100ppm	53	12	K.AQYEEIAQR.S K.ADTLTDEINFLR.A K.QCANLQAAIADAEQR.G R.QNLEPLFEQYINNLR.R	4.8 0.03 8 0.41 0.00 6	11 30 19 38	40	9	4(2)	0.7
	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	71	49900	32.1	27	100ppm	53	11	K.TRLEQEIATYR.S R.LQSSSASYGGGFGGGSCQLGGGR.G K.EFSNQVVGQVNVEMDATPGIDLTR.V	3.1 0.02 1 0.03 5	12 31 28	34	12	3(2)	0.7
N8	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	61	51872	46	24	100ppm	38	9	K.IRDWYQR.Q K.NHEEEMNALR.G K.TRLEQEIATYR.R R.GQVGGDVNVEMDAAPGVDLSR.I	0.43 0.00 24 0.09 9 0.00 045	20 43 26 48	64	10	4(2)	0.7
N9	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	79	51872	39	26	100ppm	55	12	K.IRDWYQR.Q K.NHEEEMNALR.G K.TRLEQEIATYR.R K.ILTATVDNANVLLQIDNAR.L R.GQVGGDVNVEMDAAPGVDLSR.I	0.16 0.00 51 7.2 0.09 9.9e -06	25 40 8 23 65	77	14	5(2)	0.7
N12	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	62	66170	37.6	17	100ppm	22	7	K.YEELQITAGR.H K.WELLQQVDTSTR.T K.QISNLQQSISDAEQR.G R.GGGGGGYGSGGSSYGSGGGSYGSGGGGGGGR.G	18 3.3 1.5 0.15	4 11 14 22	0		4(0)	0.7

1DE-Mass spectrometry analysis for sample 1657T (Fig. 7, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
T5	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	139	60293	44.5	28	100ppm	40	14	K.FASFIDK.V K.AQYEEIAQR.S K.YEELQVTAGR.H K.WTLLQEQGTK.T K.ADTLTDEINFLR.A R.GSGGLGGACGGAGFGSR.S R.SRAEAESWYQTK.Y R.ISIGGGSCAISGGYGSR.A K.QCANLQAAIADAEQR.G R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	0.042 0.0004 0.00011 2.2 3e-08 1.3 1.6 0.00056 0.85 1.5e-05 0.24 1e-11	27 47 54 12 90 8 10 45 12 63 21 119	291	26	12(7)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	139	60273	44.5	26	100ppm	40	13							
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	121	60315	44.5	27	100ppm	40	13							
T6	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	125	60293	43.4	26	100ppm	28	11	K.AQYEEIAQR.S K.YEELQVTAGR.H K.LALDVEIATYR.K K.ADTLTDEINFLR.A K.QCANLQAAIADAEQR.G R.QNLEPLFEQYINNLR.R R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	1.8 0.29 0.2 0.0015 0.46 0.00022 1.7e-10	11 20 23 43 16 51 106	150	17	7(3)	0.7

T7	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	76	60293	49.6	17	100ppm	22	8	K.AQYEEIAQR.S K.YEELQVTAGR.H K.ADTLTDEINFLR.A R.ISIGGGSCAISGGYGSR.A K.QCANLQAAIADAEQR.G	0.0045 0.064 0.049 4.7 0.5	37 26 28 6 15	39	11	5(2)	0.7
T8	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	151	51872	64.1	36	100ppm	34	15	K.DYSPYFK.T K.IRDWYQR.Q K.DAEEWFFTK.T K.NHEEEMNALR.G K.TRLEQEIATYR.R K.ILTATVDNANVLLQIDNAR.L R.GQVGGDVNVEMDAAPGVDLSR.I R.LLEGEDAHLSSSQFSSGSQSSR.D	2.3 0.029 0.14 6.7e-05 0.072 0.00089 1.9e-08 0.028	9 31 21 52 27 45 88 25	142	22	8(5)	0.7
	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	84	51578	58.7	21	100ppm	34	10	K.DYSPYFK.T K.IRDWYQR.Q K.TRLEQEIATYR.R K.IIAATIENAQPILQIDNAR.L R.LLEGEDAHLSSQQASGQSYSSR.E	2.3 0.029 0.072 3.8e-07 0.00029	9 31 27 79 45	106	13	5(3)	0.7
T9	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	197	51872	52.6	48	100ppm	47	20	K.IRDWYQR.Q K.NHEEEMNALR.G K.TRLEQEIATYR.R R.ILNEMRDQYEK.M R.QRPAEIKDYSPYFK.T K.DAEEWFFTKTEELNR.E K.ILTATVDNANVLLQIDNAR.L R.GQVGGDVNVEMDAAPGVDLSR.I R.LLEGEDAHLSSSQFSSGSQSSR.D	0.048 0.088 0.35 0.23 0.0029 6.4e-05 0.025 0.00067 0.78	30 24 21 22 40 56 30 46 14	97	27	9(5)	0.7
	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	105	51578	43.3	33	100ppm	47	13	K.IRDWYQR.Q K.TRLEQEIATYR.R R.QRPSEIKDYSPYFK.T K.IIAATIENAQPILQIDNAR.L R.LLEGEDAHLSSQQASGQSYSSR.E	0.048 0.35 8.4 1.2e-08 0.92	30 21 6 94 13	96	15	5(2)	0.7
	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	117	48361	45.4	33	100ppm	47	14	K.IRDWYQR.Q R.TKFETEQALR.L K.NHEEEMNALR.G K.TRLEQEIATYR.R R.ILNEMRDQYEK.M K.DAEDWFFSKTEELNR.E R.GQVGGEINVEMDAAPGVDLSR.I	0.048 0.8 0.088 0.35 0.23 0.12 0.00092	30 18 24 21 22 22 44	46	19	7(2)	0.7
T10	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	132	51578	62.5	36	100ppm	42	15	K.DYSPYFK.T K.IRDWYQR.Q K.NHEEEMLALR.G K.IIAATIENAQPILQIDNAR.L R.GQTGGDVNVEMDAAPGVDLSR.I R.LLEGEDAHLSSQQASGQSYSSR.E	2.3 0.084 0.27 7e-08 0.005 1	11 28 20 86 37 13	96	18	6(2)	0.7
	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	62	51872	43.2	24	100ppm	42	10	K.DYSPYFK.T K.IRDWYQR.Q K.DAEEWFFTK.T K.NHEEEMNALR.G K.ILTATVDNANVLLQIDNAR.L R.GQVGGDVNVEMDAAPGVDLSR.I	2.3 0.084 0.37 0.14 0.0017 0.00096	11 28 18 21 42 44	59	15	6(2)	0.7
	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	61	48361	42.9	18	100ppm	42	9	R.DYSQYYR.T K.IRDWYQR.Q K.NHEEEMNALR.G	1.8 0.084 0.14	10 28 21	0	5	3(0)	0.7

1DE-Mass spectrometry analysis for sample 1686CM (Fig. 7, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
CM2	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	68	66170	33.8	20	100ppm	35	9							
CM4	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	60	60293	38.8	24	100ppm	46	9	R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	0.83 0.00021 0.004	16 52 36	63	7	3(2)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	60	60273	38.8	24	100ppm	46	9	R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	0.83 0.00021 0.004	16 52 36	63	7	3(2)	0.7
CM5	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	63	60293	32.9	24	100ppm	51	9	K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLRR.Q R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	0.72 0.0055 0.11	18 37 21	37	9	3(1)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	63	60273	32.9	24	100ppm	51	9	K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLRR.Q R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	0.72 0.0055 0.11	18 37 21	37	9	3(1)	0.7
CM7	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	109	51578	47.2	35	100ppm	43	13	K.IRDWYQR.Q K.IIAATIENAQPILQIDNAR.L R.NKIIAATIENAQPILQIDNAR.L	1.3 0.011 1.8	16 34 11	34	5	3(1)	0.7
	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	135	51872	45.3	41	100ppm	43	15							

1DE-Mass spectrometry analysis for sample 1686T (Fig. 7, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
T2	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	57	66170	17.8	16	70ppm	35	7	R.THNLEPYFESFINNLR.R	0.045	28	28	2	1(1)	0.7
T3	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	137	66170	68	37	100ppm	45	15	K.SLNNQFASFIDKVR.F R.FSSCGGGGGSFGAGGGFGSR.S R.THNLEPYFESFINNLR.R R.THNLEPYFESFINNLRR.R R.GGGGGGYGSGGSSYGSGGGSYGSGGGGGGR.G	0.14 0.0011 0.0066 0.43 0.0096	24 40 37 18 27	57	12	5(3)	0.7
T5	Keratin, type II cytoskeletal 5	K2C5_HUMAN	P13647	67	62568	48.4	20	100ppm	46	11	K.FASFIDKVR.F R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.TEAESWYQTKYEELQQTAGR.H K.MFFDAELSQMQTHVSDTSVVLSMDNNR.N	14 0.0046 0.0075 0.58 0.0018	5 39 36 16 40	62	12	5(3)	0.7
T6	Keratin, type II cytoskeletal 5	K2C5_HUMAN	P13647	71	62568	41	23	100ppm	54	12	R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q K.MFFDAELSQMQTHVSDTSVVLSMDNNR.N	0.0097 0.00086 0.82	36 45 13	53	7	3(2)	0.7
	Keratin, type I cytoskeletal	K1C10_HUMAN	P13645	58	59020	6.7	28	100ppm	54	9							

	10																	
T7	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	157	60293	50.7	38	100ppm	66	20	K.FASFIDKVR.F K.AQYEEIAQR.S K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.AEAESWYQTKYEELQVTAGR.H R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	0.69 0.14 0.00065 0.006 2e-05 0.0055 3.7e-06	18 26 48 38 61 37 66	144	16	7(5)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	132	60273	50.7	34	100ppm	66	18	K.FASFIDKVR.F K.AQYEEIAQR.S K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.AEAESWYQTKYEELQVTAGR.H R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	0.69 0.14 0.00065 0.006 2e-05 0.0055 3.7e-06	18 26 48 38 61 37 66	144	16	7(5)	0.7
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	144	60315	48.2	36	100ppm	66	19								
T8	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	80	60293	41.3	30	100ppm	59	12	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.AEAESWYQTKYEELQVTAGR.H R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	12 1.7 0.005 0.0015 0.86 0.00014	6 14 38 43 15 51	79	14	6(3)	0.7
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	80	60273	41.3	30	100ppm	59	12	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.AEAESWYQTKYEELQVTAGR.H R.ALYDAELSQMQTHISDTSVVLSMDNNR.N	12 1.7 0.005 0.0015 0.86 0.00014	6 14 38 43 15 51	79	14	6(3)	0.7
T9	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	231	51872	40.8	57	100ppm	65	25	K.IRDWYQR.Q R.TKYETELNLR.M K.DAEEWFFTKTEELNR.E K.ILTATVDNANVLLQIDNAR.L R.YCMQLAQIQEMIGSVEEQLAQLR.C	1.4 2.3 2.3e-06 4.3e-06 1.7e-08	16 13 71 68 91	177	15	5(3)	0.7
	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	166	51578	42.4	44	100ppm	65	20	K.IRDWYQR.Q K.IIAATIENAQPILQIDNAR.L R.TDLEMQIEGLKEELAYLR.K R.YCMQLSQIQGLIGSVEEQLAQLR.C	1.4 2.1e-07 0.0034 2.5	16 81 39 10	94	14	4(2)	0.7
T10	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	118	51578	42.2	45	100ppm	63	16	K.IRDWYQR.Q K.IIAATIENAQPILQIDNAR.L R.TDLEMQIEGLKEELAYLR.K R.LLEGEDAHLSSQQASGQSYSSR.E	0.074 8.4e-06 0.35 2.4	28 65 19 10	65	13	4(1)	0.7
	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	149	48361	42.5	53	100ppm	63	18	R.DYSQYYR.T K.IRDWYQR.Q K.ILTATVDNANILLQIDNAR.L R.GQVGGEINVEMDAAPGVDLSR.I	1.4 0.074 3.2e-05 0.17	13 28 59 23	59	12	4(1)	0.7
	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	152	51872	44	53	100ppm	63	19	K.IRDWYQR.Q R.QRPAEIKDYSPYFK.T K.DAEEWFFTKTEELNR.E K.ILTATVDNANVLLQIDNAR.L	0.074 0.24 1.1 0.00035	28 21 14 49	49	11	4(1)	0.7

1DE-Mass spectrometry analysis for sample 1725N (Fig. 7, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
N1	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	66	66170	34.4	25	100ppm	54	11	K.WELLQQVDTSTR.T R.THNLEPYFESFINNLR.R R.GGGGGGYGSGGSSYGSGGGSYGSGGGGGGR.G	1.4 0.002 0.0046	14 41 26	46	9	3(2)	1
N3	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	90	66170	48	34	100ppm	58	13	K.YEELQITAGR.H K.WELLQQVDTSTR.T R.THNLEPYFESFINNLR.R R.GGGGGGYGSGGSSYGSGGGSYGSGGGGGGR.G	0.16 0.00018 2.3e-07 2.9e-05	25 54 81 52	137	10	4(3)	1
N4	Keratin, type I cytoskeletal 10	K1C10_HUMAN	P13645	70	59020	11.9	30	100ppm	52	10							
N5	Keratin, type II cytoskeletal 4	K2C4_HUMAN	P19013	68	57649	29.2	25	100ppm	45	10	R.NLDLDSIIAEVR.A K.NLEPLFETYLSVLR.K	0.25 2.9e-05	22 60	60	4	2(1)	0.7
N8	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	47	49900	10.6	24	100ppm	47	8	R.LQSSSASYGGGFGGGSCQLGGGR.G	0.0062	37	37	5	1(1)	1
N9	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	63	66170	36.1	24	100ppm	48	10	R.THNLEPYFESFINNLR.R	0.0002	51	51	2	1(1)	1
N11	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	82	66170	51.1	35	100ppm	66	13	K.WELLQQVDTSTR.T R.FSSCGGGGGSFGAGGGFGSR.S R.GGGGGGYGSGGSSYGSGGGSYGSGGGGGGR.G	0.41 5.4e-08 0.17	19 78 8	78	9	3(1)	1
N15	Keratin, type II cytoskeletal 2 epidermal	K22E_HUMAN	P35908	59	65678	32	25	100ppm	68	10							

1DE-Mass spectrometry analysis sample 1725T (Fig. 7, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
T1	Keratin, type II cytoskeletal 2 epidermal	K22E_HUMAN	P35908	64	65678	28.5	26	100ppm	51	10							
	Keratin, type I cytoskeletal 10	K1C10_HUMAN	P13645	64	59020	11.9	23	100ppm	51	10							
T2	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	86	66170	47.9	28	100ppm	43	11							
T5	Keratin, type II cytoskeletal 5	K2C5_HUMAN	P13647	76	62568	37.9	23	100ppm	75	13	K.YEELQQTAGR.H K.QCANLQNAIADAEQR.G R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	4.4 0.77 3.6e-06 1.6	9 15 70 13	70	6	4(1)	1
T6	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	121	60293	53.5	32	100ppm	64	16	K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R	0.49 0.0097	19 35	35	4	2(1)	1
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	106	60315	50.8	30	100ppm	64	15	K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R	0.49 0.0097	19 35	35	4	2(1)	1
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	105	60273	53.5	32	100ppm	64	15	K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R	0.49 0.0097	19 35	35	4	2(1)	1
T8	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	144	60293	59.7	36	100ppm	66	21	K.ADTLTDEINFLR.A K.QCANLQAAIADAEQR.G R.QNLEPLFEQYINNLR.R	0.0007 0.79 2.6e-07	48 16 81	101	7	3(2)	1
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	124	60315	56.4	33	100ppm	66	19	K.ADTLTDEINFLR.A K.QCANLQAAIADAEQR.G R.QNLEPLFEQYINNLR.R	0.0007 0.79 2.6e-07	48 16 81	101	7	3(2)	1
	Keratin, type II cytoskeletal 5	K2C5_HUMAN	P13647	76	62568	38.9	24	100ppm	66	15	K.YEELQQTAGR.H R.QNLEPLFEQYINNLR.R	0.28 2.6e-07	22 81	81	4	2(2)	1
T10	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	78	51578	31.3	37	100ppm	98	16	K.IIAATIENAQPILQIDNAR.L R.TDLEMQIEGLKEELAYLR.K	0.000827	45 6	45	7	2(1)	1
T11	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	124	51578	42	42	100ppm	94	18	K.IIAATIENAQPILQIDNAR.L R.TDLEMQIEGLKEELAYLR.K	4.9e-06 0.52	67 17	67	7	2(1)	1
	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	138	51872	36.1	48	100ppm	94	20	K.ILTATVDNANVLLQIDNAR.L	0.044	27	27	4	1(1)	1
	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	91	48361	34.7	43	100ppm	94	17							
T12	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	103	51578	52.4	36	100ppm	82	16	K.IIAATIENAQPILQIDNAR.L	0.00044	47	47	4	1(1)	1

1DE-Mass spectrometry analysis sample 1726T (Fig. 8, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
T4	Keratin, type II cytoskeletal 2 epidermal	K22E_HUMAN	P35908	59	65678	12.5	27	100ppm	59	10							
T6	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	91	60293	37.9	29	100ppm	72	15	K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.17 0.0027 0.38	24 41 19	41	4	3(1)	1
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	80	60315	37.4	29	100ppm	72	14	K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.17 0.0027 0.38	24 41 19	41	4	3(1)	1
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	81	60273	37.9	26	100ppm	72	14	K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.17 0.0027 0.38	24 41 19	41	4	3(1)	1
T9	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	66	48361	37.4	27	100ppm	58	10							
T10	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	78	66170	58.4	30	100ppm	72	13	R.THNLEPYFESFINNLR.R R.THNLEPYFESFINNLRR.R	0.0084 7.4	36 6	36	2	2(1)	1

1DE-Mass spectrometry analysis for sample 1731T (Fig. 8, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
T6	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	65	60315	38.5	19	100ppm	49	10	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.1 0.0032 0.0037 0.00086	26 41 40 45	70	6	4(3)	1
T10	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	66	51578	57.8	26	100ppm	58	11	K.IRDWYQR.Q R.QRPSEIKDYSPYFK.T R.ILNEMRDQYEQMAEK.N K.IIAATIENAQPILQIDNAR.L	0.051 0.56 0.066 1e-10	30 18 27 114	116	11	4(2)	1
	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	64	51872	45.6	25	100ppm	58	11	K.IRDWYQR.Q R.QRPAEIKDYSPYFK.T K.DAEEWFFTKTEELNR.E K.ILTATVDNANVLLQIDNAR.L	0.051 0.57 0.01 0.2	30 17 35 20	36	11	4(2)	1
T11	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	56	51578	46.9	26	100ppm	59	11	K.IRDWYQR.Q R.QRPSEIKDYSPYFK.T K.IIAATIENAQPILQIDNAR.L	0.05 1.3 1.4e-10	29 14 109	113	8	3(2)	1
	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	68	51872	39.4	27	100ppm	59	12	K.IRDWYQR.Q R.QRPAEIKDYSPYFK.T K.ILTATVDNANVLLQIDNAR.L R.GQVGGDVNVEMDAAPGVDLSR.I	0.05 0.0065 0.016 5.6e-06	29 36 29 67	82	12	4(4)	1
	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	53	60293	17.9	18	100ppm	59	10	K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.31 2.7e-05 0.026	20 60 30	63	4	3(2)	1

T12	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	50	51578	30.9	20	100ppm	47	9	K.IIAATIENAQPILQIDNAR.L R.TDLEMQIEGLKEELAYLR.K	1.8e-09 0.044	93 25	97	7	2(2)	1
T15	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	66	66170	22.4	20	100ppm	35	9	R.THNLEPYFESFINNLR.R	0.011	35	35	2	1(1)	1
T16	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	67	66170	42.1	25	100ppm	37	9	K.WELLQQVDTSTR.T K.SLNNQFASFIDKVR.F R.FSSCGGGGGSFGAGGGFGSR.S R.THNLEPYFESFINNLR.R	1.1 0.1 0.17 0.003	15 24 23 40	40	9	4(1)	1

1DE-Mass spectrometry analysis for sample 1735N (Fig. 8, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
N4	Keratin, type II cytoskeletal 5	K2C5_HUMAN	P13647	113	62568	46.2	26	100ppm	73	17	K.FASFIDKVR.F R.VSLAGACGVGGYGSR.S R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.TEAESWYQTKYEELQQTAGR.H	0.27 0.079 0.0031 0.0051 0.019	18 26 39 35 31	54	10	5(3)	1
	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	71	60293	37.7	28	100ppm	73	15	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.27 0.67 0.0031 0.0051	18 16 39 35	48	6	4(2)	1
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	91	60315	37.8	29	100ppm	73	15	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.27 0.67 0.0031 0.0051	18 16 39 35	48	6	4(2)	1
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	81	60273	37.7	29	100ppm	73	16	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.27 0.67 0.0031 0.0051	18 16 39 35	48	6	4(2)	1
N5	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	211	60293	58.9	45	100ppm	100	27	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.ISIGGGSCAISGGYGSR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.AEAESWYQTKYEELQVTAGR.H	0.037 0.00069 0.32 0.00041 0.0011 0.25	27 46 21 48 42 20	85	13	6(4)	1
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	196	60273	58.1	43	100ppm	100	26	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.ISIGGGSCAISGGYGSR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.AEAESWYQTKYEELQVTAGR.H	0.037 0.00069 0.32 0.00041 0.0011 0.25	27 46 21 48 42 20	85	13	6(4)	1
N7	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	123	49900	50.3	42	100ppm	87	19	K.QSPASPERDYSPYYK.T R.VLAEMREQYEAMAER.N R.LQSSSASYGGGFGGGSCQLGGGR.G	0.0047 0.0011 0.0016	39 45 43	72	11	3(3)	1
N8	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	180	51578	48.1	52	100ppm	90	23	R.QRPSEIKDYSPYFK.T K.IIAATIENAQPILQIDNAR.L	1.1 0.042	14 25	25	6	2(1)	1
	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	127	51872	44.2	48	100ppm	90	20							
N10	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	60	66170	19.7	22	100ppm	44	9	K.WELLQQVDTSTR.T K.SLNNQFASFIDKVR.F R.THNLEPYFESFINNLR.R R.GGGGGGYGSGGSSYGSGGGSYGSGGGGGGGR.G	4.9 0.35 0.061 1.2	7 17 26 13	26	11	4(1)	1

1DE-Mass spectrometry analysis for sample 1736T (Fig. 8, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
T5	Keratin, type II cytoskeletal 5	K2C5_HUMAN	P13647	104	62568	37.4	29	100ppm	63	16							
	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	73	60293	36	24	100ppm	63	12	K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.44 0.031 0.0067	20 30 37	39	4	3(2)	1
	Keratin, type II cytoskeletal 2 epidermal	K22E_HUMAN	P35908	62	65678	23.5	26	100ppm	63	11							
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	73	60273	36	24	100ppm	63	12	K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.44 0.031 0.0067	20 30 37	39	4	3(2)	1

1DE-Mass spectrometry analysis for sample 1737N (Fig. 8, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
N3	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	100	60293	42.7	27	100ppm	46	13	K.FASFIDKVR.F K.AQYEEIAQR.S K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.055 0.64 0.00017 1.9e-05 0.043	29 16 52 61 28	88	8	5(3)	1
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	100	60273	42.7	27	100ppm	46	13	K.FASFIDKVR.F K.AQYEEIAQR.S K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.055 0.64 0.00017 1.9e-05 0.043	29 16 52 61 28	88	8	5(3)	1
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	77	60315	42.7	23	100ppm	46	11							
	Keratin, type II cytoskeletal 4	K2C4_HUMAN	P19013	56	57649	23.9	21	100ppm	46	9	R.AQYEEIAQR.S K.NLEPLFETYLSVLR.K K.NLEPLFETYLSVLRK.Q K.FASFIDKVQFLEQQNK.V	0.64 7.3e-05 0.079 0.27	16 56 27 19	56	7	4(1)	1
N4	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	91	49900	46.1	32	100ppm	41	11	K.MIGFPSSAGSVSPR.S R.VLAEMREQYEAMAER.N R.LQSSSASYGGGFGGGSCQLGGGR.G	14 0.00041 0.00014	4 48 51	73	11	3(2)	1
N5	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	130	49900	68.4	41	100ppm	55	18	R.LKYENELALR.Q K.MIGFPSSAGSVSPR.S K.QSPASPERDYSPYYK.T R.VLAEMREQYEAMAER.N R.LQSSSASYGGGFGGGSCQLGGGR.G	0.97 0.069 0.47 2.3e-05 2e-11	17 28 18 61 120	155	16	5(2)	1

1DE-Mass spectrometry analysis for sample 1737T (Fig. 8, 10, 11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
T2	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	52	60293	39.2	24	100ppm	60	9	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R	0.067 0.077 8.6e-05	29 27 56	56	6	3(1)	1
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	52	60273	39.2	24	100ppm	60	9	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R	0.067 0.077 8.6e-05	29 27 56	56	6	3(1)	1
T4	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	68	51578	36	26	100ppm	74	13	K.IRDWYQR.Q R.QRPSEIKDYSPYFK.T R.ILNEMRDQYEQMAEK.N K.IIAATIENAQPILQIDNAR.L	0.86 2 0.62 0.0052	18 12 17 37	37	11	4(1)	1
	Keratin, type II cytoskeletal 2 epidermal	K22E_HUMAN	P35908	56	65678	13	20	100ppm	74	11							
	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	110	48361	39.5	40	100ppm	74	17							
	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	66	51872	36.2	31	100ppm	74	14							
T6	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	58	51578	31.5	21	100ppm	43	9	K.IIAATIENAQPILQIDNAR.L	0.024	30	30	4	1(1)	1

1DE-Mass spectrometry analysis for sample 1740N (Fig. 9-11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
N1	Keratin, type II cytoskeletal 4	K2C4_HUMAN	P19013	57	57649	30.2	18	100ppm	34	8	K.NLEPLFETYLSVLR.K	1e-05	64	64	2	1(1)	1
N3	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	100	49900	55.7	30	100ppm	53	14	K.QSPASPERDYSPYYK.T R.VLAEMREQYEAMAER.N R.LQSSSASYGGGFGGGSCQLGGGR.G	3 0.0025 0.41	11 41 19	41	11	3(1)	1

1DE-Mass spectrometry analysis for sample 1740T (Fig. 9-11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
T1	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	140	60293	59.1	32	100ppm	39	15	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.087 0.57 0.0066 0.73	27 19 37 16	37	6	4(1)	1
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	112	60315	59.1	26	100ppm	39	13	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.087 0.57 0.0066 0.73	27 19 37 16	37	6	4(1)	1
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	140	60273	59.1	30	100ppm	39	15	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.087 0.57 0.0066 0.73	27 19 37 16	37	6	4(1)	1
T2	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	57	60293	40	25	100ppm	62	10	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.AIGGGLSSVGGGSSTIKYTTTSSSSR.K	0.1 0.77 0.00098 0.0051 1.6	26 17 46 38 12	56	11	5(2)	1
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	57	60273	40	25	100ppm	62	10	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q R.AIGGGLSSVGGGSSTIKYTTTSSSSR.K	0.1 0.77 0.00098 0.0051 1.6	26 17 46 38 12	56	11	5(2)	1
T3	Keratin, type II cytoskeletal 5	K2C5_HUMAN	P13647	36	62568	62.4	16	100ppm	48	7	R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.38 0.0011	20 44	44	2	2(1)	1
T4	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	63	51872	21.5	33	100ppm	71	12	R.QRPAEIKDYSPYFK.T K.DAEEWFFTKTEELNR.E	2.9 0.00096	11 45	45	6	2(1)	1
T5	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	78	48361	45.7	24	100ppm	39	10							
	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	60	51578	36.2	23	100ppm	39	9							

1DE-Mass spectrometry analysis for sample 1741N (Fig. 9-11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
N4	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	54	60293	69.2	11	100ppm	17	6	K.FASFIDKVR.F R.QNLEPLFEQYINNLRR.Q	1.4 0.0042	15 38	53	4	2910	1
N6	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	87	49900	66.1	28	100ppm	46	12							

1DE-Mass spectrometry analysis for sample 1741T (Fig. 9-11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
T3	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	59	60293	62	20	100ppm	33	8	R.QNLEPLFEQYINNLRR.Q	0.043	28	28	2	1(1)	1
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	59	60273	62	20	100ppm	33	8	R.QNLEPLFEQYINNLRR.Q	0.043	28	28	2	1(1)	1
T4	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	71	60293	23	25	100ppm	78	13	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.3 0.44 0.16 0.014	22 20 23 33	33	6	4(1)	1
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	71	60273	23	25	100ppm	78	13	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.3 0.44 0.16 0.014	22 20 23 33	33	6	4(1)	1
	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	68	48361	29.2	35	100ppm	78	12							

1DE-Mass spectrometry analysis for sample 1751N (Fig. 9-11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
N1	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	70	66170	27	31	100ppm	50	10	K.SLNNQFASFIDKVR.F R.THNLEPYFESFINNLR.R	2.1e-06 0.64	72 17	72	4	2(1)	1
	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	59	60293	27	25	100ppm	50	9	K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	2.7 0.0034 0.2	12 40 22	40	4	3(1)	1
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	59	60273	29.2	25	100ppm	50	9	K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	2.7 0.0034 0.2	12 40 22	40	4	3(1)	1
N2	Keratin, type II cytoskeletal 5	K2C5_HUMAN	P13647	69	62568	75.1	21	100ppm	38	10	K.FASFIDKVR.F R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.05 0.25 0.13	29 21 23	29	4	3(1)	1
	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	62	60293	75.6	20	100ppm	38	9	K.FASFIDKVR.F R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.05 0.25 0.13	29 21 23	29	4	3(1)	1
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	62	60273	75.6	20	100ppm	38	9	K.FASFIDKVR.F R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.05 0.25 0.13	29 21 23	29	4	3(1)	1
N5	Keratin, type I	K1C13_HUMAN	P13646	142	49900	41	41	100ppm	44	15	K.MIGFPSSAGSVSPR.S R.VLAEMREQYEAMAER.N K.ILTATIENNRVILEIDNAR.L	1.2 0.0017 0.073	15 41 26	86	17	4(2)	1

	cytoskeletal 13										K.NHEEEMKEFSNQVVGQVNVEMDATPGIDLTR.V	1.3e-06	69				
	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	71	60293	38.6	23	100ppm	44	10	R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.15 0.0055	23 37	37	2	2(1)	1
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	57	60315	38.6	19	100ppm	44	9	R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.15 0.0055	23 37	37	2	2(1)	1
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	71	60273	38.6	23	100ppm	44	10	R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.15 0.0055	23 37	37	2	2(1)	1
N6	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	57	51578	11	23	100ppm	32	7							
N7	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	57	60293	42.7	18	100ppm	27	8	R.QNLEPLFEQYINNLRR.Q	0.034	30	30	2	1(1)	1
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	57	60273	42.7	18	100ppm	27	8	R.QNLEPLFEQYINNLRR.Q	0.034	30	30	2	1(1)	1

1DE-Mass spectrometry analysis for sample 1751T (Fig. 9-11)

*Gel piece No.	Protein Name	Protein ID	Accession No.	PMF							MS/MS						
				Total Score	Mass (Da)	IC (%)	SC (%)	Tolerance	Searched Peaks	Matched Peaks	Peptides Identified	Expect	Peptide Score	Total Score	SC (%)	M	Tolerence (Da)
T1	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	95	60293	63.4	26	100ppm	34	11	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.94 0.081 0.007 0.32	17 27 37 20	37	6	4(1)	1
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	68	60315	63.4	18	100ppm	34	9	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.94 0.081 0.007 0.32	17 27 37 20	37	6	4(1)	1
	Keratin, type II cytoskeletal 6C	K2C6C_HUMAN	P48668	82	60273	63.4	24	100ppm	34	10	K.FASFIDKVR.F K.ADTLTDEINFLR.A R.QNLEPLFEQYINNLR.R R.QNLEPLFEQYINNLRR.Q	0.94 0.081 0.007 0.32	17 27 37 20	37	6	4(1)	1
T4	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	62	51578	201	24	50ppm	49	11	K.IRDWYQR.Q R.QRPSEIKDYSPYFK.T K.IIAATIENAQPILQIDNAR.L	2.5 0.094 0.035	13 26 29	29	8	3(1)	1

Table A3. 2DE-PMF data of the spots from the 2DE gels of enriched keratins from Normal/cut margin and tumor gingivo buccal complex tissue samples

2DE-PMF-MS for sample 1736N (Fig. 65)

Sample No.	Spot No.	Identity	Protein ID	Accession no.	Score	MW	IC	SC	Tolerance	Searched peaks	Matched peaks
1736N	N1-N3	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	64	49900	43.5	21	100ppm	21	7

2DE-PMF-MS for sample 1737N (Fig. 29, 30, 66)

Sample No.	Spot No.	Identity	Protein ID	Accession no.	Score	MW	IC	SC	Tolerance	Searched peaks	Matched peaks
1737N	N1	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	65	49900	50.1	30	100ppm	58	8
	N2	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	228	49900	74.8	58	100ppm	66	25
	N3	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	219	49900	80	55	100ppm	68	25
	N4	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	159	49900	67	56	100ppm	64	21
	N5	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	197	49900	80.7	55	100ppm	58	23
	N6	Actin, alpha skeletal muscle	ACTS_HUMAN	P68133	172	42366	82.2	56	100ppm	53	15
		Actin, alpha cardiac muscle 1	ACTC_HUMAN	P68032	139	42334	78.7	55	100ppm	53	13
		Actin, aortic smooth muscle	ACTA_HUMAN	P62736	124	42381	78.7	53	100ppm	53	12
		Actin, gamma-enteric smooth muscle	ACTH_HUMAN	P63267	110	42249	78.7	49	100ppm	53	11
	N7	Actin, cytoplasmic 1	ACTB_HUMAN	P60709	95	42052	43.6	39	100ppm	34	10
		Actin, cytoplasmic 2	ACTG_HUMAN	P63261	95	42108	43.6	39	100ppm	34	10
	For Basic Keratin with pI 5-8 (Fig.36)										
	N1	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	82	66170	32.1	28	100ppm	29	9
	N3	Keratin, type II cytoskeletal 4	K2C4_HUMAN	P19013	58	57649	32.3	12	100ppm	16	6

2DE-PMF-MS for sample 1737CM (Fig. 29, 31, 66)

Sample No.	Spot No.	Identity	Protein ID	Accession no.	Score	MW	IC	SC	Tolerance	Searched peaks	Matched peaks
1737CM	CM1	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	78	49900	62	22	100ppm	21	9
	CM2	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	188	49900	77.1	45	100ppm	48	20
	CM3	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	118	49900	57.2	38	100ppm	46	13
	CM4	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	71	49900	60.9	21	100ppm	28	8
	CM5	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	149	49900	77.8	35	100ppm	50	19
	CM6	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	90	66170	30.2	23	100ppm	51	13
		Keratin, type I cytoskeletal 15	K1C15_HUMAN	P19012	76	49409	26.5	29	100ppm	51	11
	CM7	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	68	66170	26.5	17	100ppm	34	9
	CM9	Actin, aortic smooth muscle	ACTA_HUMAN	P62736	84	42381	76.7	33	100ppm	37	9
		Actin, alpha cardiac muscle 1	ACTC_HUMAN	P68032	84	42334	76.7	33	100ppm	37	9
		Actin, alpha skeletal muscle	ACTS_HUMAN	P68133	83	42366	76.7	33	100ppm	37	9
		Actin, gamma-enteric smooth muscle	ACTH_HUMAN	P63267	72	41877	76.7	30	100ppm	37	8
	CM10	Actin, cytoplasmic 1	ACTB_HUMAN	P60709	147	42052	87.4	42	100ppm	30	11
		Actin, cytoplasmic 2	ACTG_HUMAN	P63261	147	42108	87.4	42	100ppm	30	11
	CM12	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	246	51578	75.3	56	100ppm	66	26
	CM13	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	257	51578	70.8	53	100ppm	53	24
	CM15	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	97	51872	18.9	36	100ppm	47	11
	CM16	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	91	51872	42.9	33	100ppm	44	12
		Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	87	51578	42.6	32	100ppm	44	11
	CM17	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	156	51872	66	47	100ppm	72	20
		Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	64	48361	48.1	24	100ppm	72	12
	CM18	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	191	51578	65.8	56	100ppm	76	25
		Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	139	51872	55.8	50	100ppm	76	20
	CM19	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	68	51872	13.7	29	100ppm	41	10

	CM20	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	128	51578	54.3	39	100ppm	59	17
	CM23	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	193	51872	63.6	55	100ppm	95	29
		Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	95	51578	40	37	100ppm	95	18
		Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	78	48361	48.8	27	100ppm	95	15
	CM24	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	157	51872	70.2	48	100ppm	51	18
	CM25	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	153	51872	25.6	50	100ppm	66	20
	CM26	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	259	51872	67.2	61	100ppm	84	28
		Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	64	51578	35.1	26	100ppm	84	12
		Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	63	48361	38.6	25	100ppm	84	12
	CM28	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	161	51872	56.5	55	100ppm	80	21
		Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	79	51578	49.6	49	100ppm	80	21

2DE-PMF-MS for sample 1737T (Fig. 29, 32, 66)

Sample No.	Spot No.	Identity	Protein ID	Accession no.	Score	MW	IC	SC	Tolerance	Searched peaks	Matched peaks
1737T	T11	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	79	51578	32.4	29	100ppm	35	10
	T12	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	223	51578	66.5	53	100ppm	81	27
	T13	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	139	48361	13.6	40	100ppm	69	18
		Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	118	51872	20.1	42	100ppm	69	18
		Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	78	51578	8	28	100ppm	69	14
	T14	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	133	48361	60.9	40	100ppm	46	15

2DE-PMF-MS for sample 1740N (Fig. 29, 33, 67)

Sample No.	Spot No.	Identity	Protein ID	Accession no.	Score	MW	IC	SC	Tolerance	Searched peaks	Matched peaks
1740N	N1	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	197	49900	60.1	46	100ppm	61	20
	N3	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	141	49900	59.6	45	100ppm	71	21
	N5	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	148	49900	53.3	38	100ppm	75	18
		Keratin, type I cytoskeletal 10	K1C10_HUMAN	P13645	71	59020	22.4	24	100ppm	66	12
	N18	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	102	51872	28.6	35	100ppm	48	14
	N22	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	83	51872	36.5	31	100ppm	39	11
	N23	Keratin, type I cytoskeletal 10	K1C10_HUMAN	P13645	68	59020	17.7	18	100ppm	42	10
	N24	Keratin, type I cytoskeletal 10	K1C10_HUMAN	P13645	98	59020	24.5	31	100ppm	87	18
	N26	Actin, cytoplasmic 2	ACTG_HUMAN	P63261	59	42108	41.5	34	100ppm	33	7
	N27	Actin, gamma-enteric smooth muscle	ACTH_HUMAN	P63267	73	41877	45	34	100ppm	36	8
		Actin, aortic smooth muscle	ACTA_HUMAN	P62736	72	42381	45	34	100ppm	36	8
		Actin, alpha cardiac muscle 1	ACTC_HUMAN	P68032	72	42334	44.4	34	100ppm	36	8
		Actin, alpha skeletal muscle	ACTS_HUMAN	P68133	59	42366	44.4	29	100ppm	36	7
	N28	Actin, cytoplasmic 1	ACTB_HUMAN	P60709	138	42052	84.1	49	100ppm	42	14
		Actin, cytoplasmic 2	ACTG_HUMAN	P63261	138	42108	84.1	49	100ppm	42	14
	N29	Actin, cytoplasmic 1	ACTB_HUMAN	P60709	105	42052	73.8	45	100ppm	53	12
		Actin, cytoplasmic 2	ACTG_HUMAN	P63261	91	42108	73.8	40	100ppm	53	11

2DE-PMF-MS for sample 1740CM (Fig. 29, 34, 67)

Sample No.	Spot No.	Identity	Protein ID	Accession no.	Score	MW	IC	SC	Tolerance	Searched peaks	Matched peaks
1740CM	CM1	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	178	49900	58.2	48	100ppm	65	22
	CM2	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	209	49900	65.6	55	100ppm	72	26
	CM3	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	187	49900	60.7	54	100ppm	78	25
	CM4	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	86	49900	55.5	29	100ppm	29	11
	CM6	Actin, cytoplasmic 1	ACTB_HUMAN	P60709	153	42052	79.3	43	100ppm	36	15
		Actin, cytoplasmic 2	ACTG_HUMAN	P63261	153	42108	79.3	43	100ppm	36	15
	CM7	Actin, cytoplasmic 2	ACTG_HUMAN	P63261	83	42108	56.2	39	100ppm	33	9
		Actin, cytoplasmic 1	ACTB_HUMAN	P60709	70	42052	56.2	34	100ppm	33	8
	CM8	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	96	49900	45.6	30	100ppm	35	13
	CM10	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	91	49900	37.8	31	100ppm	40	12
	CM12	Keratin, type I cytoskeletal 15	K1C15_HUMAN	P19012	57	49409	46	19	100ppm	14	5

2DE-PMF-MS for sample 1740T (Fig. 29, 35, 67)

Sample No.	Spot No.	Identity	Protein ID	Accession no.	Score	MW	IC	SC	Tolerance	Searched peaks	Matched peaks
1740T	T1	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	205	51578	46.6	53	100ppm	64	23
	T2	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	116	51872	36.7	41	100ppm	48	15
	T3	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	114	51872	42.6	39	100ppm	57	15
	T4	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	99	51872	32	37	100ppm	44	12
	T5	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	79	48361	32.9	32	100ppm	52	11
	T6	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	118	48361	61.4	34	100ppm	47	14
	T7	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	177	48361	50.7	43	100ppm	46	19
	T8	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	80	51872	48.2	30	100ppm	49	12
	T9	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	175	51578	36.6	47	100ppm	81	23
		Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	138	51872	20.3	43	100ppm	81	20
		Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	117	48361	33.9	37	100ppm	81	19
	T10	Keratin, type I cytoskeletal 9	K1C9_HUMAN	P35527	59	62255	16.7	18	100ppm	44	8
	T12	Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	88	51578	52.2	25	100ppm	42	12
	T13	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	79	48361	32.6	29	100ppm	38	11
	T15	Keratin, type I cytoskeletal 17	K1C17_HUMAN	Q04695	121	48361	61.7	54	100ppm	100	30
		Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	96	51872	59.4	58	100ppm	100	30
		Keratin, type I cytoskeletal 16	K1C16_HUMAN	P08779	73	51578	49.9	44	100ppm	100	24
	T16	Actin, cytoplasmic 1	ACTB_HUMAN	P60709	57	42052	64.9	21	100ppm	31	6
		Actin, cytoplasmic 2	ACTG_HUMAN	P63261	57	42108	64.9	21	100ppm	31	6

Table A4. LC-MS/MS analysis of protein in the boxed portion in Fig. 46.

Protein Name	Protein ID	Accession No.	Mass (Da)	Peptides Identified	Expect	Peptide Score	Total Score	Matches
Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	49900	K.TIEELRDK.I	0.0015	45	212	14 (7)
				K.YENELALR.Q	0.013	35		
				K.TEITELRR.T	0.065	29		
				R.VILEIDNAR.L	2.6e-05	61		
				K.ILTATIENNR.V	2.1	14		
				R.LKYENELALR.Q	2.8e-08	90		
				R.QSVEADINGLRR.V	0.048	29		
				K.TRLEQEIATYR.S	0.15	24		
				K.MIGFPSSAGSVSPR.S	0.37	21		
				K.AGLENTVAETECR.Y	0.52	19		
				K.VRALEEANADLEVK.I	10	5		
				K.QSPASPERDYSPYYK.T	5.2e-05	58		
				R.LQSSSASYGGGFGGGSCQLGGGR.G	2.9-05	60		
				K.ILTATIENNRVILEIDNAR.L	0.18	21		
Keratin, type I cytoskeletal 15	K1C15_HUMAN	P19012	49395	K.TIEELRDK.I	0.0015	45	146	8 (4)
				K.YENELALR.Q	0.013	35		
				R.VLDELTLAR.T	0.5	19		
				R.VILEIDNAR.L	2.6e-05	61		
				R.LKYENELALR.Q	2.8e-08	90		
				K.TRLEQEIATYR.S	0.15	24		
				K.AGLENSLAETECR.Y	0.52	19		
				K.VRALEEANADLEVK.I	10	5		

Table A5. 2DE-PMFdata of the spots from the 2DE gels for Figures. 48-E, 50-E and 50-F

*Gel spot No.	Protein Name	Protein ID	Accession No.	PMF			
				Total Score	Mass (Da)	pI	SC (%)
(Fig. 48-E) MDA-MB-468							
1.	Keratin, type I cytoskeletal 18	K1C18_HUMAN	P05783	194	48029	5.2	46.7
2.	Heterogeneous nuclear ribonucleoprotein F	HNRPF_HUMAN	P52597	110	45985	5.3	40.5
3.	Keratin, type I cytoskeletal 18	K1C18_HUMAN	P05783	75	48029	5.2	24.7
4.	Keratin, type I cytoskeletal 18	K1C18_HUMAN	P05783	75	48029	5.2	24.7
5.	Keratin, type I cytoskeletal 18	K1C18_HUMAN	P05783	237	48029	5.2	53.7
6.	Actin, cytoplasmic 2	ACTG_HUMAN	P63261	163	42108	5.2	55.2
7.	Actin, cytoplasmic 2	ACTG_HUMAN	P63261	143	42108	5.2	44.5
8.	Actin, cytoplasmic 2	ACTG_HUMAN	P63261	152	42108	5.2	54.4
9.	Actin, cytoplasmic 1	ACTB_HUMAN	P60709	167	42052	5.2	49.6
10.	Actin, cytoplasmic 1	ACTB_HUMAN	P60709	177	42052	5.2	58.4
11.	Actin, cytoplasmic 1	ACTB_HUMAN	P60709	185	42052	5.2	50.9
12.	Actin, cytoplasmic 2	ACTG_HUMAN	P63261	209	42108	5.2	55.2
13.	Actin, cytoplasmic 1	ACTB_HUMAN	P60709	87	42052	5.2	28.8
14	Actin, cytoplasmic 1	ACTB_HUMAN	P60709	170	42052	5.2	54.1
15	Actin, cytoplasmic 2	ACTG_HUMAN	P63261	117	42108	5.2	50.1
(Fig. 50-E) Breast tissue (B1)							
1.	Actin, cytoplasmic 1	ACTB_HUMAN	P60709	96	42052	5.2	37.6
2.	Actin, cytoplasmic 1	ACTB_HUMAN	P60709	143	42052	5.2	46.1
3.	Actin, cytoplasmic 1	ACTB_HUMAN	P60709	156	42052	5.2	40.3
4.	Keratin, type I cytoskeletal 18	K1C18_HUMAN	P05783	246	48029	5.2	49.5
(Fig. 50-F) 1740 T							
16.	Calpain-9	CAN9_HUMAN	O14815	74	79731	5.3	12.5
	Actin, cytoplasmic 1	ACTB_HUMAN	P60709	57	42052	5.2	21.1
17.	Actin, cytoplasmic 1	ACTB_HUMAN	P60709	41	42052	5.2	20.0

(Appendix Table A4 is published as a supplimentary Table. 3S in Journal of proteomics, Amit Fulzele et. al. 2012.)

Figure A1. Micrographs of H&E stained tissue sections (Images are shown with respective sample number of normal (N) , cut margin (CM) and tumor (T) tissues).

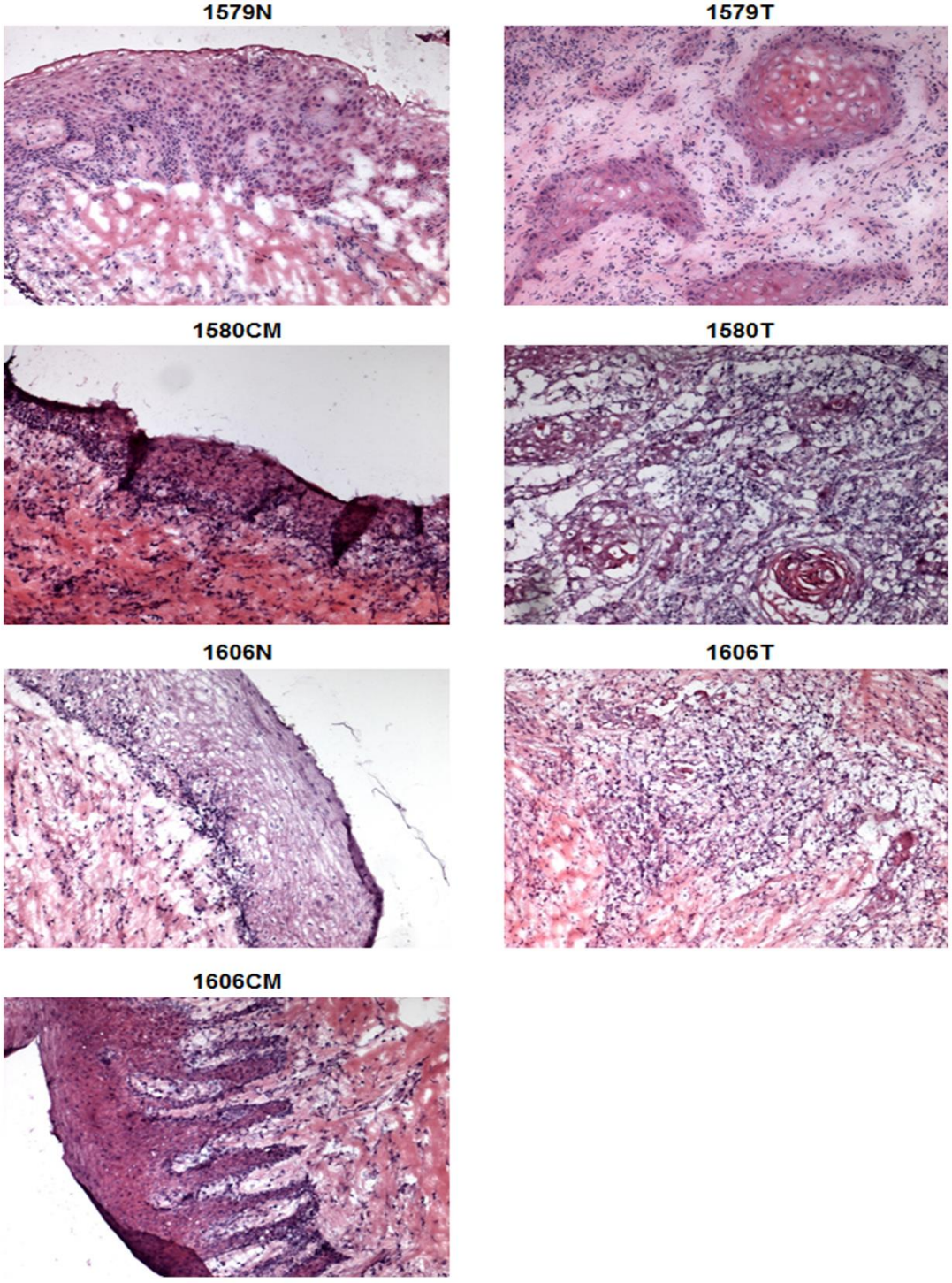

Figure A1. Micrographs of H&E stained tissue sections. continued…..

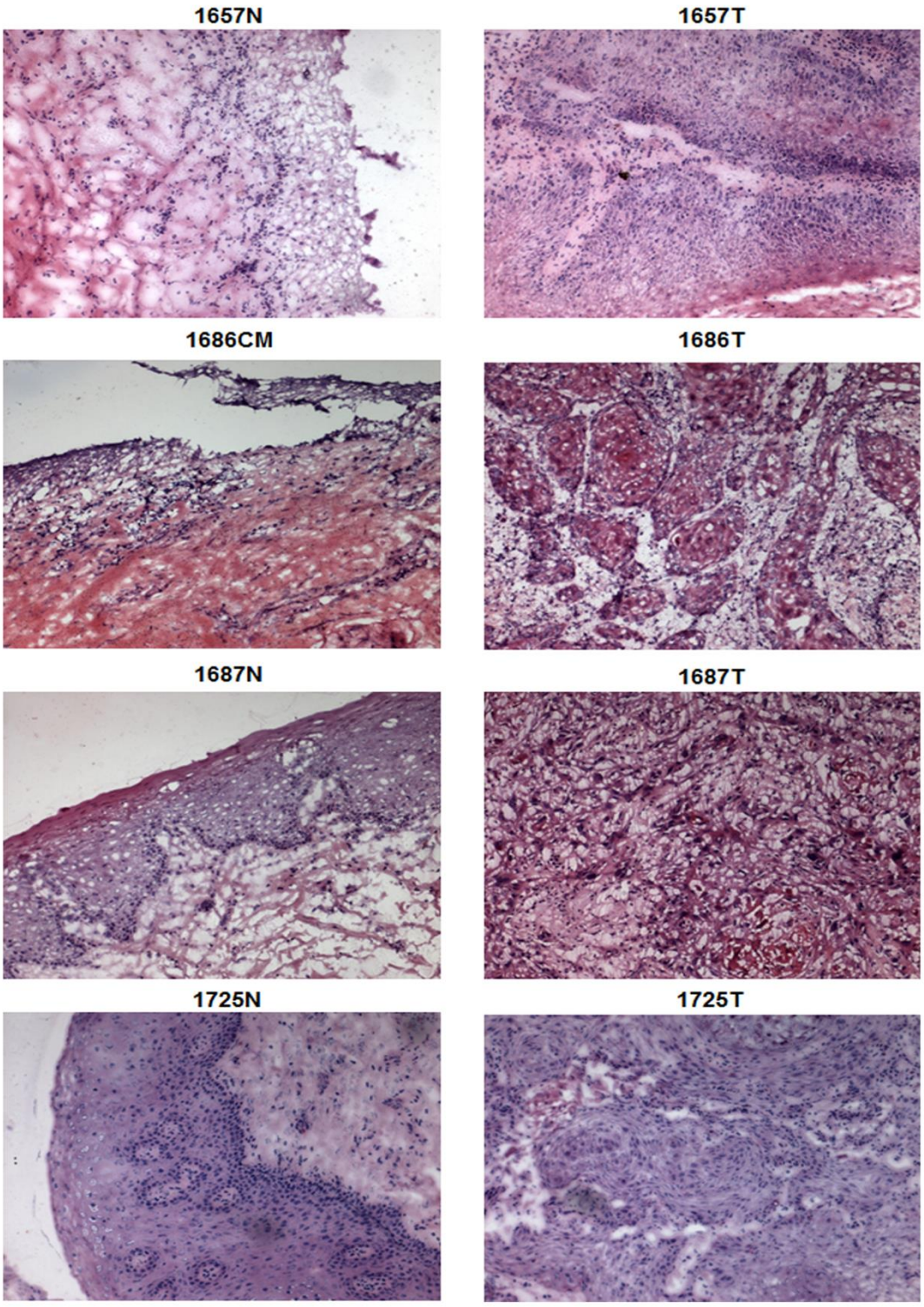

Figure A1. Micrographs of H&E stained tissue sections. continued…..

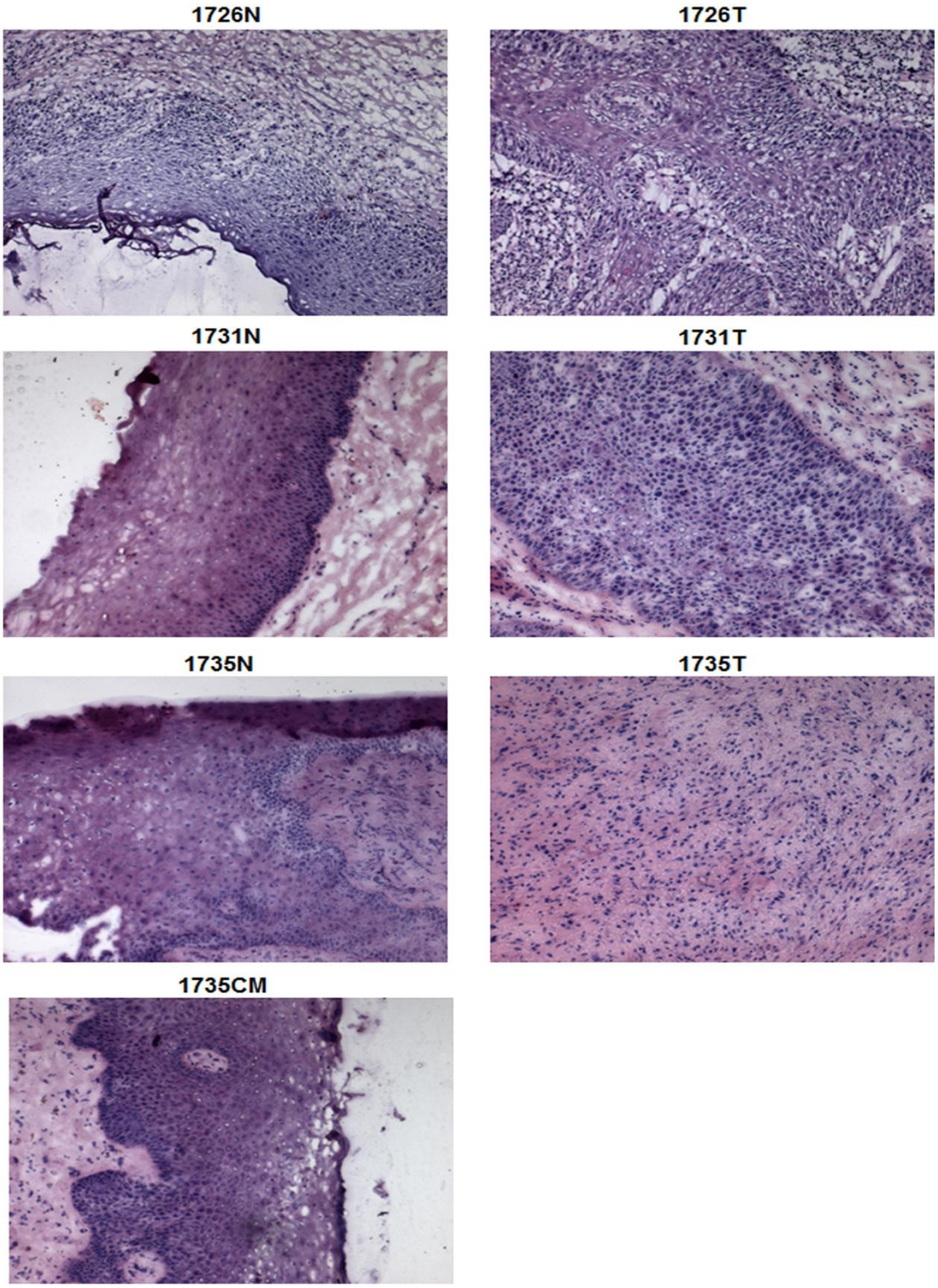

Figure A1. Micrographs of H&E stained tissue sections. continued…..

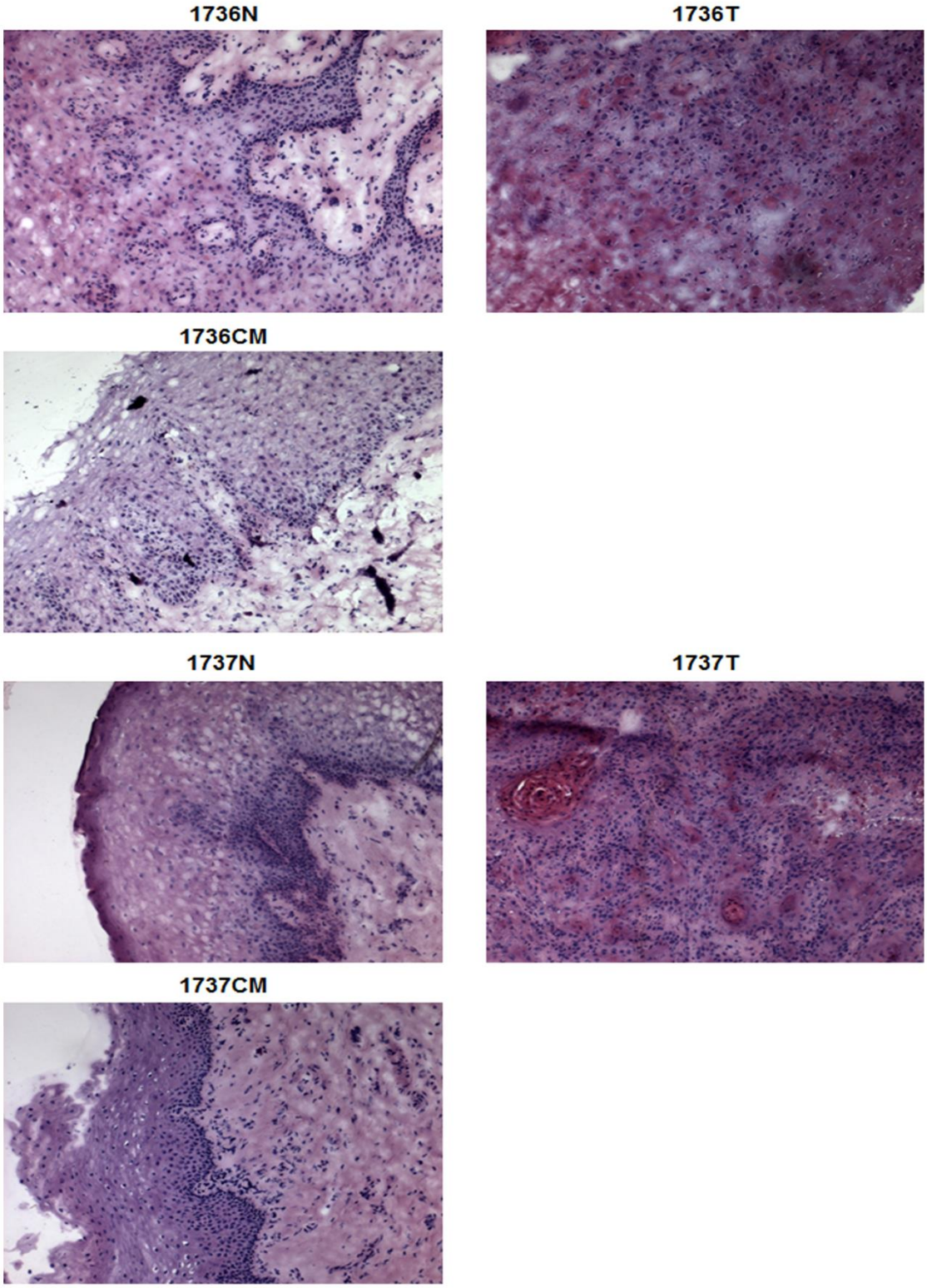

Figure A1. Micrographs of H&E stained tissue sections. continued…..

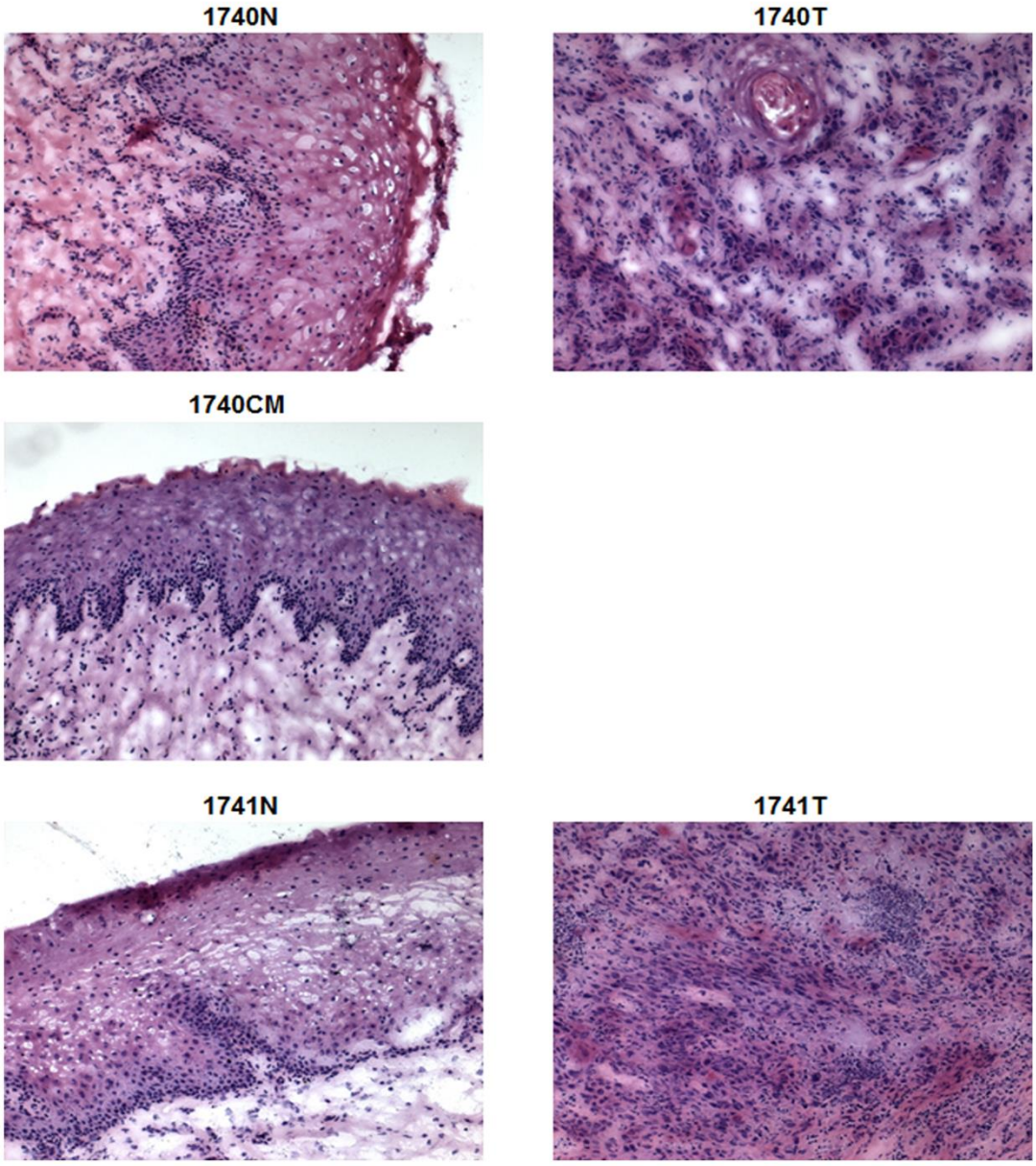

Figure A1. Micrographs of H&E stained tissue sections. continued…..

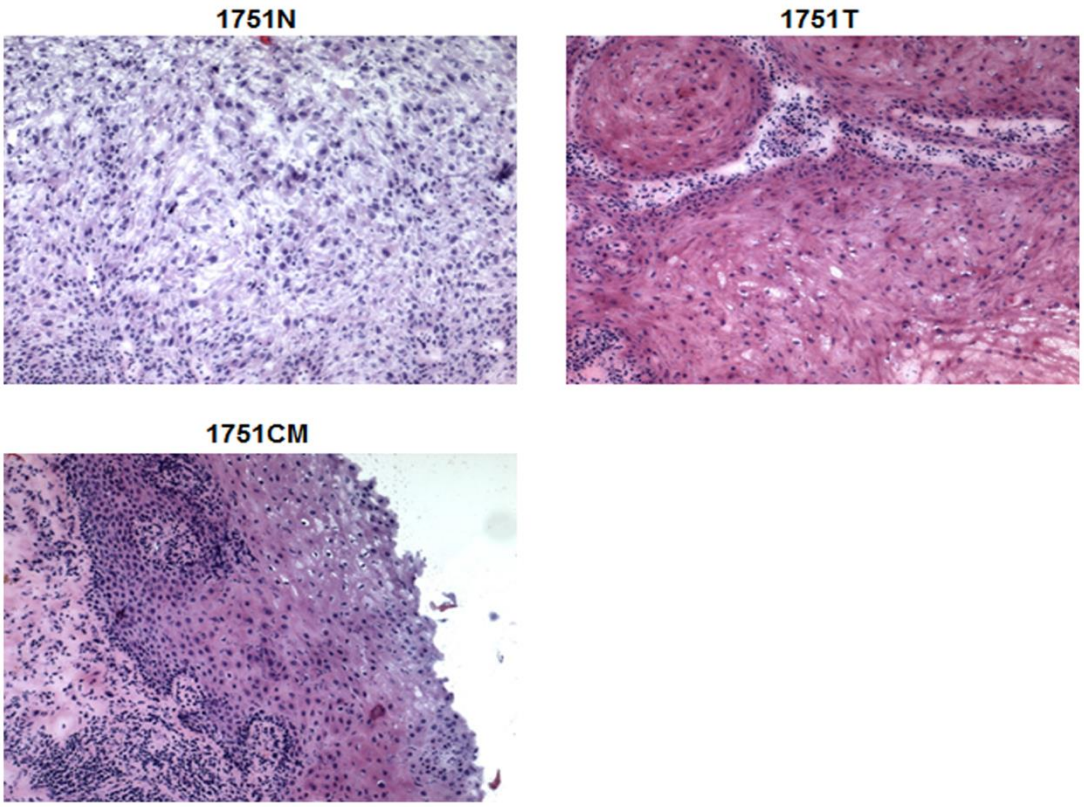

Figure A2. Comparison of the expression pattern obtained by mass spectrometry, silver staining, western blotting and immunohistochemistry.

Figure A2.1

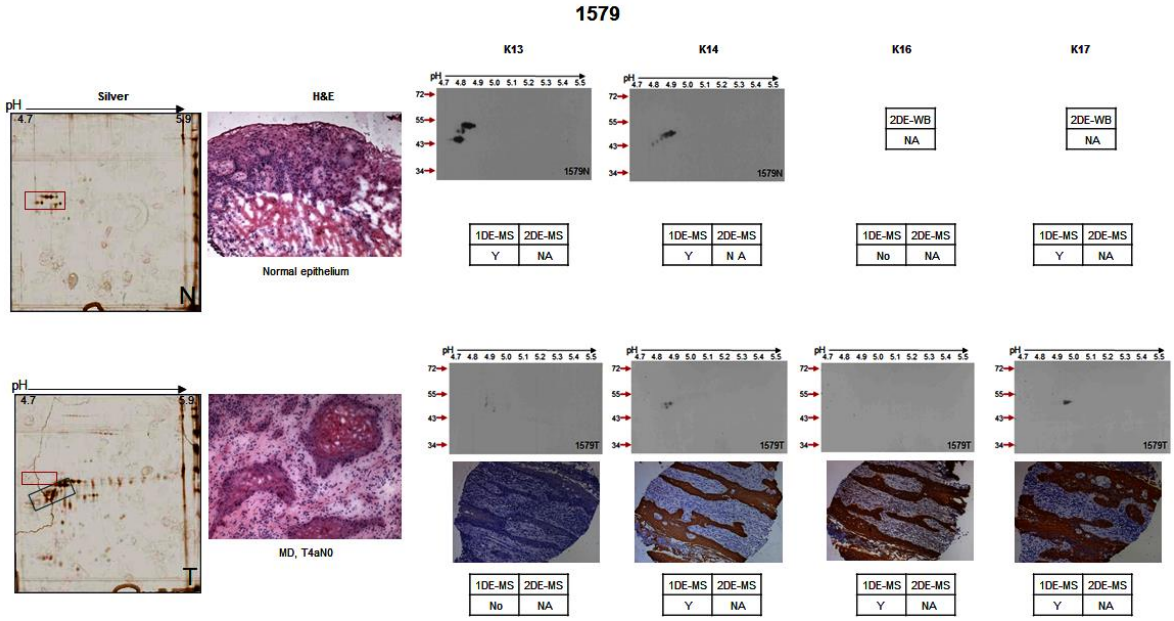

Fig. A2.1. Comparison of the expression pattern obtained by 1DE/2DE- mass spectrometry, 2DE-silver staining, 2DE western blotting and immunohistochemistry staining for Sample No. 1579. Tissue was not available for IHC and keratin enriched lysate was not available for 2DE-WB for K16 and K17 for Sample No. 1579 N.

Figure A2.2

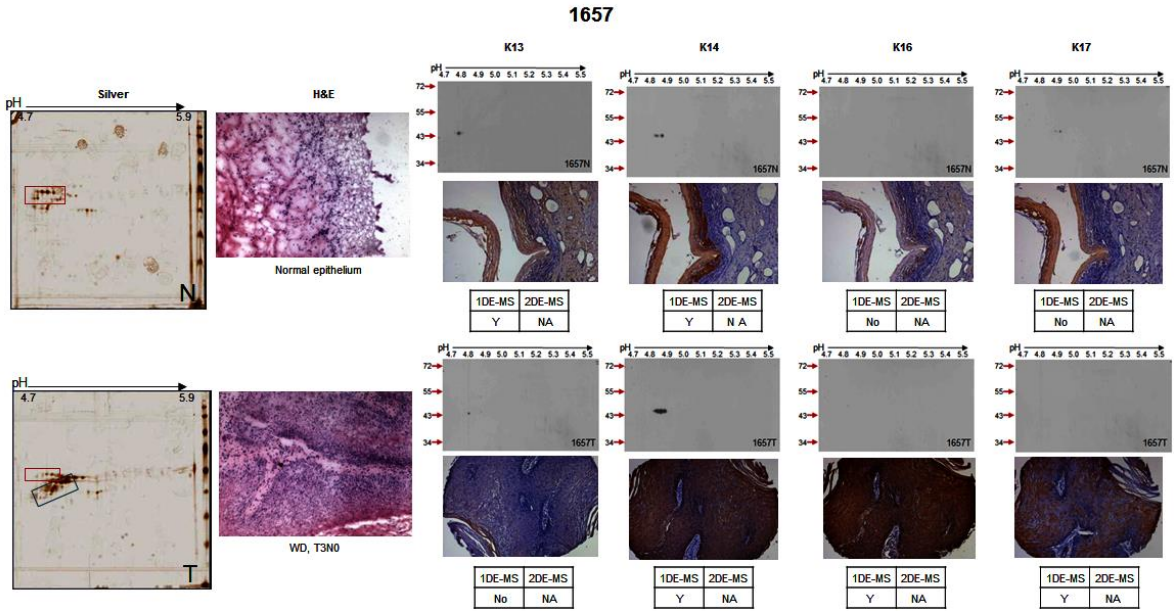

Fig. A2.2. Comparison of the expression pattern obtained by 1DE/2DE- mass spectrometry, 2DE-silver staining, 2DE western blotting and immunohistochemistry staining for Sample No. 1657.

Figure A2.3

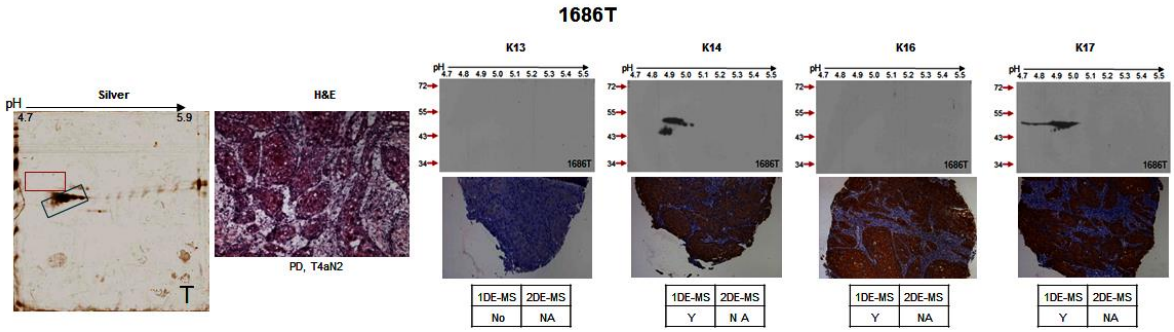

Fig. A2.3. Comparison of the expression pattern obtained by 1DE/2DE- mass spectrometry, 2DE-silver staining, 2DE western blotting and immunohistochemistry staining for Sample No. 1686T.

Figure A2.4

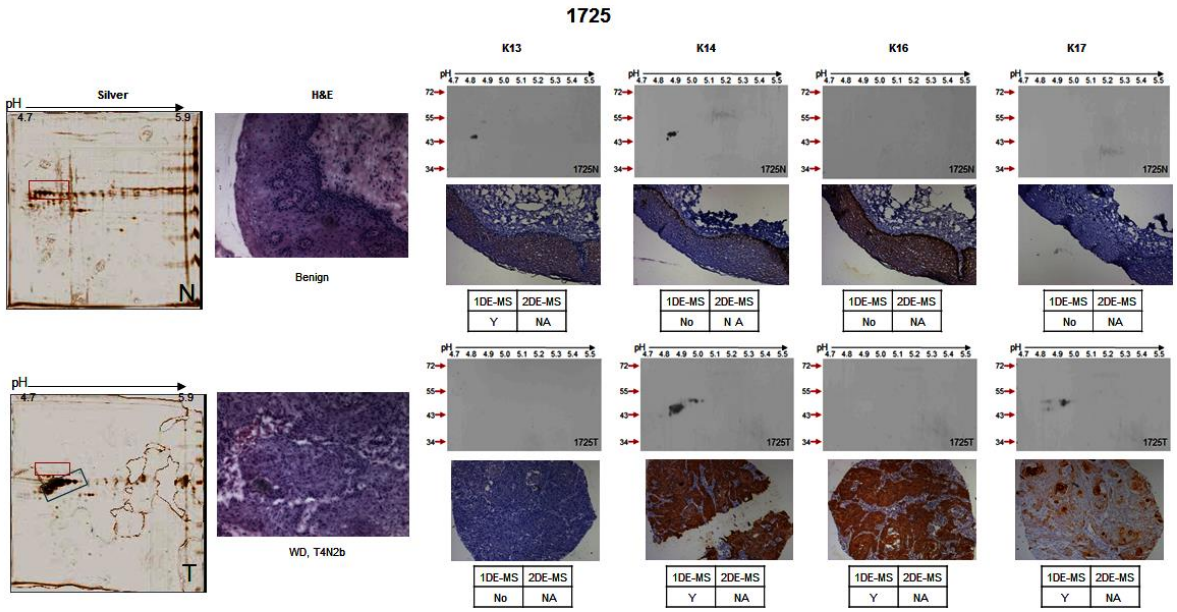

Fig. A2.4. Comparison of the expression pattern obtained by 1DE/2DE- mass spectrometry, 2DE-silver staining, 2DE western blotting and immunohistochemistry staining for Sample No. 1725.

Figure A2.5

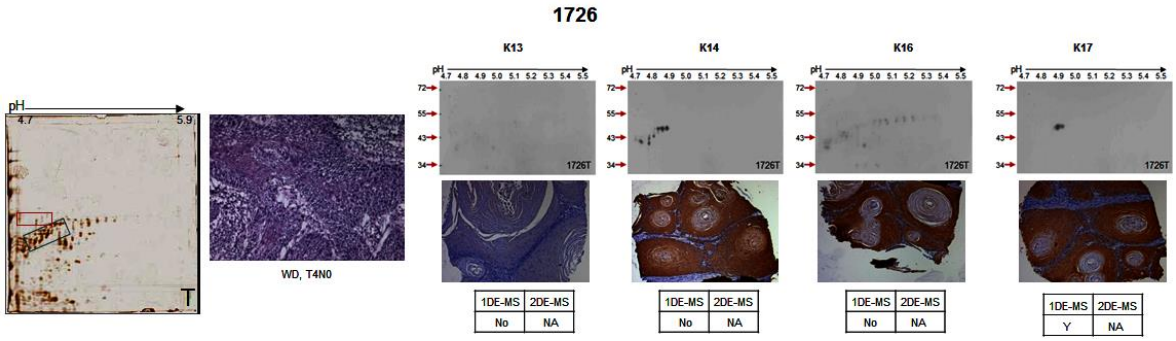

Fig. A2.5. Comparison of the expression pattern obtained by 1DE/2DE- mass spectrometry, 2DE-silver staining, 2DE western blotting and immunohistochemistry staining for Sample No. 1726T.

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Keratins in oral cancer: Necessity of mass spectrometry for validation of antibody based identifications

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ABSTRACT

Keratins are intermediate filament family proteins which are predominantly expressed in the epithelial cells. Most of the studies which evaluate the status of keratins in clinical samples of the oral cavity are based on the identification of their presence and localization by immunohistochemistry using monoclonal antibodies. It is very well known that many monoclonal/polyclonal antibodies show cross-reactivity with the other closely related or non-related proteins. This cross-reactivity might be the result of epitope similarity, but it is not always necessary. Therefore studies done with only antibody based techniques can mislead interpretation unless they are validated with additional techniques like mass-spectrometry.

In this investigation we have evaluated the status of keratin 18 in cancer of buccal mucosa using 1DE, 2DE and western blotting with monoclonal antibody to keratin 18. The patterns emerging showed aberrant as well as differential expression of K18 in adjacent normal versus tumor tissue samples of buccal mucosa. Mass spectrometry analysis of the immunodetected spots however revealed that it is keratin 13. Thus this study emphasizes the necessity of validation of antibody based findings when dealing with proteins of a large family having similarity/homology in amino acid sequence.

1. Introduction

Keratins (K) are intermediate filament family proteins which are predominantly expressed in the epithelial cells. The major function of the keratins is to provide mechanical support to the cell by forming a cytoskeletal network essential for structural integrity of the cell [1,2]. Keratins are also reported to perform several other functions such as regulation of melanin pigmentation in skin, protein synthesis and epithelial cell growth [3], cell signalling [4], stress response [5,6], apoptosis [7] and other keratin specific functions in the tissues [8–15]. Many of these functions are modulated by post-translational modifications such as glycosylation, phosphorylation, intra chain disulphide bonds and proteolysis of the keratins [16–21].

Buccal mucosa—a stratifying, non-keratinizing tissue—is reported to express K4 and K13 along with K5 and K14 [22]. Simple epithelial keratins K7, K8, and K18 are expressed in no more than trace amounts in normal stratified squamous epithelium [23] but have been reported to be aberrantly expressed in squamous cell carcinomas as shown by immunohistochemistry [24–28] and by 2D electrophoresis followed by western blotting using laboratory generated rabbit polyclonal antibodies to total keratins [29,30]. The aberrant expression

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has been correlated with poor prognosis in squamous cell carcinoma of the oral cavity and in oral leukoplakia [25].

Most of the studies which evaluate the status of keratins in clinical samples of the oral cavity are based on the identification of their presence and localization by immunohistochemistry using monoclonal antibodies. Many monoclonal antibodies are available for keratin detection [31–39]. It is very well known that many monoclonal/polyclonal antibodies show cross-reactivity with the other closely related or non-related proteins [40,41], which might be because of epitope similarity, but it is not always necessary.

In on-going studies we have been investigating the status of keratin 18 in cancer of the buccal mucosa. Protein lysates from micro dissected tissue and enriched preparations of keratins were resolved by 2DE and transferred to PVDF membrane and probed with the monoclonal antibody to K18, clone CY-90 from Sigma-Aldrich, USA. Differential pattern of staining was obtained between the keratins from the adjacent histologically normal tissue and the tumor tissue. Mass spectrometry analysis revealed that the protein identified by keratin 18 antibody was K13.

From these observations it is to be questioned that either K18 is aberrantly expressed as reported earlier or the antibody based studies are misleading the observations. Therefore, this study provides an example that only antibody based studies may be insufficient to draw conclusions regarding expression of proteins and caution is imperative for interpretation.

2. Materials and methods

2.1. Tissues

This study was approved by the Tata Memorial Centre Hospital ethics committee and all samples were collected with informed consent. Adjacent normal (N), cut margin (CM) and tumor (T) tissues were collected from patients with cancer of gingivo-buccal complex and breast during surgery. Tissues were quick frozen in liquid nitrogen till further use. The tissues were sectioned using a cryostat and the sections were stained with haematoxylin and eosin. The histology of the stained sections was evaluated by the pathologist for the grade of differentiation and marking tumor/normal epithelial area. Details of the samples are provided in the Supplementary Table 1S.

2.2. Reagents and antibodies

Tris-Cl, acrylamide, bis-acrylamide, glycine, trypsin, acetonitrile, iodoacetamide, urea, and thiourea were purchased from Sigma, USA. CHAPS and DTT were purchased from USB, USA. Trifluoroacetic acid (TFA) was a product of Applied Biosystems, UK. Silver nitrate and sodium bicarbonate were obtained from Fisher Scientific, India. Sodium thiosulphate and ammonium bicarbonate were purchased from SRL, India. Potassium ferricyanide was obtained from Qualigens, India. PVDF membrane was purchased from Millipore. Ampholines of pH 4.7 to 5.9 (Cat. No. 163-2097) and IPG strips pH 4 to 7, 7 cm (Cat. No. 163-2001), pH 4.7 to 5.9, 17 cm (Cat. No. 163-2021) were purchased from Bio-Rad Laboratories, USA. Mouse monoclonal antibody to keratin 18 (CY-90 clone; Cat. No. C-8541, Lot Nos. 062K4853 and 043K4839 were purchased from Sigma, USA and anti-keratin 18 (CY-90 clone; Cat. No ab49824 and LDK18, Cat. No. ab31844 were obtained from Abcam. UK. Rabbit polyclonal to keratin 13 (Cat. No. ab58744) was obtained from Abcam, UK. Mouse monoclonal antibody to β actin Cat. No. A5316 was purchased from Sigma. Anti-mouse IgG, HRP conjugated secondary antibody from sheep (Cat. No. NA931V), anti-rabbit IgG, HRP conjugated secondary antibody from donkey (Cat. No. NA934V) and ECL plus western blotting detection kits (RPN2132) were purchased from Amersham Biosciences (GE Healthcare), UK.

2.3. Preparation of lysates from micro-dissected tissues

Normal tissues, cut margin and tumor tissues were sectioned using a cryostat to obtain sections of 5 μm thickness. Normal epithelium and tumor areas were manually scraped as per the areas marked by the pathologists with reference to their respective stained master haematoxylin and eosin stained slides. The dissected tissue areas were then dissolved in 2DE buffer (8 M Urea, 2 M Thiourea, 2% CHAPS, 1% DTT). The homogenate was centrifuged at 55,000 rpm, at 4 °C for 1 h in Beckman optima TLX™ ultracentrifuge and the supernatant was used for further analysis.

2.4. Preparation of enriched keratin fractions

Enriched keratin fractions were made from the collected tissues using essentially the protocol of Achtstaetter et al. [42]. Briefly, tissues were thoroughly washed with chilled 10 mM Tris HCl and then homogenized in 10 ml buffer (96 mM NaCI, 8 mM KH_2PO4, 5.6 mM $Na_2HPO_4{\cdot}2H_20$, 1.5 mM KCl, 10 mM EDTA, 0.1 mM DTT, pH 6.8) using the Yellow^{Line} DI 25 basic homogenizer, Germany. The homogenate was centrifuged at 4500 rpm for 30 min, 4 °C in a Rota 4R centrifuge from Plasto Crafts, India. The supernatant (sup.1) was collected in another tube. Chilled detergent buffer A (10 mM Tris–HCl, 140 mM NaCI, 5 mM EDTA, 1% Triton X-100, pH 7.6) was added to the pellet and the mixture was incubated for 30 min at 4 °C. It was then centrifuged for 30 min, at 4 °C at 4500 rpm. The supernatant (sup.2) was collected in another tube and the pellet was suspended in high salt buffer B (10 mM Tris–HCl, 140 mM NaCl, 1.5 M KCI, 5 mM EDTA, 0.5% (w/v) Triton X-100, pH 7.6). The homogenate was stirred for 1 h at 4 °C. Homogenate was then centrifuged at 4500 rpm at 4 °C. The final pellet was dissolved in 2DE buffer and the supernatant (sup.3) was collected in a tube. Protein content of the samples was measured as described by Peterson [43].

2.5. 1DE and 2DE, immunostaining and silver staining

The micro dissected tissue lysates and/or enriched keratin preparations were resolved by 1D and 2D SDS-PAGE essentially according to the Laemmli protocol [44]. For 2DE, 7 cm or 17 cm length IPG strips (Bio-Rad, USA) of pH range 4 to 7 or 4.7 to 5.9, respectively were used. For 1DE and 2DE studies, 3 μg and 30 μg of enriched keratins were used respectively. After 1DE and 2DE, proteins from the gel were transferred on to PVDF membrane essentially according to Towbin [45] . For immunostaining, the blots were blocked in 3% BSA dissolved in TBST. The position of the keratins was detected using mouse monoclonal antibody to

keratin 18 clone CY90 from Sigma and abcam, LDK 18 or rabbit polyclonal antibody to keratin 13 at 1:2000 dilution in 1% BSA dissolved in TBST. The primary antibodies were detected using the HRP conjugated secondary anti-mouse antibody at 1:2000 dilution or the anti-rabbit antibody at 1:5000 dilution in 1% BSA dissolved in TBST. ECL plus western blotting detection system from GE Healthcare was used for detecting the bound antibodies. As required some of the gels were stained with silver essentially according to Oakley et al. [46].

2.6. *Mass spectrometry*

Silver stained protein spots were processed for mass spectrometry with/without separation using LC. The proteins were identified by mass spectrometry. The in-gel digestion of silver stained spots with trypsin was carried out essentially according to Shevchenko et al. [47]. 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. Proteins in the 1DE resolved gel plugs were reduced using 10 mM DTT and then alkylated with 55 mM iodoacetamide. For protein in 2DE silver stained gels reduction and alkylation is done in the equilibration buffers used for 2DE [44]. The proteins in the plugs were then trypsinized overnight with 10 ng/μl trypsin in 25 mM ammonium bicarbonate in water and the peptides were recovered by extraction with 50% ACN and 5% TFA. Tryptic protein digests were reconstituted in 10% ACN with 0.1% TFA solvent before subjecting them to mass spectrometry analysis.

To confirm the identities obtained in the gel spots, the trypsin digests were resolved on a Dionex, C-18 column attached to the Agilent 1200 chromatography system. Five microliters of the sample was loaded on the column through the Agilent autosampler. The bound peptides were eluted with a gradient of increasing concentration of acetonitrile containing 0.1% TFA. The gradient programme is given in the Supplementary Table 2S.

The eluate was collected on a Prespotted Anchor Chip™ (PAC) and analyzed by mass spectrometry in an automated mode.

Mass calibration was carried out using peptide mixture of five known peptides spanning mass range of 757–3147 *m/z* and error was kept to less than 10 ppm. Accelerating voltage of 25 kV was applied to the first TOF tube. The MS data were acquired in an automated manner using a solid state NdYAG laser at 337 nM. The resulting MS data was analyzed using Flex analysis 3.0 (Brucker Daltonik, Germany) software. Five most intense peaks for protein identity obtained in MS analysis were subjected to MS/MS. The MS peaklist and the MS/MS ions of the chosen peptides were searched against SwissProt database version 2011_07 onwards and version 57.15 for LC-MS/MS analysis using MASCOT search engine for protein ID with precursor tolerance of 100 ppm for MS and fragment tolerance of 0.7 Da for MS/MS.

3. Results

3.1. *K18 profile in micro-dissected tissue lysates*

Keratin 18 is reported to be aberrantly expressed in cancers of the buccal mucosa [24–30]. Tissue lysates made from micro-dissected epithelium were resolved by 1DE and 2DE, and the proteins were blotted on to the PVDF membranes. The blots were probed with antibody to keratin 18. Figs. 1 and 2 show the autographs of micro dissected tissue lysates from histological normal and tumor samples, resolved by 1DE and 2DE followed by immunostaining with keratin 18 antibody (CY90). It was observed that the antibody recognizes bands at ~48 kDa and ~45 kDa in the normal tissue and either both ~48 kDa band and a lower band at ~45 kDa or only the lower band ~45 kDa was observed in tumor tissues. Based on these observations it was hypothesized that keratin 18 might be post-translationally modified in the buccal mucosa tumors leading to the formation of the additional lower ~45 kDa band.

3.2. *Keratin 18 in enriched keratin preparations of normal and tumor buccal mucosa tissues*

To study the changes in the keratin 18 molecular weight and to identify the post-translational modifications if any, the preparations of enriched keratins were resolved by 1DE and 2DE, transferred to PVDF membrane and probed with antibody to keratin 18 (CY90). Fig. 3 shows autographs of the signals obtained for K18. The antibody staining pattern was similar to the total tissue lysates as seen in Figs. 1 and 2 and therefore enrichment protocol was followed for further studies.

3.3. *Mass spectrometry of spot detected by antibody to keratin 18 reveals that it is keratin 13*

To confirm the identity of K18 in the 2DE gels by mass spectrometry the corresponding immunoreactive bands for K18

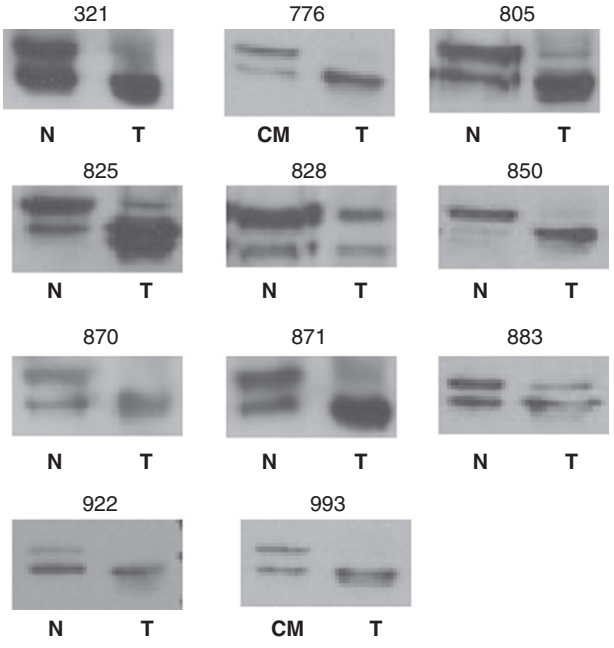

Fig. 1 – Autographs of proteins from micro dissected normal (N) cut margin (CM) and tumor tissue lysates, resolved by 10%. 1DE, blotted on membranes and probed with antibody to K18 (CY90 clone). The sample number is indicated above each panel. Histopathological information for the samples is given in supplementary Table 1S.

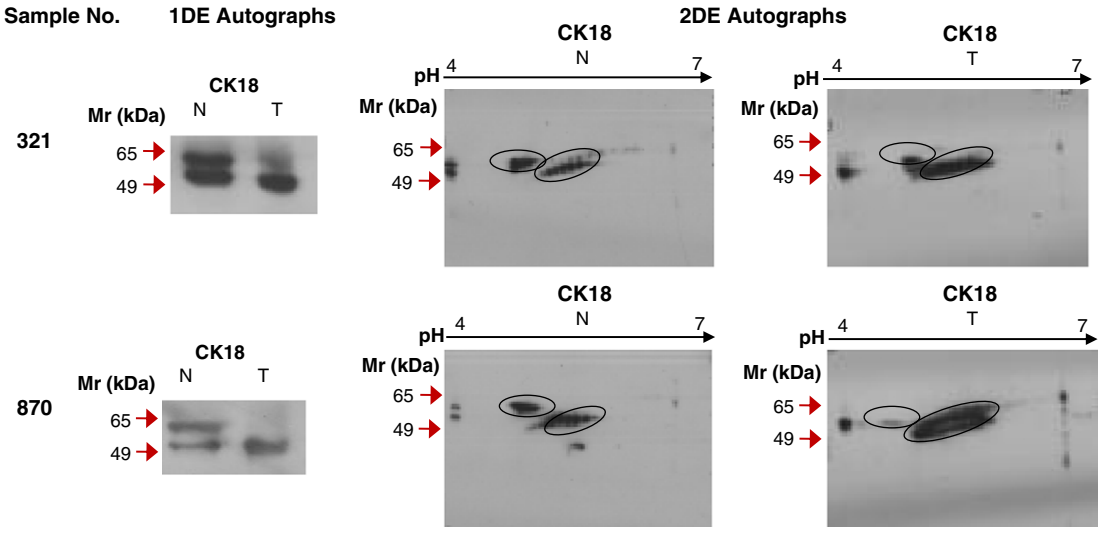

Fig. 2 – Autographs of proteins from adjacent normal and tumor tissue lysates resolved by 1DE and 2DE using 7 cm IPG strips with pH range of 4–7, transferred to membranes and probed with antibody to K18 (CY90 clone).

antibody were plugged out from silver stained gels. Fig. 4 shows a silver stained gel profile of enriched keratins from histologically normal epithelium of 1737 CM resolved by 2DE. The four darkly silver stained spots near 48 kDa corresponded to the autographic signal obtained on a parallel gel which was blotted and immunostained with antibody to keratin 18. Colloidal stained 2DE blot shows the relative position of the spots. The silver stained gel spots were subjected to trypsin digestion after destaining and the eluted peptides were analyzed by mass spectrometry on the Brucker Ultraflex II MALDI TOF-TOF. Proteins were identified by Peptide Mass Fingerprinting and 5 matched peptides of high intensity were further selected for MS/MS analysis. Spot Nos. 1, 2, 3 and 5 were identified as keratin 13 (Table 1).

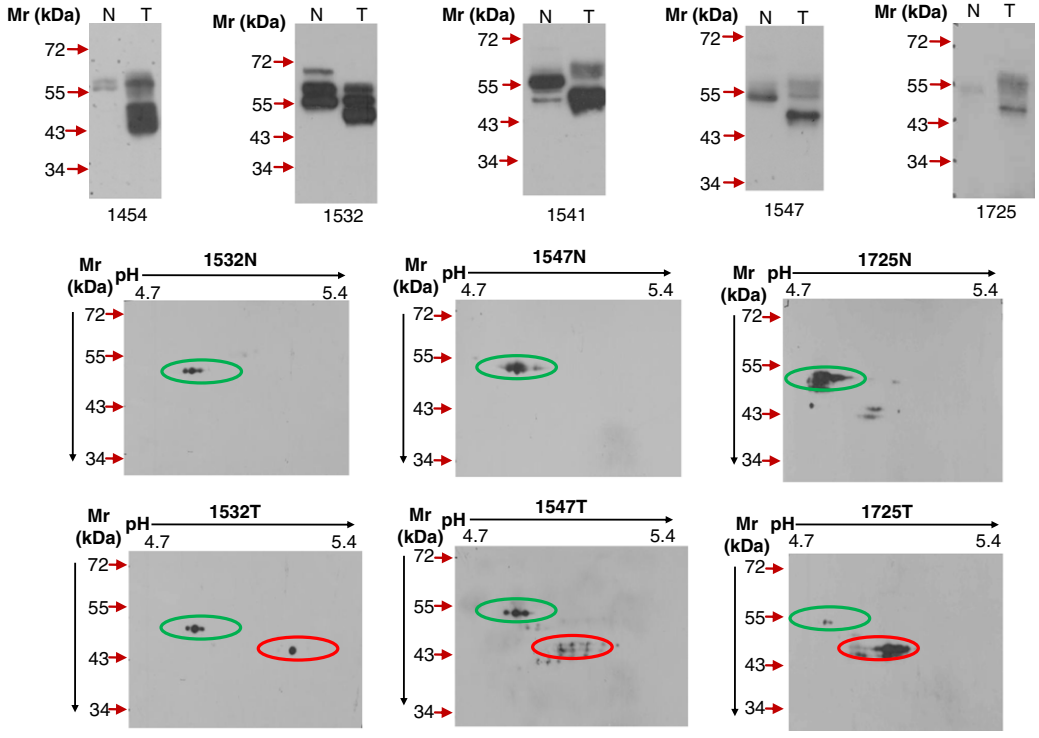

Fig. 3 – Autographs of enriched keratins from adjacent normal and tumor tissue lysates resolved by 1DE and 2DE using 17 cm IPG strips with pH range of 4.7–5.9, transferred to membranes and probed with antibody to K18 (CY90 clone). The 2DE blot was cut beyond pH 5.4 to minimise antibody requirements.

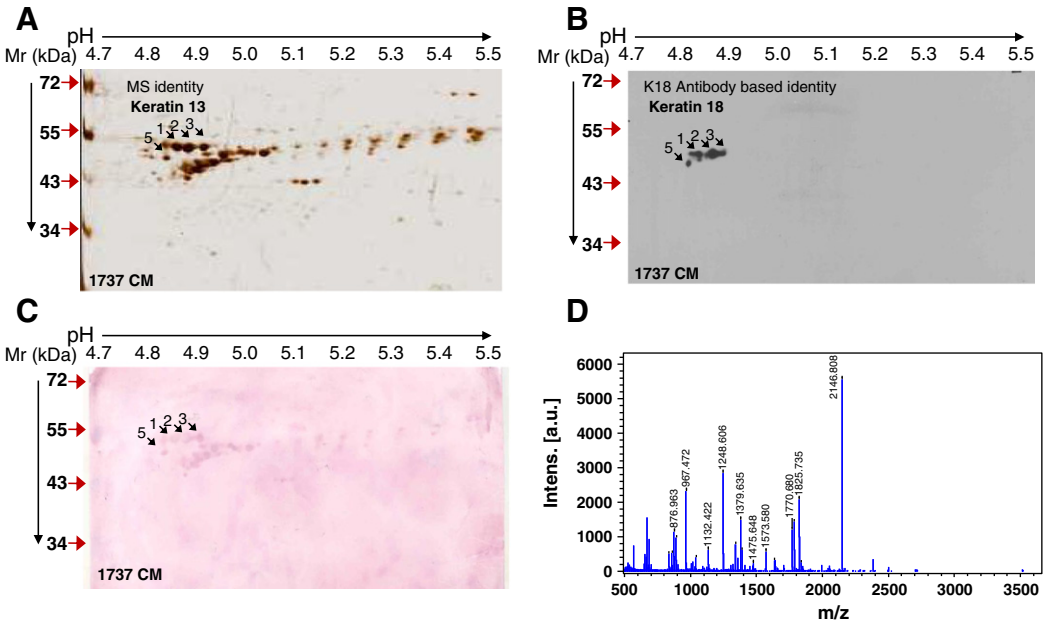

Fig. 4 – Profiles of enriched keratin preparation from a histologically normal cut margin tissue sample (1737 CM) resolved by 2DE on a 17 cm IPG strip of pH range 4.7–5.9. A) Silver stained gel; (B) Autographic profile of the blot stained with antibody to K18 (CY-90); C) Blot stained with Colloidal gold; D) MS peak profile of spot 1 identifies it as keratin 13. The 2DE blots were cut beyond pH 5.5 to minimize antibody requirements.

3.4. *Confirmation of the identity of the keratin 13 by 2DE-LC-MS/MS*

To confirm if keratin 18 was entirely absent, enriched keratin preparation from another histologically normal epithelium of 1574 CM was resolved by 2DE. Fig. 5 shows the 2DE profile of enriched keratins from the sample 1574 CM. The silver stained spots in the boxed area were subjected to trypsin digestion and the eluted peptides resolved by liquid chromatography and analyzed by mass spectrometry. Only keratin 13 along with keratin 15 was detected and K18 identity was not obtained (Table 2).

3.5. *Mass spectrometry analysis of the enriched keratin preparations after resolution by 1DE*

Fig. 6 shows profiles of enriched keratins from histologically normal 1579 N tissue sample resolved by 1DE and 2DE. It is

Table 1 – Mass spectrometry analysis of silver stained spots from Fig. 4A.

Spot no.	Protein name	Protein ID	Accession no.	PMF			MS/MS				
				Total score	Mass (Da)	SC (%)	Peptides identified (peptide score)	Expect	Total score	SC (%)	M
1	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	93	49900	22	K.IRDWHLK.Q (19) R.LKYENELALR.Q (66) R.VLAEMREQYEAMAER.N (46) R.LQSSSASYGGGFGGGSCQLGGGR.G (95)	0.53 1.2e-05 0.0005 3.1e-09	226	12	4 (3)
2	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	188	49900	45	R.LKYENELALR.Q (45) K.MIGFPSSAGSVSPR.S (38) R.VLAEMREQYEAMAER.N (54) R.LQSSSASYGGGFGGGSCQLGGGR.G (127)	0.0016 0.0051 9.1e-05 2.6e-12	186	13	4 (4)
3	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	118	49900	38	K.MIGFPSSAGSVSPR.S (14) K.QSPASPERDYSPYYK.T (18) R.VLAEMREQYEAMAER.N (35) R.LQSSSASYGGGFGGGSCQLGGGR.G (186)	1.5 0.63 0.011 7.2e-18	193	14	4 (2)
5	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	149	49900	35	R.LKYENELALR.Q (21) K.MIGFPSSAGSVSPR.S (41) R.VLAEMREQYEAMAER.N (27) R.LQSSSASYGGGFGGGSCQLGGGR.G (67)	0.43 0.0027 0.05 3.4–06	83	13	4 (3)

Abbreviations: SC—Sequence coverage, M—Matches (No. of peptides with $p \leq 0.05$).

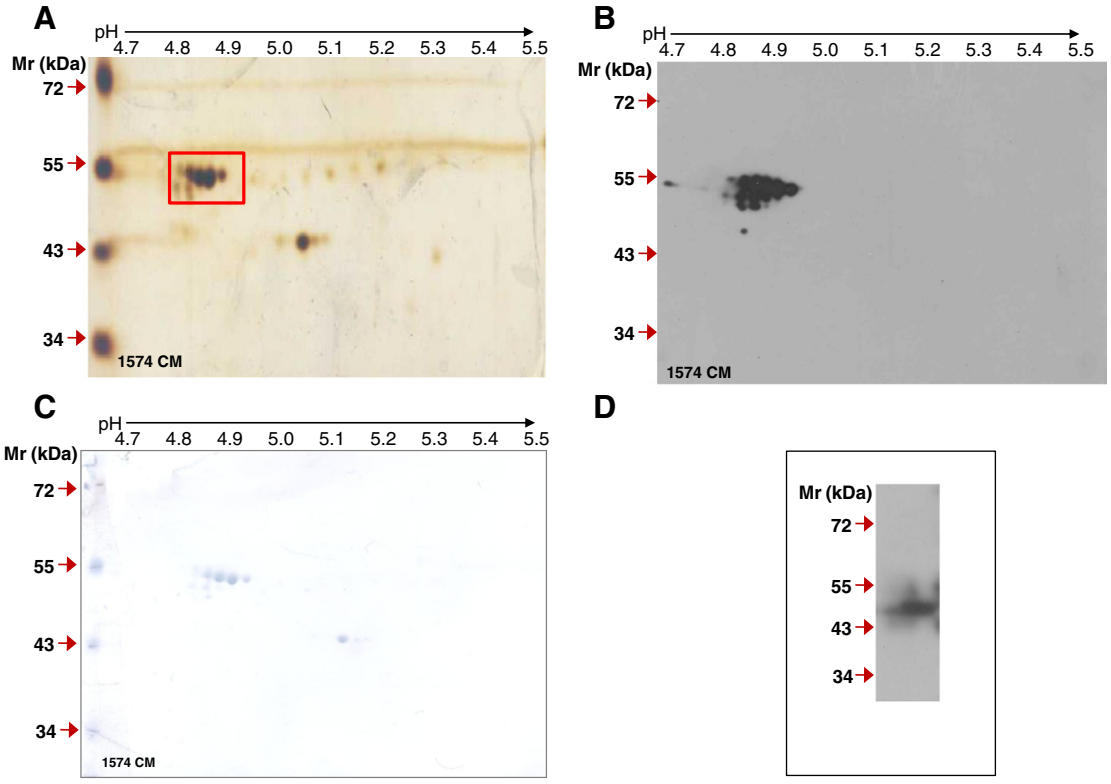

Fig. 5 – Profiles of enriched keratin preparation from histologically normal cut margin tissue sample (1574 CM) resolved by 2DE on a 17 cm IPG strip of pH range 4.7 to 5.9. A) Silver stained gel of enriched keratin preparation (30 μg). Boxed area shows the spots taken for LC-MS/MS analysis; B) Autographic profile obtained by probing with antibody to K18 (CY-90); C). Amido stained 2DE blot for comparison of position of spots on silver stained gels and blots probed with antibody to K18; D) 1DE autoradiographic profile of keratin preparation stained with antibody to K18. The 2DE blots were cut beyond pH 5.5 to minimise antibody requirements.

Table 2 – LC-MS/MS analysis of protein in the boxed portion in Fig. 5A.

Protein name	Protein ID	Accession no.	Mass (Da)	Peptides identified (peptide score)	Expect	Total score	Matches
Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	49900	K.TIEELRDK.I (45)	0.0015	212	14 (7)
				K.YENELALR.Q (35)	0.013		
				K.TEITELRR.T (29)	0.065		
				R.VILEIDNAR.L (61)	2.6e-05		
				K.ILTATIENNR.V (14)	2.1		
				R.LKYENELALR.Q (90)	2.8e-08		
				R.QSVEADINGLRR.V (29)	0.048		
				K.TRLEQEIATYR.S (24)	0.15		
				K.MIGFPSSAGSVSPR.S (21)	0.37		
				K.AGLENTVAETECR.Y (19)	0.52		
				K.VRALEEANADLEVK.I (5)	10		
				K.QSPASPERDYSPYYK.T (58)	5.2e-05		
				R.LQSSSASYGGGFGGGSCQLGGGR.G (60)	2.9—05		
				K.ILTATIENNRVILEIDNAR.L (21)	0.18		
Keratin, type I cytoskeletal 15	K1C15_HUMAN	P19012	49395	K.TIEELRDK.I (45)	0.0015	146	8 (4)
				K.YENELALR.Q (35)	0.013		
				R.VLDELTLAR.T (19)	0.5		
				R.VILEIDNAR.L (61)	2.6e-05		
				R.LKYENELALR.Q (90)	2.8e-08		
				K.TRLEQEIATYR.S (24)	0.15		
				K.AGLENSLAETECR.Y (19)	0.52		
				K.VRALEEANADLEVK.I (5)	10		

Matches (no. of peptides with $p \leq 0.05$).

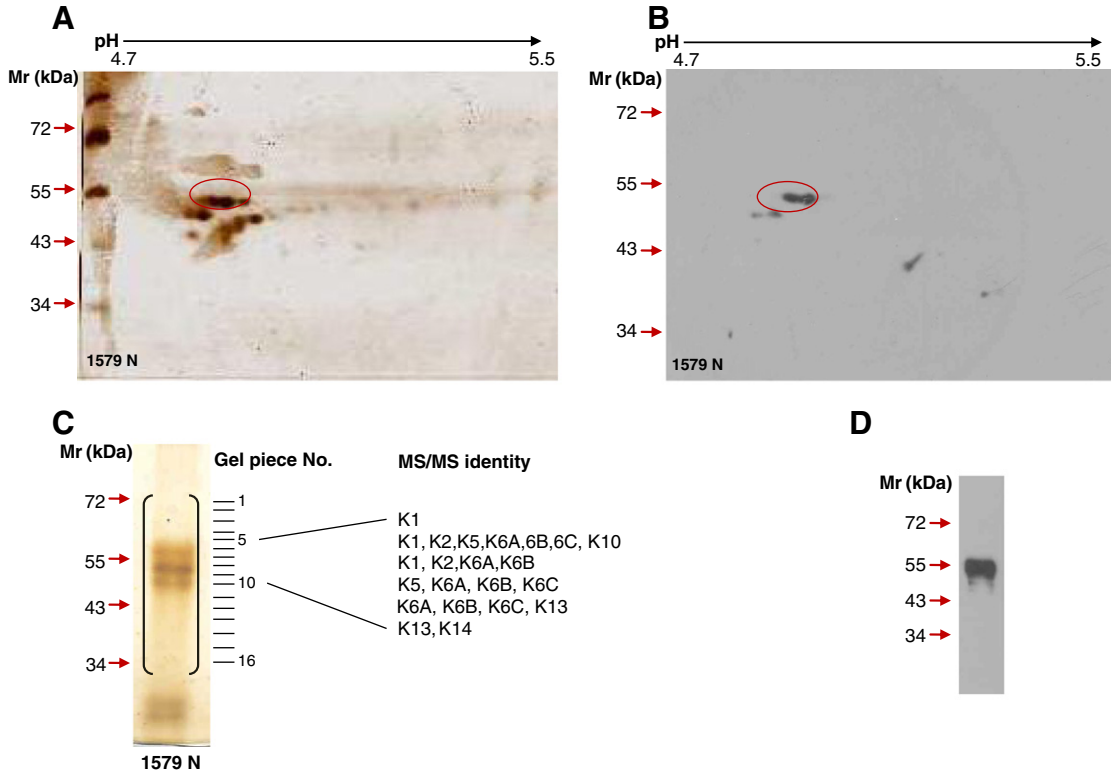

Fig. 6 – Profiles of enriched keratin preparation from histologically normal sample 1579 N resolved by 2DE on a 17 cm IPG strip of pH range 4.7 to 5.9. A) Silver stained profile; B) Autographic profile of the keratins resolved by 2DE and blots probed with antibody to K18; C) Keratin preparation resolved by 1DE and stained with silver. The entire area from 72 kDa to 34 kDa was cut into pieces and the peptides obtained after tryptic digestion was subjected to mass spectrometry analysis. D) Keratins separated by 1DE and probed with K18 antibody for the same sample. The 2DE blots were cut beyond pH 5.5 to minimize antibody requirements.

seen that as in Fig. 5B the antibody to keratin 18 detects a major single diffuse spot on the autograph (Fig. 6B). The area over riding this signal is indicated on the silver stained gel (Fig. 6A). To confirm that keratin 18 was indeed absent in the sample, the enriched keratin preparation was resolved by 1DE and the entire area indicated in Fig. 6C was cut into 16 pieces. All gel plugs were subjected to in-gel digestion, and then subjected to mass spectrometry analysis. Protein identities were obtained in gel piece nos. 5 to 10. Keratins 1, 2, 5, 6A, 6B, 6C, 13 and 14 were identified as shown in Fig. 6C with details in Table 3. However, keratin 18 was not detected in the sample.

3.6. *Identification of keratin 13 and keratin 18 in the enriched keratin preparations for normal and tumor samples by immunoblotting*

To identify the relative position of K13 and K18, enriched keratin preparations were resolved by 2DE using 17 cm IPG strips pH 4.7 to 5.9 and the resolved proteins were immunostained with respective antibodies after blotting on PVDF membranes. Fig. 7 shows 2DE autographs of blots probed with antibody to keratin 18 and keratin 13 for samples 1736 and 1740. It is seen that in the normal/cut margin samples, antibody to K18 reacts only with the 48 kDa band. In the tumor samples, antibody to K18 recognizes lower 45 kDa band. Antibody to K13 also recognizes only the 48 kDa band and does not recognize the lower band.

3.7. *Evaluation of the reactivity of keratin 18 antibody from different sources*

To determine whether the clone CY 90 from another source other than Sigma also detected K13, the experiment as described in Section 3.6 was repeated. The blots were probed with clone CY 90 from Abcam and LDK18 (an antibody to the C-terminal end of keratin 18). The results obtained are given in Fig. 8. The same signals were seen with the anti-keratin 18 antibody from Abcam as were seen with the Sigma antibody shown in Fig. 7. Another antibody to keratin 18 i.e. LDK 18 does not detect any protein at 48 kDa or 45 kDa in the keratin preparations from 1736 N and T and 1740 CM and T samples.

3.8. *Evaluation of the reactivity of clone CY90 with breast cancer cells and breast cancer tissues which express keratin 18*

Breast cancer cells MDA-MB-468 are reported to express keratin 18 [48]. Cell lysates were resolved by 2DE and the proteins transferred to PVDF membranes. The blots were probed with anti-keratin 18 clone CY 90 from Sigma and Abcam, LDK 18 antibody, K13 antibody and actin specific antibody. The data are given in the supplementary. Fig. 1S-A to G show the autographic profiles obtained for each of the antibodies.

Table 3 – Mass spectrometry analysis of protein in boxed area in Fig. 6C.

*Gel piece no.	Protein name	Protein ID	Accession no.	PMF			MS/MS				
				Total score	Mass (Da)	SC (%)	Peptides Identified (Peptide Score)	Expect	Total score	SC (%)	M
5	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	76	66170	36	K.YEELQITAGR.H (60) R.FLEQQNQVLQTK.W (45) K.QISNLQQSISDAEQR.G (51) R.FSSCGGGGGSFGAGGGFGSR.S 56) R.GGGGGGYGSGGSSYGSGGGSYGSG GGGGGGR.G (177)	4.9e-05 0.0014 0.00027 2.9e-05 9.1e-18	289	13	5 (5)
6	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	138	66170	41	K.IEISELNR.V (25) K.YEDEINKR.T (38) K.YEELQITAGR.H 62) K.NMQDMVEDYR.N (26) R.FLEQQNQVLQTK.W (41) R.GGGGGGYGSGGSSYGSGGGSY GSGGGGGGGR.G (110)	0.19 0.0071 3.3e-05 0.058 0.0035 1.4e-10	167	12	6 (4)
	Keratin, type II cytoskeletal 5	K2C5_HUMAN	P13647	121	62568	34	K.FASFIDK.V (31) R.GRLDSELR.N (15) K.YEDEINKR.T (38) K.AQYEEIANR.S (32) K.LALDVEIATYR.K (59) R.VSLAGACGVGGYGSR.S (14)	0.031 2.1 0.0071 0.03 5.6e-05 1.5	73	9	6 (4)
	Keratin, type I cytoskeletal 10	K1C10_HUMAN	P13645	109	59020	30	R.LENEIQTYR.S (21)-R.QSVEADINGLR.R (9) R.SQYEQLAEQNRK.D (34)	0.34 6 0.00019	54	5	3 (1)
	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	105	60293	33	K.FASFIDK.V (31) R.GRLDSELR.G (15) K.YEDEINKR.T (38) K.AQYEEIAQR.S (27) K.YEELQVTAGR.H (29) K.LALDVEIATYR.K (59)	0.031 2.1 0.0071 0.1 0.058 5.6e-05	71	9	6 (4)
	Keratin, type II cytoskeletal 6 C	K2C6C_HUMAN	P48668	105	60273	33	K.FASFIDK.V (31) R.GRLDSELR.G (15) K.YEDEINKR.T (38) K.AQYEEIAQR.S (27) K.YEELQVTAGR.H (29) K.LALDVEIATYR.K (59)	0.031 2.1 0.0071 0.1 0.058 5.6e-05	71	9	6 (4)
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	97	60315	32	K.FASFIDK.V (31) R.GRLDSELR.N (15) K.YEDEINKR.T (38) K.AQYEEIAQR.S (27) K.YEELQITAGR.H (62) K.LALDVEIATYR.K (59)	0.031 2.1 0.0071 0.1 3.3e-05 5.6e-05	103	9	6 (4)
	Keratin, type II cytoskeletal 2	K22E_HUMAN	P35908	74	65678	26	K.FASFIDK.V (31) K.IEISELNR.V (25) K.YEDEINKR.T (38) K.AQYEEIAQR.S (27) K.LALDVEIATYR.K (59) R.FLEQQNQVLQTK.W (41)	0.031 0.19 0.0071 0.1 5.6e-05 0.0035	82	8	6 (4)
7	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	91	66170	37	K.YEELQITAGR.H (42) R.FLEQQNQVLQTK.W (37) R.SGGGFSSGSAGIINYQR.R (68) R.FSSCGGGGGSFGAGGGFGSR.S (91) R.GGGGGGYGSGGSSYGSGGGSYGSG GGGGGGR.G (172)	0.0028 0.0077 5.8e-06 2.2e-08 1.3e-16	306	13	5 (5)
	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	63	60293	29	K.FASFIDK.V (41) K.AQYEEIAQR.S (18) K.YEELQVTAGR.H (23) R.QNLEPLFEQYINNLR.R (57)	0.0033 0.92 0.22 7.1e-05	69	7	4 (2)
	Keratin, type II cytoskeletal 6 C	K2C6C_HUMAN	P48668	63	60273	29	K.FASFIDK.V (41) K.AQYEEIAQR.S (18) K.YEELQVTAGR.H (23) R.QNLEPLFEQYINNLR.R (57)	0.0033 0.92 0.22 7.1e-05	69	7	4 (2)

(continued on next page)

Table 3 (*continued*)

*Gel piece no.	Protein name	Protein ID	Accession no.	PMF			MS/MS				
				Total score	Mass (Da)	SC (%)	Peptides Identified (Peptide Score)	Expect	Total score	SC (%)	M
	Keratin, type II cytoskeletal 2	K22E_HUMAN	P35908	58	65678	26	K.FASFIDK.V (41) K.AQYEEIAQR.S (18) R.FLEQQNQVLQTK.W (37)	0.0033 0.92 0.0077	53	4	3 (2)
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	56	60315	27	K.FASFIDK.V (41) K.AQYEEIAQR.S (18) K.YEELQITAGR.H (42) R.QNLEPLFEQYINNLR.R (57)	0.0033 0.92 0.0028 7.1e-05	83	7	4 (3)
8	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	120	60293	37	K.FASFIDK.V (51) K.AQYEEIAQR.S (7) R.QNLEPLFEQYINNLR.R (48)	0.00032 11 0.0005	71	5	3 (2)
	Keratin, type II cytoskeletal 6 C	K2C6C_HUMAN	P48668	110	60273	34	K.FASFIDK.V (51) K.AQYEEIAQR.S (7) R.QNLEPLFEQYINNLR.R (48)	0.00032 11 0.0005	71	5	3 (2)
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	112	60315	35	K.FASFIDK.V (51) K.AQYEEIAQR.S (7) R.QNLEPLFEQYINNLR.R (48)	0.00032 11 0.0005	71	5	3 (2)
	Keratin, type II cytoskeletal 5	K2C5_HUMAN	P13647	80	62568	26	K.FASFIDK.V (51) K.AQYEEIANR.S (34) K.YEELQQTAGR.H (27) R.QNLEPLFEQYINNLR.R (48)	0.00032 0.021 0.088 0.0005	78	6	4 (3)
9	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	78	49900	37	R.LKYENELALR.Q (31) R.LQSSSASYGGGFGGGSCQLGGGR.G (72)	0.059 1.4e-06	75	7	2 (2)
	Keratin, type II cytoskeletal 6A	K2C6A_HUMAN	P02538	64	60293	31	K.FASFIDK.V (10) R.QNLEPLFEQYINNLR.R (90)	3.7 6.7e-08	90	3	2 (1)
	Keratin, type II cytoskeletal 6 C	K2C6C_HUMAN	P48668	64	60273	32	K.FASFIDK.V (10) R.QNLEPLFEQYINNLR.R (90)	3.7 6.7e-08	90	3	2 (1)
	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	P04259	64	60315	32	K.FASFIDK.V (10) R.QNLEPLFEQYINNLR.R (90)	3.7/ 6.7e-08	90	3	2 (1)
10	Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	122	51872	41	K.IRDWYQR.Q (28) K.NHEEEMNALR.G (57) K.TRLEQEIATYR.R (56) R.GQVGGDVNVEMDAAPGVDLSR.I (96)	0.086 7.4e-05 0.00011 6.5e-09	158	10	4 (3)
	Keratin, type I cytoskeletal 13	K1C13_HUMAN	P13646	105	49900	34	K.IRDWHLK.Q (12) R.LKYENELALR.Q (48) K.TRLEQEIATYR.S (56)	3 0.00073 0.00011	76	6	3 (2)

Abbreviations: SC—Sequence coverage, M—Matches (No. of peptides with $p \leq 0.05$). * Protein identities were obtained only in gel piece numbers 5 to 10 shown in Fig. 6C.

Sigma and Abcam anti-K18 antibodies (CY90) detected a band overlapping the position of actin Fig. 1S-A and B while LDK 18 antibody reacted more specifically to the spots above actin. No signal was seen in the position of K13 and this was confirmed as shown by the autographic profile obtained with K13 antibody (Fig. 1S-D). Actin specific antibody reacted with actin (Fig. 1S-E).

Fig. 2S-A to D show a close up of the autographs in Fig. 1S with the corresponding colloidal stained profiles. It is seen in A and B that the autographic signals obtained with antibody CY90 overlaps with actin and the spots immediately above it. The LDK 18 signal in C is also very close to actin with focus on the upper spots. Autographic signal for actin in D is directly on the intensely stained protein and there is no signal for K18 in the autograph.

MDA-MB-468 lysate (50 μg protein) was also mixed with 30 μg of enriched keratin preparation from sample 1740 CM. The blotted proteins were probed with anti-K13 which detected a band in the upper left hand corner Fig. 1S-F. The blot was stripped and reprobed with Sigma clone CY 90. The profile in Fig. 1S-G now shows that the clone CY90 from Sigma has detected the additional upper band at the position of K13 even in the presence of K18.

To identify the spots detected by CY90 in the MDA-MB-468 cells, 400 μg of total cell lysate proteins were resolved on 2DE and silver stained. Fig. 2S-E shows the silver stained profile and the stained spots which were subjected to in-gel digestion followed by mass spectrometry of the peptides generated. The MS results given in Table 3S showed the identity for actin in spot numbers 6 to 15, while K18 was detected in spot numbers 1, 3, 4 and 5.

As another example breast cancer tissue which is known to express K18 was processed for keratin enrichment, 2DE, western blotting and mass spectrometry as described above.

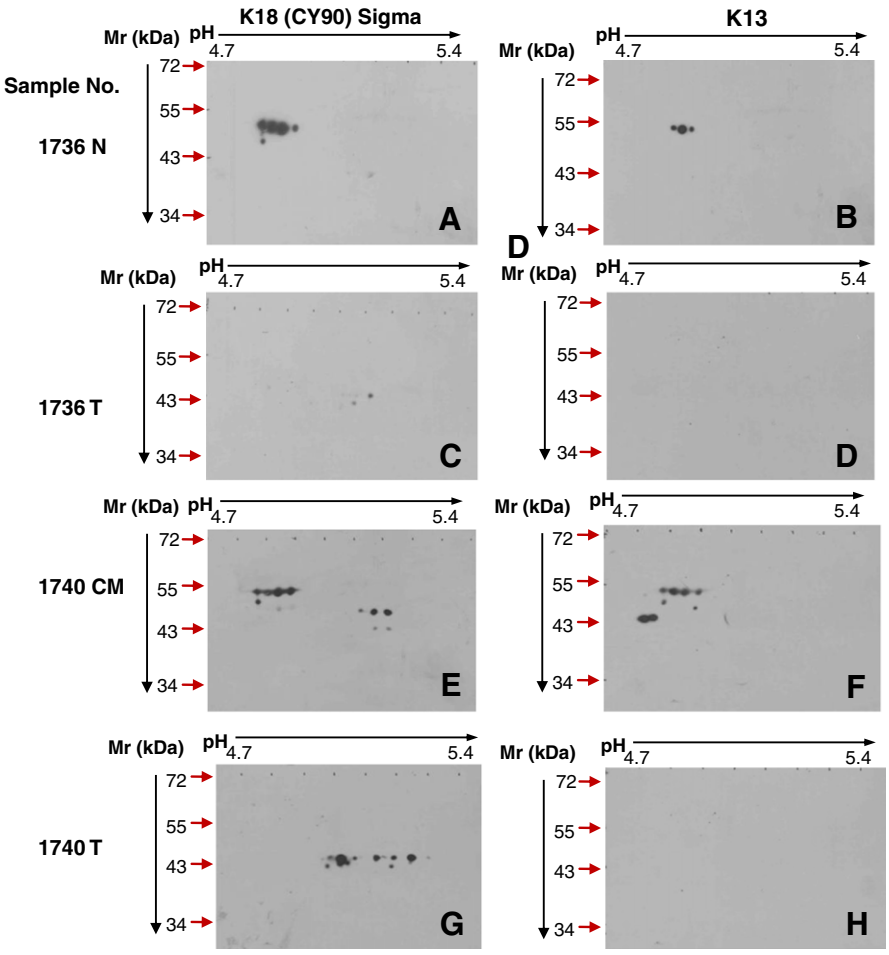

Fig. 7 – Autographic profiles of enriched keratin preparation from 1736 normal (N) and tumor (T) and 1740 cut margin (CM) and tumor (T) samples resolved by 2DE on a 17 cm IPG strip of pH range 4.7 to 5.9 and blots probed with antibodies to K18 (CY-90) and K13 antibodies. Autographs A, C, E, and G are for antibody to K18 and autographs B, D, F, and H are for antibody to K 13. The 2DE blots were cut beyond pH 5.4 to minimize antibody requirements.

Fig. 3S shows the profile of enriched keratin preparations (30 μg) resolved by 2DE and the transferred proteins probed with CY 90 from Sigma (Fig. 3S-A) and Abcam (Fig. 3S-B). It is seen that the antibodies detect a band to the left of actin. A third blot was probed with anti-K18, LDK 18. Fig. 3S-C shows that the antibody detects a spot on actin and one slightly above actin. Another blot was probed for K13 (Fig. 3S-D) and the same blot probed with antibody to actin after stripping (Fig. 3S-E). There is no signal for K13 in Fig. 3S-D, while the position for actin is clearly seen in Fig. 3S-E.

Fig. 4S A to D shows a close up of the autographs in Fig. 3S with the corresponding colloidal stained profile. It is seen that the autographic signals obtained with antibody CY90 are to the left of the intense actin protein band and there are few faint signals above actin shown by an arrow (a), The LDK 18 signal shows two spots, one on actin (a) and one above (K18). In the blot probed with anti-actin, the signal is directly on the intensely stained protein.

Fifty microgram of enriched keratins from the breast cancer tissue was mixed with 30 μg of enriched keratins from oral tissue sample 1740 CM and the same resolved by 2DE. One blot was probed with anti-K13, Fig. 3S-F. The same blot was stripped and reprobed with anti-K18 clone CY90 from Sigma Fig. 3S-G. Fig. 3S-F shows that anti-K13 detects the upper band which we know is K13 by mass spectrometry. Anti-K18 (CY90) from Sigma detects the upper band (K13) and shows a signal which spans from the left of the intense colloidal band for actin and across it encompassing K18 as seen in Figs. 3S-A and 4S-A. CY90 therefore detects both K13 and K18 when they are present.

The antibody staining pattern was verified by mass spectrometry. Fig. 4S-E shows a silver stained profile of enriched keratins (400 μg) from the breast cancer tissue and 400 μg keratins from oral tissue 1740 T (Fig. 4S-F). It is seen in Fig. 4S-E that there are clearly defined protein spots above actin very similar to those seen in MDA-MB-468 in Fig. 2S-E. These spots are not seen in the oral tissue gel in Fig. 4S-F.

The spots corresponding to the position of K18 and actin from these silver stained gels were processed for PMF and the identities obtained by mass spectrometry are shown in Table 3S. It is seen that the breast tissue contains K18 in spot 4.

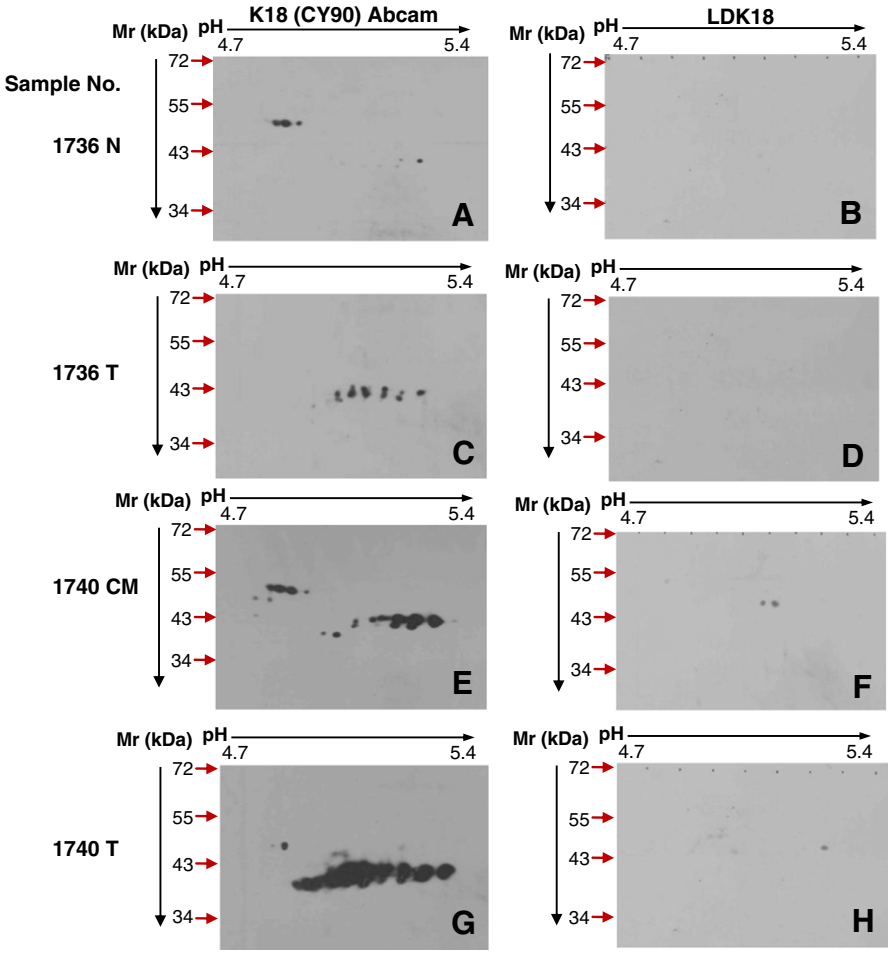

Fig. 8 – Autographic profiles of enriched keratin preparation from 1736 normal (N) and tumor (T) and 1740 cut margin (CM) and tumor (T) samples resolved by 2DE on a 17 cm IPG strip of pH range 4.7 to 5.9 and blots probed with antibodies to K18 (CY-90 from Abcam) and LDK 18 from Abcam. Autographs A, C, E and G are for antibody to K18, CY 90 from abcam and autographs B, D, F and H are for antibody to K 18, LDK18 from abcam. The 2DE blots were cut beyond pH 5.4 to minimize antibody requirements.

3.9. *Immunofluorescence and immunohistochemistry*

To further validate the reactivity of clone CY90 as seen above with 2DE and western blotting, MDA-MB-468 cells were stained with clone CY90 from Sigma and Abcam, anti-K13 and anti-actin (Fig. 5S-A to G) The immunofluorescence staining procedure is given in the supplementary. As these cells are known to express K18, clear staining profile of this molecule is seen Fig. 5S-A, B and C. K13 is absent in these cells (Fig. 5S-D) and anti-actin shows a distinct profile from that for K18 (Fig. 5S-E).

The blocks of the oral tissue samples processed for 2DE of enriched keratin preparations were sectioned and the sections stained with the antibodies to keratins 18, keratin13, β- actin (Materials and methods in supplementary). Fig. 6S shows the stained profiles of the CM and T tissues for samples 1736 and 1740. It is seen in Fig. 6S-A and -B that in the oral tissue samples CY 90 clone shows a signal for K18, while LDK 18 does not and anti-K13 lights up only in the histologically normal cut margin samples, indicating that clone CY90 is giving a non-specific signal.

Keratin 18 is known to be present in breast tissues. Fig. 7S shows the IHC stained profiles of three tissues. It is clearly seen that CY 90 from both the sources and LDK 18 stain the breast cancer tissue, while there is no signal for K13. The staining pattern for β actin is quite distinct from that of the K18 signal.

4. Discussion

To investigate the status of expression of keratin 18 in squamous cell carcinoma of buccal mucosa, protein lysates of micro dissected epithelial cells and enriched preparations of keratin were analyzed by immunostaining after resolution by 1DE and 2DE. The gel spots corresponding to the autographic signals were then analyzed by mass spectrometry. It is apparent from this study that the antibody K18 (CY-90) against K18 from Sigma as well as Abcam is identifying K13. It is also to be noted that the sensitivity of ECL plus detection system used for immunodetection is in the picomolar range and that of the MALDI-TOF machine is in the femtomolar range and therefore the chance of missing K18 if present is minimal, by mass spectrometry. The BLAST analysis for the K18 epitope of 312–356 amino-acid residues against which the K18 (CY-90) antibody is raised, showed 42% identity and

78% positivity with K13. Therefore, in the absence of K18 protein in the sample, the antibody may detect other keratins such as K13 resulting in misinterpretation. This antibody has been used in some investigations with oral cancer [24,28,49,50]. The results in the study were further confirmed using breast cancer cells and breast cancer tissue which express keratin 18. Clone CY 90 gave a signal with actin in both the cells and tissue preparations. It also detected other keratins adjacent to actin in the enriched keratin preparations from breast cancer tissue. The immunohistochemistry staining pattern on the tissue sections therefore needs to be evaluated with caution. Observations in this study therefore emphasize the necessity to specifically verify the antigen recognized by the antibody using mass spectrometry. Keratins are abundant proteins in epithelial cells. They have conserved rod domains and show several epitope similarities with other members of the family. It is likely that specific monoclonal/polyclonal antibodies to other keratins may also show cross reactivity with family member proteins and caution is imperative in interpretation.

5. Conclusion

Keratin 18, which was identified by probing 1DE/2DE blots with keratin specific monoclonal antibody to K18 (CY-90 clone) from Sigma, was not detected by mass spectrometry. Instead, mass spectrometry revealed its identity as K13 suggesting that anti-K18 (CY-90 clone) cross reacts with keratin 13 and actin. This study emphasizes the necessity to specifically verify the antigen recognized by the antibody by mass spectrometry. It is therefore necessary to be cautious in our conclusions arising from antibody based techniques such as immunohistochemistry, immunoblotting, etc. wherein the antibody exhibits cross reactivity to another related molecule.

Supplementary materials related to this article can be found online at doi:10.1016/j.jprot.2012.02.016.

Author contribution

The research work was conceptualized by SZ. The appropriate tissue samples were identified and provided by AD and PC following surgical resection. The histopathological analysis was done by AP and SK. The experimental work with micro dissected tissue samples and immunohistochemistry was done by SM. RG contributed to the analysis and interpretation of the data. AF performed the experimental work with enriched keratins and wrote the manuscript under the guidance of SZ.

Conflicts of interest statement

All authors state no conflicts of interest.

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Proteomic profile of keratins in cancer of the gingivo buccal complex: Consolidating insights for clinical applications

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ABSTRACT

Keratins play a major role in several cellular functions. Each tissue type expresses a specific set of keratins. The immense potential of keratins as diagnostic and prognostic markers for different cancers is emerging. Oral cancer is the fifteenth most common cancer worldwide. However, comprehensive information on the profile of keratins in the oral cavity is not available. Several independent reports have identified keratins using antibody based techniques which have pitfalls due to the cross reactivity of the antibodies to this set of very homologous proteins. A few recent proteomic studies have reported the identification of keratins in head and neck cancer. Majority of the studies have used tissues from the head and neck region without specifying subsites. This study reports the analysis of enriched preparations of keratins from cancer of the gingivo buccal complex (GBC) using MS, 2DE, WB, silver staining of 2DE gels and IHC. Our study reveals the absence of K4 and K13 and presence of K14, K16, and K17, in cancers of the GBC and combination of these expression patterns in the cut margins. This report also shows that K13 is glycosylated. This well characterized profile of keratins may have potential to be used in clinics.

Biological significance

In recent years the immense potential of keratins as diagnostic and prognostic markers for different cancers is emerging. However, comprehensive information on the profile of keratins in the oral cavity is not available. Several independent reports have identified keratins using only antibody based techniques which have pitfalls due to the cross reactivity of the antibodies to this set of very homologous proteins. This study reports the analysis of enriched preparations of keratins from a subsite of the oral cavity, the gingivo buccal complex (GBC) using mass spectrometry, 2DE, western blotting, silver staining of 2DE gels and IHC. The proteomic analysis shows the absence of K4 and K13 and presence of K14,

Abbreviations: IHC, immunohistochemistry; IF, immunofluorescence; SCC, squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; OSF, oral submucous fibrosis; GBC, gingivo buccal complex; N, adjacent normal; CM, cut margin; T, tumor; 1DE, 1-dimensional polyacrylamide gel electrophoresis; 2DE, 2-dimensional polyacrylamide gel electrophoresis; MS, mass spectrometry; H&E, haematoxylin and eosin staining; WB, western blotting; PVDF, polyvinylidene fluoride; IPG, immobilised pH gradient; ACN, acetonitrile; TFA, trifluoroacetic acid; ECL, enhanced chemiluminescence.

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K16, and K17 in cancers of the GBC and combination of these expression patterns in the cut margins. This well characterized profile of keratins from the gingivo buccal complex provides defined markers which may have potential to be used in the clinics.

1. Introduction

Oral cancer is the fifteenth most common cancer worldwide and the second largest malignancy in males in India [1]. The major factors responsible for incidence of oral cancer are smoking and/or chewing of tobacco and heavy alcohol consumption. Keratins which are intermediate filament proteins are receiving intense attention in recent times due to their multifarious functions other than the role of providing structural stability to a cell. Keratins are reported to be involved in regulating protein synthesis and epithelial cell growth [2] signalling [3], apoptosis [4], organelle transport [5–7] cell motility [8,9], cell proliferation [10] and stress [11,12]. There are 54 genes coding for type I (28) and type II (26) keratins which are expressed predominantly in epithelial cells [13]. The epithelial keratins form obligate heterodimers of one acidic (type I) and one basic (type II) keratin and exhibit tissue specific expression. Several recent, comprehensive and in-depth reviews have described the expression and role of specific keratins in different tissues [14–19]. None of these reviews provide much information on the expression profile for the keratins in the head and neck region.

There are several independent reports on keratins from the head and neck region. Majority of these studies have used antibody based techniques such as immunohistochemistry, immunofluorescence and immunoblotting. A few proteomic and microarray studies show the presence/expression of keratins in tissues from the head and neck region.

1.1. *Keratin expression profile by immunohistochemistry*

K1:K10; K4:K13; K5:K14; K6:K15, K16, K17; K7:K19; and K8:K18 form obligate pairs in epithelial cells [17,18]. Analysis of the studies shows that in several of these studies the keratins have been reported using in house generated antibody clones (e.g. IC7, RCK107, LHK15), pan keratin antibodies (e.g. KL1, AE2) and those that recognize two keratins (e.g. DE-K13, CK8.16, D5/16B4) thereby leaving an ambiguity about their expression. The immunogens used for raising the antibodies are either whole tissue/epithelial cell lysates or cytoskelatal preparations, e.g. for 34βB4, DE-K10, RKSE60, CK8.6, 35βH11. Few antibodies are prepared using recombinant proteins, purified keratin or synthetic peptides specific to the keratins e.g. KS13.1, XM26. The specificity of several of the antibodies used is evaluated by 1DE-WB and is reported to recognize a band at a particular molecular weight which is attributed to a keratin. Some examples are DE-K10, RKSE60, CK8.60, C6B10, KS1A3 and 35βH11. Very few of the studies provide details of antigen retrieval steps which are important for optimal reactivity of the antibodies. In addition to the issue of antibodies most of the studies have analysed keratins in tissues from different head and neck sites without due attention for subsites.

Keratin 1 has been shown to be expressed in normal oral tissues [20–22]. Ranganathan et al. [21] have used the monoclonal antibody 34βB4 and reported the down regulation of K1 from normal tissues (40%), oral submucous fibrosis (OSF) (32%) to oral cancer (20%) in cancer of the buccal mucosa. Lalli et al. [23] have used an in house antibody, LHK1 and shown its over expression specifically in the suprabasal layer in oral submucous fibrosis of tissues from the buccal mucosa, tongue and gingiva. Its pair, Keratin 10 is over expressed in OSF in the suprabasal layer in all sites taken together [23], and hyperkeratotic lesions of the oral cavity (from the gingival, palate, tongue and buccal mucosa) [24]. It is reported to be same in normal and cancer tissues from keratinizing and non-keratinizing sites of the oral cavity [25]. The antibodies used for the study were DE-K10 [23] RKSE60 [23,24] and CK8.60 [25] respectively. CK8.60 recognizes K10 and K11. Keratin 4 expression is observed in normal, OSF and oral cancer tissues to nearly the same extent using antibody C6B10 [21,23]. Its pair K13 is reported to be expressed in normal and OSF tissues using the antibodies KS1A3 [20,26], KS13.1 [22], DE-K13 [27] and IC7 [23] respectively. Expression of K13 is down regulated in lesions of oral mucosa as shown using clone 2D7 [24], and the oral cancer with clones KS1A3 and DE-K13 [26,27] and with an antibody for CK13 from Dako [28] while it is reported to be same as normal using the antibody CK8.12 which recognizes K13/K16 [25]. On the contrary, Xu et al. [29] have reported its expression in normal, dysplastic as well as in HNSCC to the same extent using monoclonal antibody KS13.1 which is reported to recognize a cytokeratin at 54 kDa. Keratin 5 expression has been reported in normal and oral cancer tissues [20–22,30] using antibodies KL1, XM26 and D5/16B4. Keratin 14 is over-expressed in oral cancer [21,25,26,31] as shown using clones CKB1 [21,25], RCK107 [31] and an antibody to synthetic peptides which recognizes mouse proteins of 59 kDa and 67 kDa [26]. Keratin 15 expression is less studied by IHC and Lalli et al. [23] have shown its expression in OSF tissue samples using an in-house generated antibody, LHK15. Keratin 5/6 is expressed in normal tissues with a pan keratin antibody KL1 [20] and clone D5/16B4 [22]. K6 is over expressed in the basal and suprabasal layers in OSF and suprabasal layer of normal tissue as shown with clone LHK6B [23]. Comparison of the status of K6 in normal and oral cancer tissue is not very well documented by IHC. Keratin 16 expression is reported in normal tissue with a pan keratin clone KL1 [20] and an in-house clone LL025 [23]. It is reported to be expressed to the same extent in OSF samples and normal tissues [23]. Kannan et al. [25] have reported its presence in normal oral tissue, leukoplakia and carcinoma to the same extent using CK8.12 which recognizes K13/16. Keratin 17 is over expressed in OSF [23] and oral cancer [27,32,33] as seen using clone E3 for K17. The simple epithelium associated Keratin 8 has been shown to be aberrantly expressed in oral cancer in several investigations [20,21,29,34–37] with the antibodies 35βH11, M20, and those that recognize K8 and K18 i.e. CAM5.2 and CK8/18. Similarly its pair K18 is reported to be aberrantly expressed in oral cancer using clones CY90 [21], LE61 and RCK106 [34] and CK8/18 [35]. Of these, CY90 clone exhibits non-specificity [38], CK8/18 recognize two proteins and LE61

Table 1 – Details of primary and secondary antibodies used for western blotting and IHC.

Sr. no.	Antibody	Clonality	Clone	Catalogue no.	Company	Dilution for WB (1° Ab)	Dilution for WB (2° Ab)	Dilution for IHC (1° Ab)
1.	Cytokeratin 4	Rabbit MAb	EP1599Y	1994-1	Epitomics (USA)	1:1000	1:5000 [a]	1:1000
2.	Cytokeratin 13	Rabbit polyclonalAb	Polyclonal	Ab58744	Abcam (UK)	1:2000	1:5000 [a]	1:300
3.	Cytokeratin 14	Rabbit MAb	EP1612Y	2001-1	Epitomics (USA)	1:20,000	1:5000 [a]	1:3500
4.	Cytokeratin 16	Rabbit MAb	EP1615Y	2141-1	Epitomics (USA)	1:10,000	1:5000 [a]	1:2500
5.	Cytokeratin 17	Rabbit MAb	EPR1624Y	1960-1	Epitomics (USA)	1:10,000	1:5000 [a]	1:1500
6.	O-linked N-acetyl glucosamine	Mouse MAb	RL2	MA1-072	Affinity BioReagents (USA)	1:3000	1:2000 [b]	

WB—western blotting; 1°—primary antibody; 2°—secondary antibody.
As an isotype control, primary monoclonal antibody was replaced by rabbit IgG from Epitomics (Cat. ISO-3855) at 1:1000 dilution for IHC.
[a] Secondary antibody used is anti-rabbit IgG, HRP conjugated secondary antibody from donkey (Cat. no. NA934V), Amersham Biosciences (GE Healthcare), UK.
[b] Secondary antibody used is anti-mouse IgG, HRP conjugated secondary antibody from sheep (Cat. no. NA931V), Amersham Biosciences (GE Healthcare), UK.

and RCK106 were in-house antibodies. Balm et al. [33] have used an in house monoclonal antibody DE-K18 and verified the data with monoclonal antibodies, KsB17.2 and RGE 53 and shown that majority of the cancer tissues from larynx and oropharynx are positive for CK18 and the keratin is present to a lower extent in normal tissues from the same subsites, while sporadic positivity is seen for SCC of the oral cavity. Xu et al. [29] have used the antibody BA17 and shown over expression of K19 from normal (13%) to dysplastic (71%) to head & neck squamous cell carcinoma (HNSCC) (82%). Keratin 19 is also reported to be over expressed from leukoplakia without dysplasia to oral squamous cell carcinoma (OSCC) [35]. However, in this study the antibody used was not specified. Crowe et al. [39] have reported the down regulation of K19 in OSCC cell lines with increasing invasive potential using anti-human K19 primary antibody from Sigma and details were not given. Keratin 19 expression is reported in inflamed normal oral tissues while in both hyperplastic and hyperkeratotic lesions it is absent as seen with antibody RCK108. [24].

The other keratins less reported in oral epithelial cells are K2, K7 and K9. Gires et al. [20] and Mai et al. [22] have used pan keratin antibodies KL1 and AE2 and seen positive reactions. Keratin 7 expression is reported in the study by Gires et al. [20] wherein it is expressed in normal tissues using the pan keratin

A

Keratins	Normal/Cut Margin (n=12)			Tumor (n=13)		
	PMF	PMF-MS/MS	%	PMF	PMF-MS/MS	%
K1	2	7	75	1	6	53
K2	2	1	25	4	0	30
K4	0	4	33	0	0	0
K5	0	3	25	2	4	46
K6A	0	10	83	0	13	100
K6B	2	6	66	2	8	76
K6C	2	7	75	1	11	92
K10	1	2	25	3	1	30
K13	1	9	83	0	0	0
K14	2	2	33	1	8	69
K16	1	2	25	1	9	77
K17	1	0	8	5	5	77

B

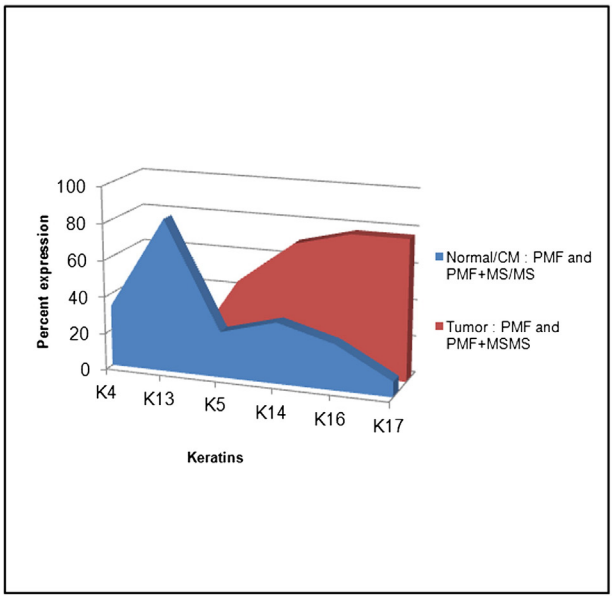

Fig. 1 – Keratins in N/CM and T tissues as identified by mass spectrometry. (A) Table shows the percentage of different keratins present in normal/cut margin (n = 12) and tumor (n = 13) samples as identified by mass spectrometry. The keratins identified in each of the samples are given in Supplementary Fig. 2S. The numbers in the PMF and PMF–MS/MS columns indicate the number of samples from which the data was obtained. (B) Graphical representation of the major keratins K4, K13, K14, K16 and K17 present in normal/cut margin and tumor samples. K5 has been shown as a keratin which is near equally identified in normal and transformed tissues. Detailed mass spectrometry analysis is given in Table 2S.

antibody KL1, while Su et al. [34] have reported no expression in normal tissues and weak expression in dysplastic and SCC of oral cavity using in-house antibodies RCK105 and LP 5K.

1.2. *Keratin expression by other methods*

Early studies by Vaidya et al. [40] using 2DE and western blotting with in-house generated rabbit polyclonal antibodies to total keratins have shown the presence of K4, K5, K13 and K14 in normal oral tissue and K1, K4, K14, K16, K17, and K18 in oral cancer tissues. Lowered expression of K5 was reported in leukoplakia, OSF, SCC of tongue and SCC of oral mucosa, and expression of K2, K4, K14 and K19 along with aberrant expression of K8/18 and reduced expression of K13 were also reported in oral cancer [40–43]. There are a few reports which use global proteomic analyses of tissues from the head and neck region and show the differential expression or presence of keratins K1, K2, K4, K5, K6, K8, K9, K10, K13, K14, K15, K16, K17, K18 and K19 [44–48].

Genomic studies have shown the expression of K4, K13 [49] and K15 in normal mucosa and its down regulation in tumor [50]. Odani et al. has reported over expression of K2 and K10 in oral leukoplakia as compared to SCC [51]. Toyoshima et al. have shown the over expression of K17 in OSCC as compared to normal mucosa [52].

Most of the studies have analysed keratins in tissues from the head and neck without due attention to subsites. The oral subsite, buccal mucosa is biologically and molecularly different from the tongue [53–60] which is also apparent from the K13 expression profile in tongue tissues in which K13 is over expressed in tumor tissues [48]. Further, as the focus of the investigations was not on keratins some of the proteomic studies [61–63] made tissue lysates in buffers without urea in which keratins are not soluble. Some of the studies reporting 2DE–mass spectrometry data have focussed on regions below 50 kDa thereby overlooking the keratins [44,54,64].

In a recent study from our laboratory we have shown the pitfalls in using antibodies to very homologous proteins such as the keratins and the need to use mass spectrometry to verify their identities [38]. This study was therefore undertaken to comprehensively catalogue keratins in normal and tumor tissues from the gingivo buccal complex using the MS-based approach and multiple techniques. An enriched preparation of keratins from each of the tissue samples was

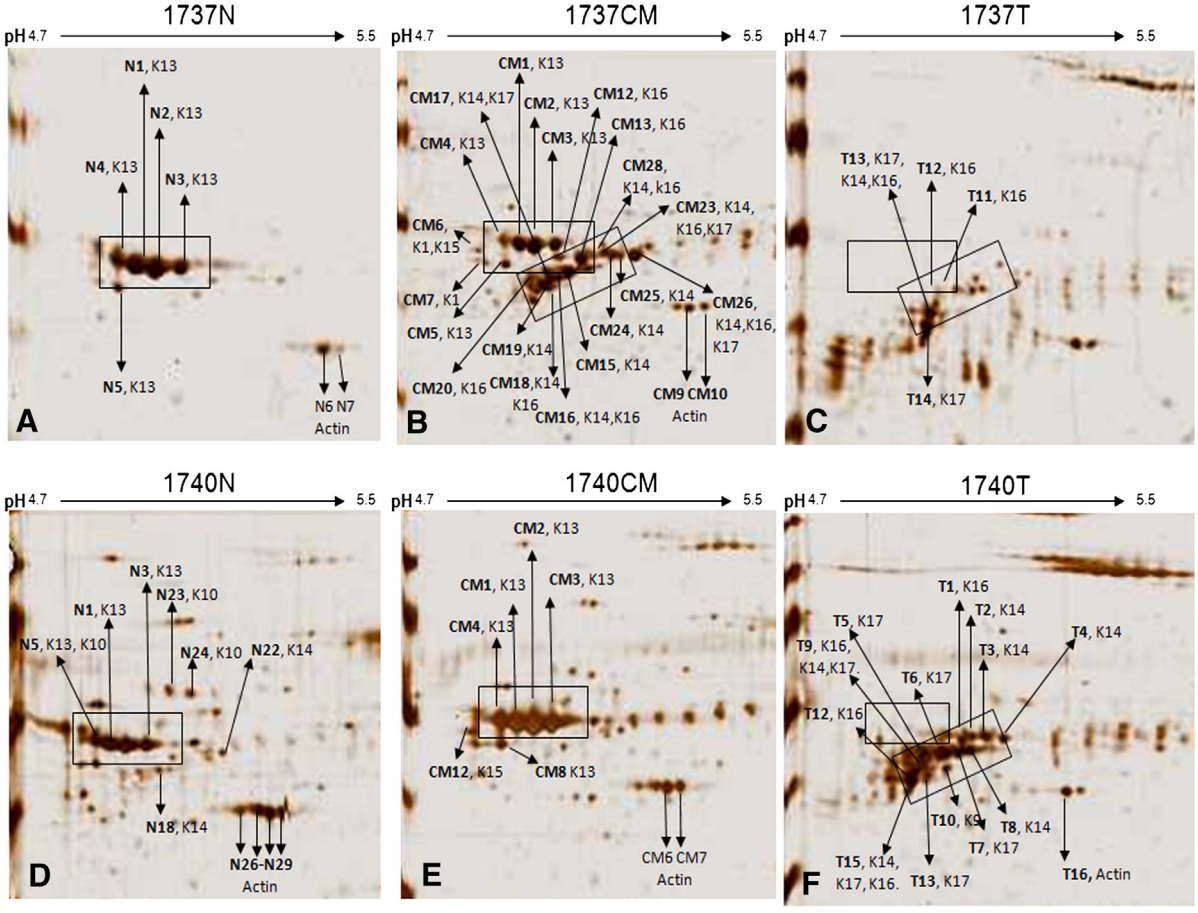

Fig. 2 – 2DE-silver stained gel patterns for the keratins showing the position of the different keratins identified by mass spectrometry. Subpanels A, B and C are the profiles for sample 1737 N, CM and T respectively. Subpanels D, E and F show the profiles for sample 1740 N, CM and T respectively. The pH range for IEF is shown above the gel profile. The spot numbers (NX), (CMX) and (TX) are followed by name of the keratin as identified by mass spectrometry. The details of the mass spectrometry data are given in Supplementary Table 3S.

used for the analysis so as to be able to identify the keratins even if they are present in low amounts. We report the presence of K4 and K13 in normal tissues and that of K14, K16 and K17 in the tumor tissues using 1DE followed by mass spectrometry. This data is further confirmed by 2DE silver staining followed by MS and western blotting. Analysis of the enriched keratin preparation by 2DE and silver staining reveals distinct patterns between the normal and tumor tissues and from the cut margins (CM). IHC shows the locations of the major keratins K4, K13, K14, K16 and K17 and their differential expression in the tumor tissues. The data also shows that K13 is glycosylated. In summary, the study provides a comprehensive and well characterized profile of the keratins which may have potential in clinics.

2. Material and methods

2.1. Tissues

This study was approved by the Tata Memorial Centre, Hospital Ethics committee. Adjacent normal (N), cut margin (CM) and tumor (T) tissues were collected from patients with cancer of the gingivo buccal complex undergoing surgery as part of their treatment. Tissues were quick frozen in liquid nitrogen till further use. The histology of the tissues was confirmed by the pathologist. Fifteen sets of tumor (T) tissue, adjacent normal (N) tissue and the cut margin (CM) when available were collected for the analysis in this study. The

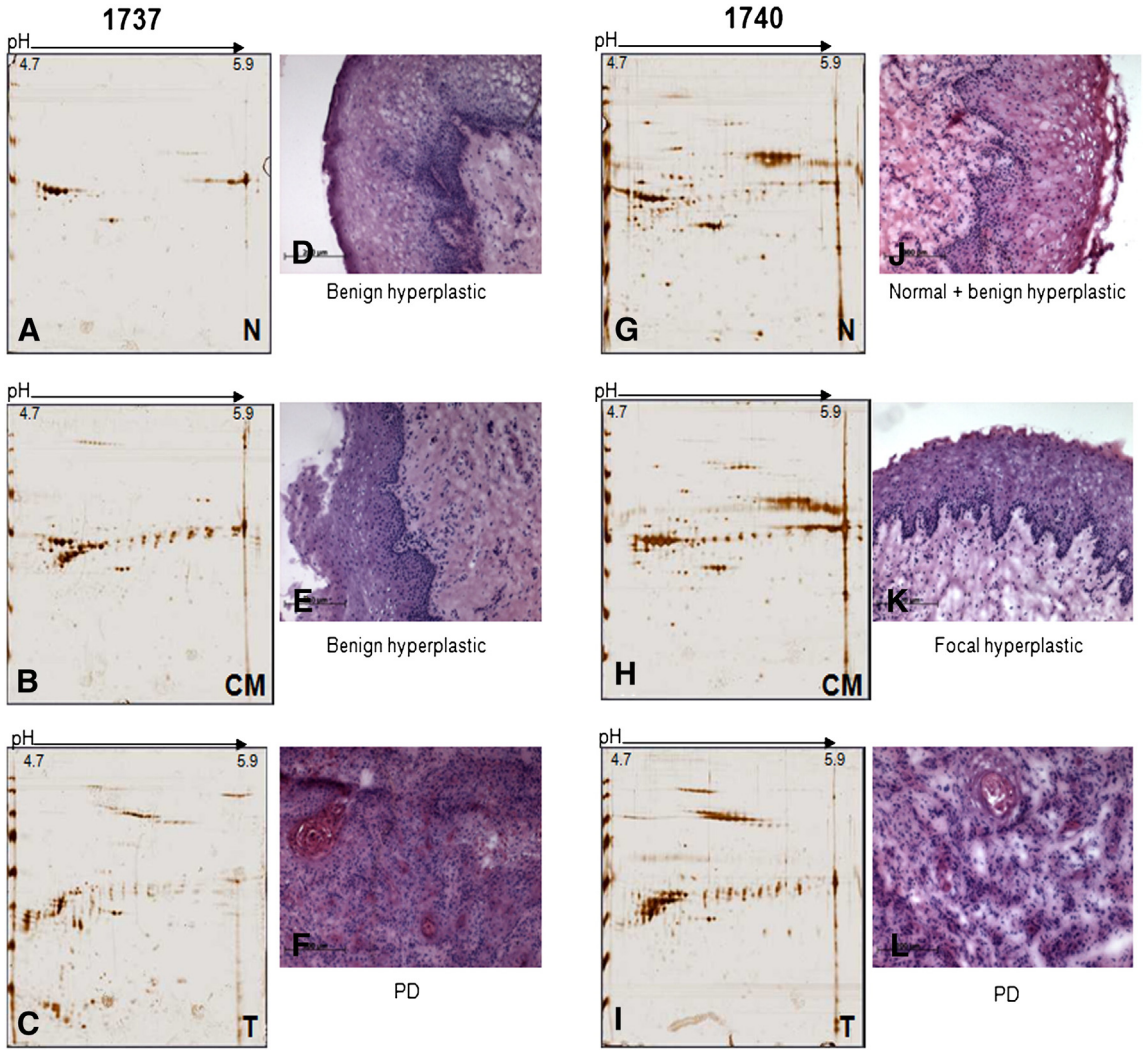

Fig. 3 – Comparison of the 2DE-silver stained pattern with the H&E stained sections for samples 1737 (N, CM and T) and 1740 (N, CM and T). Subpanels (A, B, C, G, H and I) show the silver stained gel profile of enriched keratin preparations resolved by 2DE. The pH range for IEF is indicated above each gel profile. The sample number is written above each column of the gel images. Normal (N), cut margin (CM) and tumor (T) indicate the tissue from that sample number which was used for the analysis. The images of the H&E stained section of the tissue used (D, E, F, J, K, L) are placed on the right side and adjacent to the corresponding gel image. The histological status of the tissue is given below each H&E stained section. PD; poorly differentiated.

histology of the stained sections was evaluated by the pathologist for the grade of differentiation and for marking the tumor/normal epithelial area using the World Health Organisation classification of tumors [65] and the TNM classification was according to the International Union against Cancer (UICC) [66]. Details of the samples are provided in the Supplementary Table 1S.

2.2. Reagents and antibodies

Tris–Cl, acrylamide, bis-acrylamide, glycine, trypsin, acetonitrile, iodoacetamide, urea, and thiourea were purchased from Sigma, USA. CHAPS and DTT were purchased from USB, USA. Trifluoroacetic acid (TFA) was a product of Applied Biosystems, UK. Silver nitrate and sodium bicarbonate were obtained from Fisher Scientific, India. Sodium thiosulfate and ammonium bicarbonate were purchased from SRL, India. Potassium ferricyanide was obtained from Qualigens, India. PVDF membrane was purchased from Millipore. Ampholines of pH 4.7 to 5.9 (Cat. no. 163-2097) and IPG strips pH 4.7 to 5.9, 17 cm (Cat. no. 163-2021) and pH 5 to 8, 17 cm (Cat. no. 163-2011) were purchased from Bio-Rad Laboratories, USA. Details of the primary and secondary antibodies used are given in Table 1. Anti-mouse IgG, HRP conjugated secondary antibody from sheep (Cat. no. NA931V), anti-rabbit IgG, HRP conjugated secondary antibody from donkey (Cat. no. NA934V) and ECL plus western blotting detection kits (RPN2132) were purchased from Amersham Biosciences (GE Healthcare), UK.

2.3. Preparation of enriched keratin fractions

Enriched keratin fractions were made from the collected tissues using essentially the protocol of Achtstaetter et al. [67]. The tissues were homogenized in different buffers containing detergents and high salt and then centrifuged to remove, cytosolic and membrane proteins sequentially. The

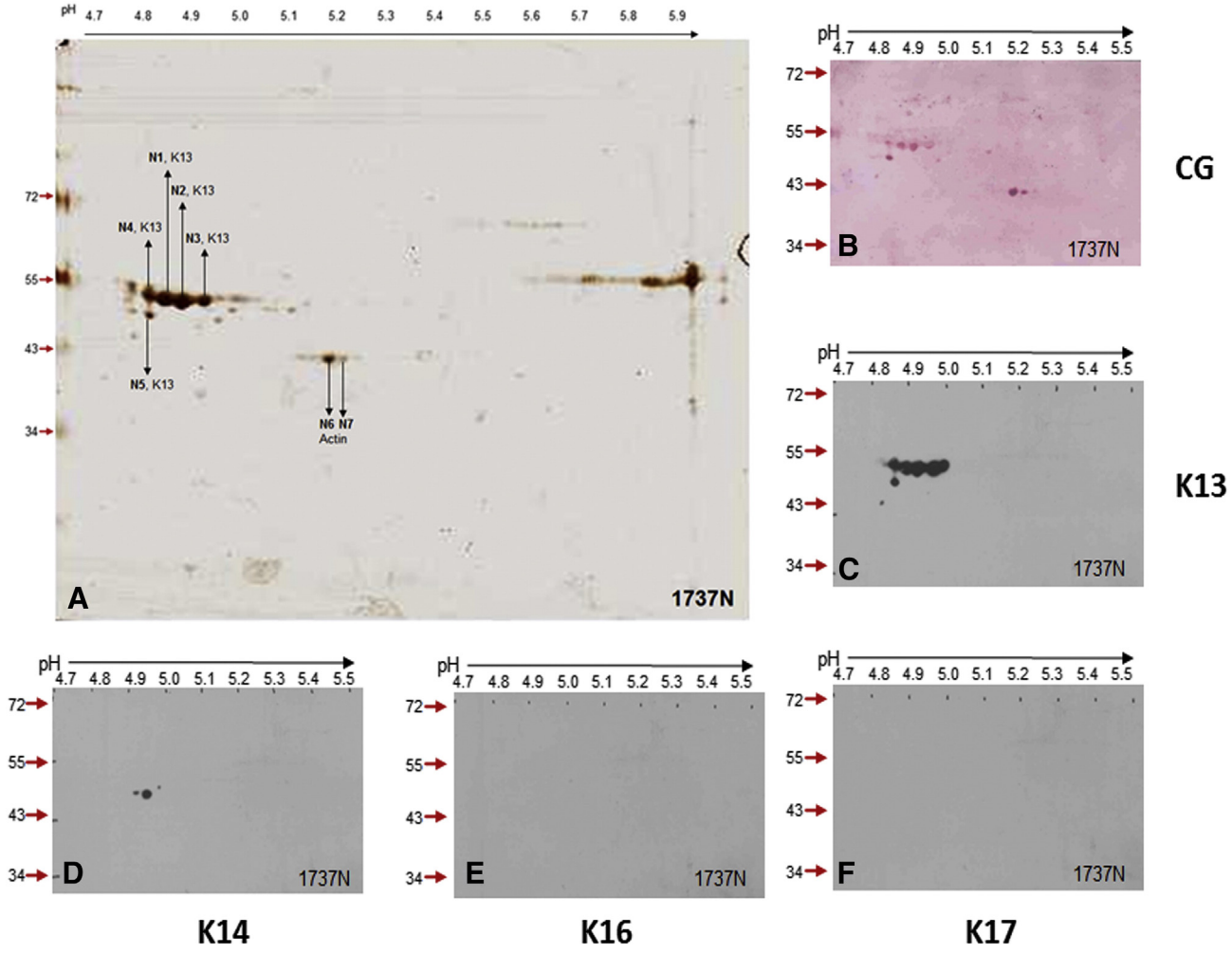

Fig. 4 – Validation of keratin identities obtained by 2DE–MS by western blotting using antibodies specific for K13, K14, K16 and K17 for sample 1737 N. Figures show the profiles obtained for sample 1737 N. (A) 2DE-silver stained gel profile and the location of the keratins. (B) Colloidal stained 2DE blot to show the position of the resolved proteins. (C) Autograph of the blot stained with antibody to K13, (D) K14, (E) K16, and (F) K17. The pH range used for IEF is given above the gels and blots. On the silver gel profile, the spot number (NX) and the identity of the keratin (KX) as per mass spectrometry data in Table 3S are shown. The 2DE blot was cut beyond pH 5.5 to minimize antibody requirements.

remaining pellet was further washed with high salt buffer to yield the enriched insoluble keratin preparation. Details are given in the Supplementary MM1S. Protein content of the enriched keratin preparations was measured as described by Peterson [68] or by the Bradford method using the BioRad protein assay Kit (Cat No.500-0006) after diluting the urea concentration in the samples to 0.8 M.

2.4. 1DE, 2DE, immunostaining and silver staining

The enriched keratin preparations were resolved by 1DE and 2DE essentially according to the Laemmli protocol [69]. For 1DE and 2DE studies, 3 μg and 30 μg of enriched keratins were used respectively. For 2DE, 17 cm length IPG strips (Bio-Rad, USA) of pH range 5–8 (for K4) and 4.7–5.9 (for other acidic keratins) were used. After 2DE, proteins from the gel were transferred on to PVDF membrane essentially according to Towbin [70]. For immunostaining, the blots were blocked in 3% BSA dissolved in 1 M Tris buffered saline (pH 7.6) with 0.01% Tween-20 (TBST). The position of the keratins was detected using keratin specific antibodies diluted in TBST with 1% BSA. The primary antibodies were detected using the HRP conjugated secondary anti-mouse antibody at 1:2000 dilution or the anti-rabbit antibody at 1:5000 dilution in 1% BSA dissolved in TBST. ECL plus western blotting detection system from GE Healthcare was used for detecting the bound antibodies. As required some of the gels were stained with silver essentially according to Oakley et al. [71]. K4 was separated on the 17 cm IPG strip of pH 5–8 using cup loading method which is used to resolve basic proteins [72].

2.5. Mass spectrometry

Proteins in the silver stained spots were identified by mass spectrometry after in-gel digestion with trypsin essentially according to Shevchenko et al. [73] and described in Fulzele

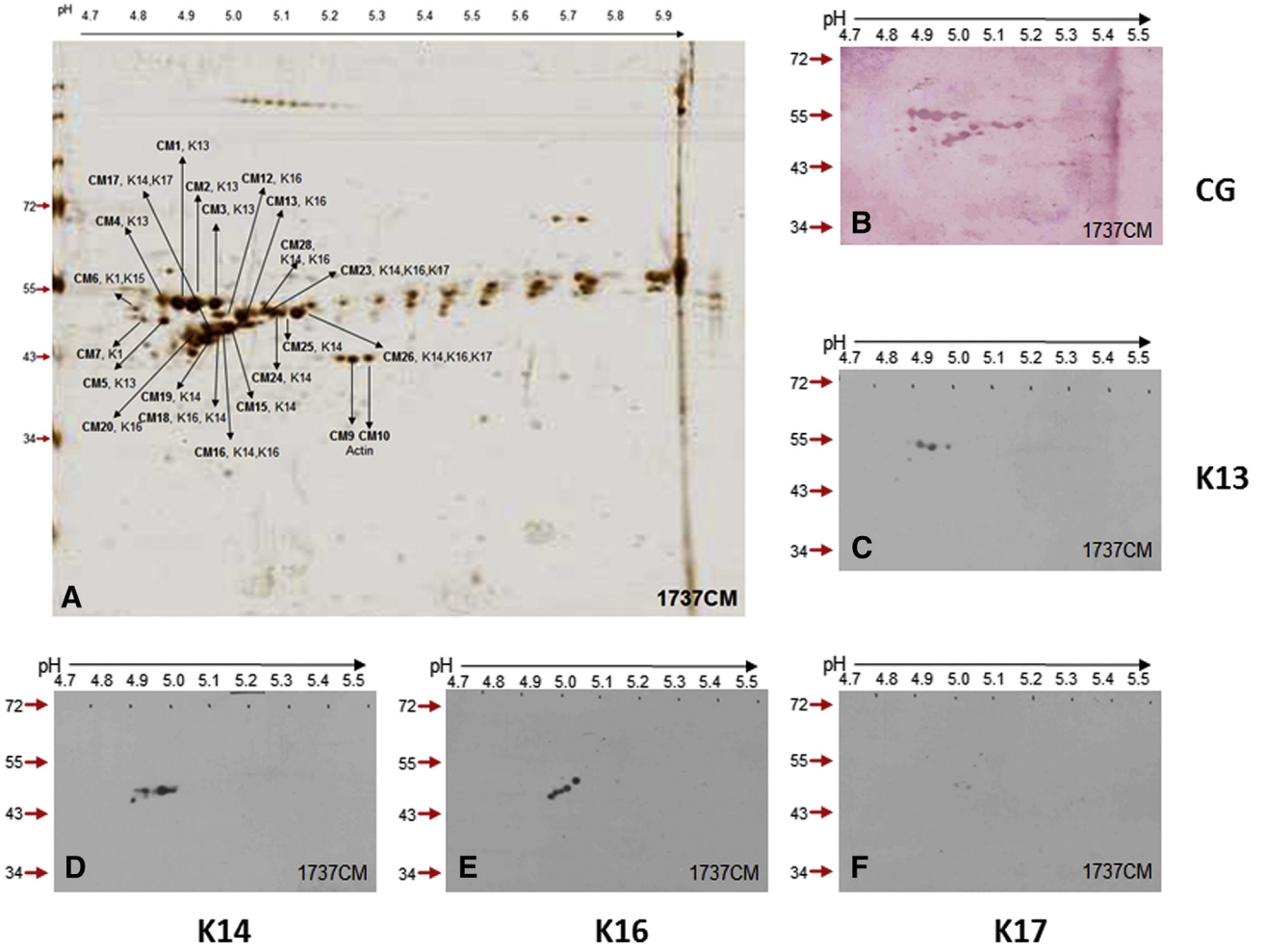

Fig. 5 – Validation of keratin identities obtained by 2DE–MS by western blotting using antibodies specific for K13, K14, K16 and K17 for sample 1737 CM. Figures show the profiles obtained for sample 1737 CM. (A) 2DE-silver stained gel profile and the location of the keratins. (B) Colloidal stained 2DE blot to show the position of the resolved proteins. (C) Autograph of the blot stained with antibody to K13, (D) K14, (E) K16, and (F) K17. The pH range used for IEF is given above the gels and blots. On the silver gel profile, the spot number (CMX) and the identity of the keratin (KX) as per mass spectrometry data in Table 3S are shown. The 2DE blot was cut beyond pH 5.5 to minimize antibody requirements.

et al. [38]. 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. Proteins in the 1DE resolved gel plugs were reduced using 10 mM DTT and then alkylated with 55 mM iodoacetamide. For protein in 2DE silver stained gels reduction and alkylation are done in the equilibration buffers used for 2DE [69]. The proteins in the plugs were then trypsinized overnight with 10 ng/μl trypsin in 25 mM ammonium bicarbonate (10 μl/plug) and the peptides were recovered by extraction with 40 μl of 50% acetonitrile (ACN) and 5% trifluoroacetic acid (TFA) (2–3 times). Tryptic protein digests were reconstituted in 10% ACN with 0.1% TFA solvent before subjecting them to mass spectrometry analysis.

Mass calibration was carried out using peptide mixture of five known peptides spanning mass range of 757–3147 m/z and error was kept to less than 10 ppm. Accelerating voltage of 25 kV was applied to the first TOF tube. The MS data was acquired in an automated manner using a solid state NdYAG laser at 337 nM. The resulting MS data was analysed using Flex analysis 3.0 (Brucker Daltonik, Germany) software and was acquired using Biotools 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 0.7 to 1 Da for MS/MS analysis. The 100 ppm tolerance permitted inclusion of all keratins and further 2DE–MS MS analysis and 2DE WB confirmed the identity of the keratin.

2.6. *Immunohistochemistry*

Tissues from normal, cut margin and tumor area were fixed in buffered formalin (10% formalin, 0.025 M sodium dihydrogen

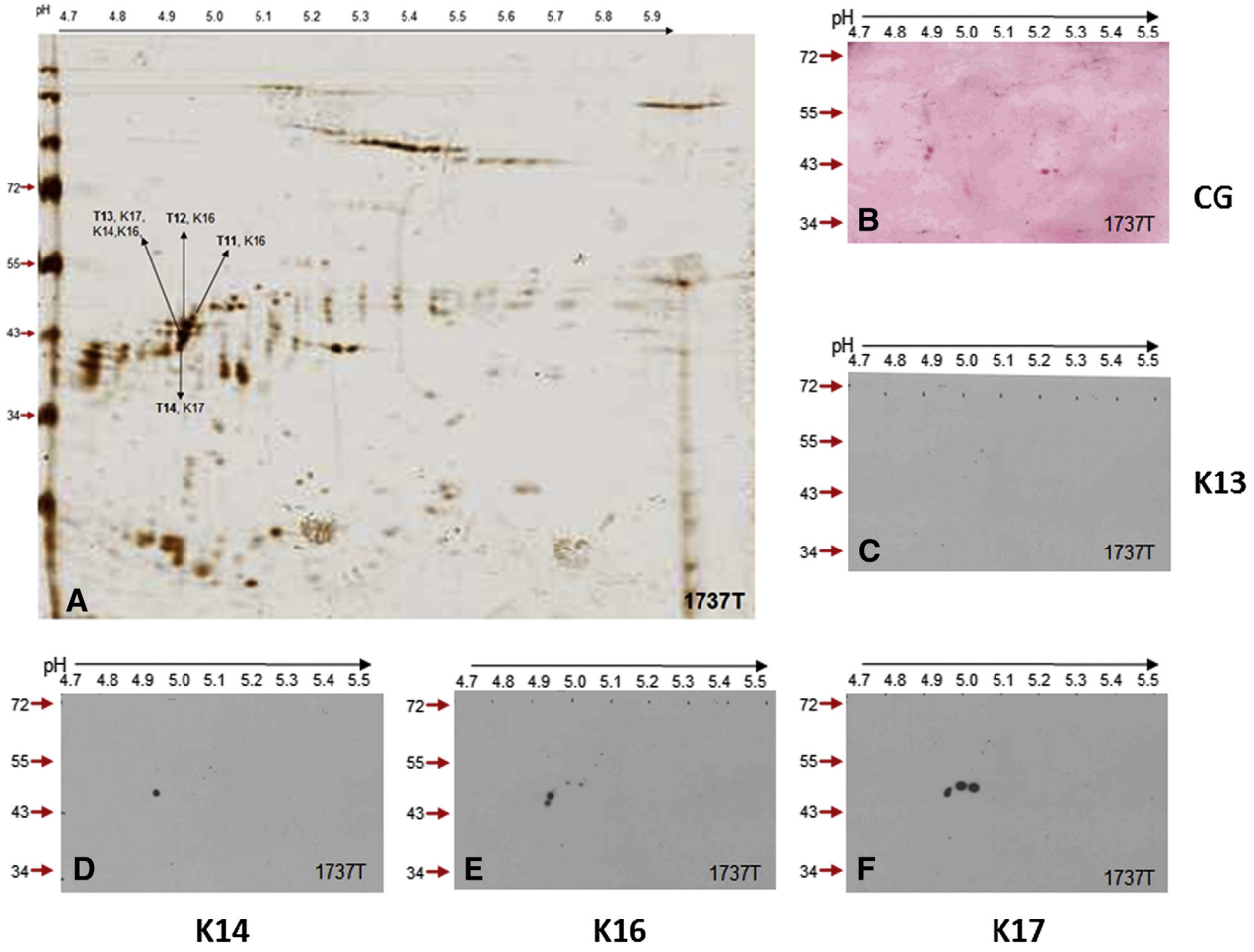

Fig. 6 – Validation of keratin identities obtained by 2DE–MS by western blotting using antibodies specific for K13, K14, K16 and K17 for sample 1737 T. Figures show the profiles obtained for sample 1737 T. (A) 2DE-silver stained gel profile and the location of the keratins. (B) Colloidal stained 2DE blot to show the position of the resolved proteins. (C) Autograph of the blot stained with antibody to K13, (D) K14, (E) K16, and (F) K17. The pH range used for IEF is given above the gels and blots. On the silver gel profile, the spot number (TX) and the identity of the keratin (KX) as per mass spectrometry data in Table 3S are shown. The 2DE blot was cut beyond pH 5.5 to minimize antibody requirements.

phosphate and 0.046 M disodium hydrogen phosphate in deionized water) overnight. Fixative was removed by keeping tissue in tap water followed by dehydration of tissues in 70% ethanol, 80% ethanol, 95% ethanol and 100% ethanol (5 min each). Tissues were kept in xylene for 30 min twice and then in mixture of xylene and paraffin (1:1) for 1 h. Tissues were paraffinized in fresh paraffin for 2 h twice and then embedded in paraffin blocks. Sections from these blocks were examined by the pathologist and areas identified for generating a tissue microarray. Cores of 1 mm size from the donor blocks were transferred in an array format on to the recipient block made of low melting paraffin wax (Gold Standard Peel-A-Way Micro Cut Paraffin from Polysciences, Inc., USA (Cat. 24198)). Five micron sections from the array were placed on poly-L-lysine coated slides. The sections were deparaffinised and rehydrated prior to endogenous blocking of peroxidase with 3% H_2O_2 in methanol. The antigenic sites were unmasked using heat induced antigen retrieval treatment using Tris EDTA buffer pH 9.0. The nonspecific antigenic sites were blocked using horse serum and the sections were then incubated at 4 °C overnight with specific antibodies for keratins K4, K13, K14, K16 and K17 individually with dilutions 1:1000, 1:300, 1:3500, 1:2500 and 1:1500 respectively. Each tissue array of the samples was incubated with antibody to one of the keratins and this was repeated for the keratins to be evaluated. The bound primary antibody was detected using Vectastain Universal ABC kit (PK-6200). The biotinylated secondary antibody was incubated at RT for 30 min, followed by incubation with avidin–biotin complex at RT for 1 h and the signal was developed using 0.025% diaminobenzidene with 0.03% H_2O_2. After every incubation the sections were washed with TBST (Tris buffered saline pH 7.6, 0.01% Tween 20). The stained sections were counterstained using Mayer's haematoxylin and finally mounted with DPX (mixture of

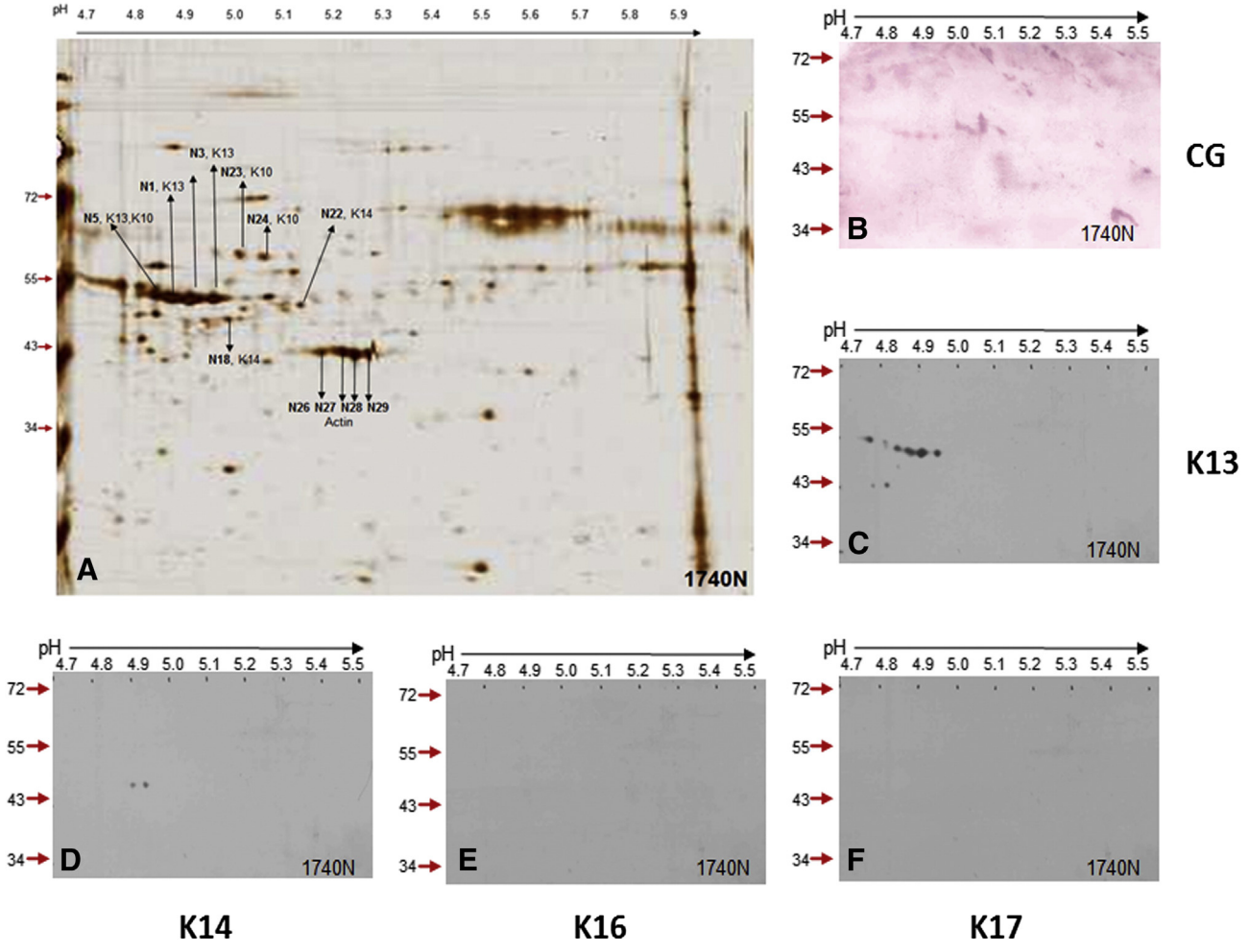

Fig. 7 – Validation of keratin identities obtained by 2DE–MS by western blotting using antibodies specific for K13, K14, K16 and K17 for sample 1740 N. Figures show the profiles obtained for sample 1740 N. (A) 2DE-silver stained gel profile and the location of the keratins. (B) Colloidal stained 2DE blot to show the position of the resolved proteins. (C) Autograph of the blot stained with antibody to K13, (D) K14, (E) K16, and (F) K17. The pH range used for IEF is given above the gels and blots. On the silver gel profile, the spot number (NX) and the identity of the keratin (KX) as per mass spectrometry data in Table 3S are shown. The 2DE blot was cut beyond pH 5.5 to minimize antibody requirements.

distyrene, a plasticizer, and xylene). In the negative control, primary monoclonal antibody was replaced by isotype control rabbit IgG from Epitomics (Cat. ISO-3855) at 1:1000 dilution. Images were captured using Zeiss AxioImager Z1 microscope.

3. Results

3.1. *Mass spectrometry analysis of enriched keratin preparations*

Enriched keratins from 12 normal (N)/cut margin (CM) and 13 tumor (T) tissue samples were resolved by 1DE and visualized by silver staining. Fig. 1S shows the silver staining pattern and mass spectrometry identity obtained for the representative Sample No. 1606. Each of the gels was sliced from Mr 72 kDa to 34 kDa as shown in the figure and the gel pieces used for in-gel digestion to obtain peptides. The peptides so obtained were subjected to MS. The proteins identified by PMF were confirmed by MS–MS by taking at least 5 most abundant peaks for that keratin. Fig. 1 shows the percentage of samples in which different keratins were identified. Fig. 2S summarises the presence of the keratins in the samples from which MS data was obtained. Detailed MS analysis is given in Supplementary Table 2S. It is seen that K4 was identified in 33% (4/12) and K13 in 83% (10/12) normal/cut margin tissues while these keratins were absent in all 13 tumor tissues. Keratin 5 was identified in 25% (3/12) normal/cut margin tissues and in 46% (6/13) tumor tissue samples. In normal/cut margin tissues K14, K16 and K17 were identified in 33% (4/12), 25% (3/12) and 8% (1/12) samples for respective keratins while in the tumor they were identified in 69% (9/13), 77% (10/13) and 77% (10/13) samples for respective keratins. There is a clear difference in the expression pattern for K4, K13, K14, K16 and K17 in the normal/cut margin and tumor samples. The presence of K1, K2, K5, K6A, K6B, K6C and K10 was observed in normal/cut margin as well as in tumor tissue samples to almost the same extent.

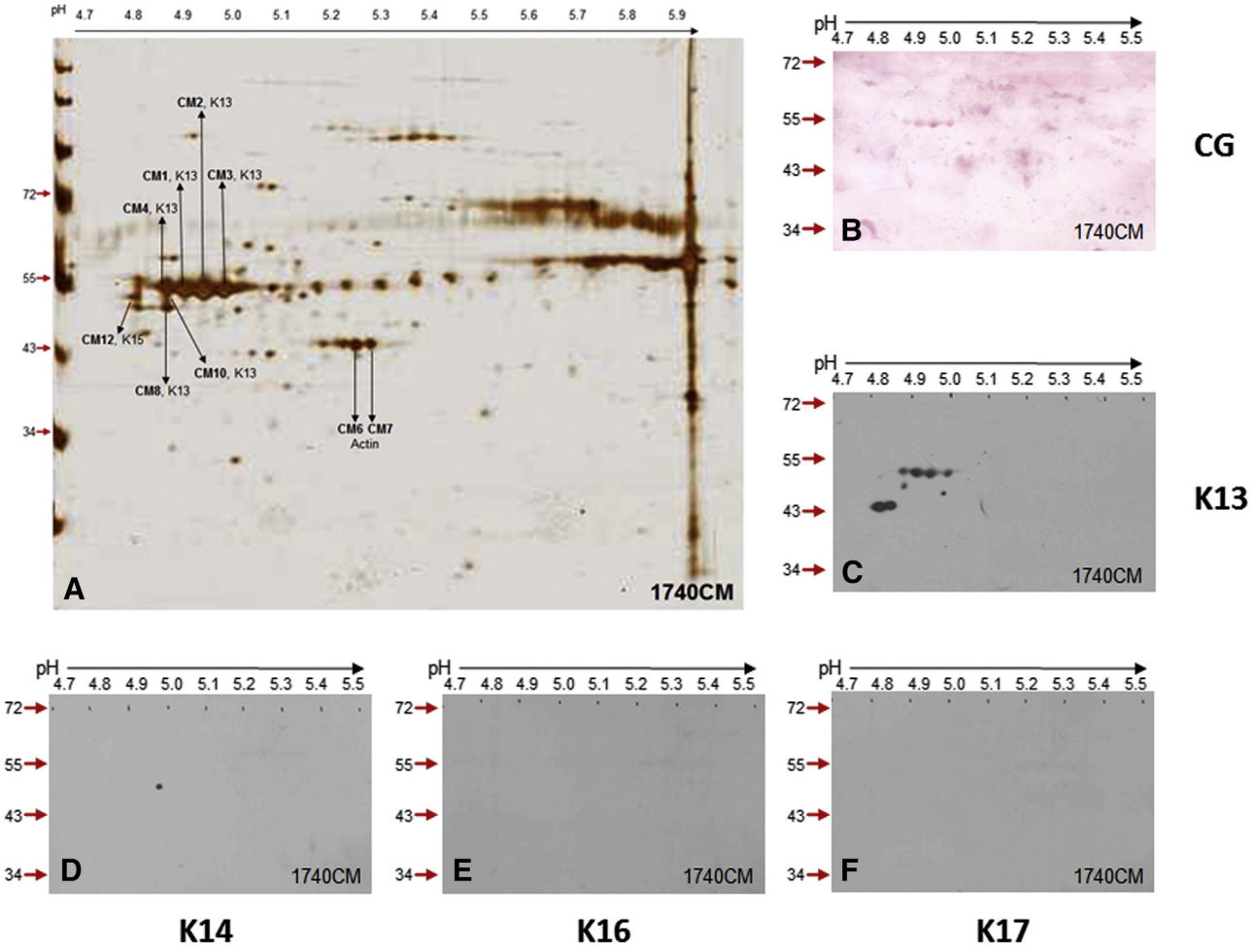

Fig. 8 – Validation of keratin identities obtained by 2DE–MS by western blotting using antibodies specific for K13, K14, K16 and K17 for sample 1740 CM. Figures show the profiles obtained for sample 1740 CM. (A) 2DE-silver stained gel profile and the location of the keratins. (B) Colloidal stained 2DE blot to show the position of the resolved proteins. (C) Autograph of the blot stained with antibody to K13, (D) K14, (E) K16, and (F) K17. The pH range used for IEF is given above the gels and blots. On the silver gel profile, the spot number (CMX) and the identity of the keratin (KX) as per mass spectrometry data in Table 3S are shown. The 2DE blot was cut beyond pH 5.5 to minimize antibody requirements.

3.2. *Localization of identified K4, K13, K14, K16 and K17 on 2DE gels by mass spectrometry and by protein blotting*

Enriched keratin preparations from samples 1737 and 1740 normal, cut margin and tumor were resolved by 2DE and the spots were subjected to mass spectrometry. In parallel, 2DE gels were run for these samples for immunoblotting with antibodies to K4, K13, K14, K16 and K17. Fig. 2(A–C) and (D–F) shows position of the acidic keratins K13, K14, K16 and K17 as determined by MS (Table 3S) in 1737 and1740 N, CM and T samples respectively. The isoelectric point and molecular weight of these keratins matched with reported literature. The position of K13 is clearly seen in the gels for samples 1737 N and 1740 N (Fig. 2A, D). In the gels for 1737 T and 1740 T shown in Fig. 2C and F, it is interesting to note that the band for K13 is absent in the tumor samples and Keratin 14, 16 and 17 are clearly seen in tumor tissue profiles. In the cut margins there are two patterns seen. In sample 1737 CM (Fig. 2B) the straight line of spots corresponding to K13 and below that, the curved band of spots corresponding to K14, 16 and 17 are seen, indicating that in the histological normal cut margins there are molecular changes which are attributable to the transformation process. In sample 1740 CM (Fig. 2E), only the spots corresponding to K13 are seen. The 2DE silver patterns and the corresponding H&E stained sections of the tissues used are shown for 1737 and 1740 (Fig. 3) and for thirteen more samples in Figs. 3S to 7S. It is seen that the silver patterns show the molecular changes which are not yet apparent in the H&E staining profiles. These results suggest that K13 is the major component in normal tissue keratins while K14, K16 and K17 are mainly present in the tumor sample.

The position of the keratins is further confirmed by 2DE western blotting. Figs. 4 to 9 (A–F) show the silver gel pattern juxtaposed to the western blots for samples 1737 N, CM and T and 1740 N, CM, and T. The signals obtained on the blots probed with antibodies to K13, K14, K16 and K17 are shown in the figures. Fig. 10 shows the position of Keratin 4 by 2DE–MS (Table 3S) and WB. This basic keratin does not resolve well and a streak

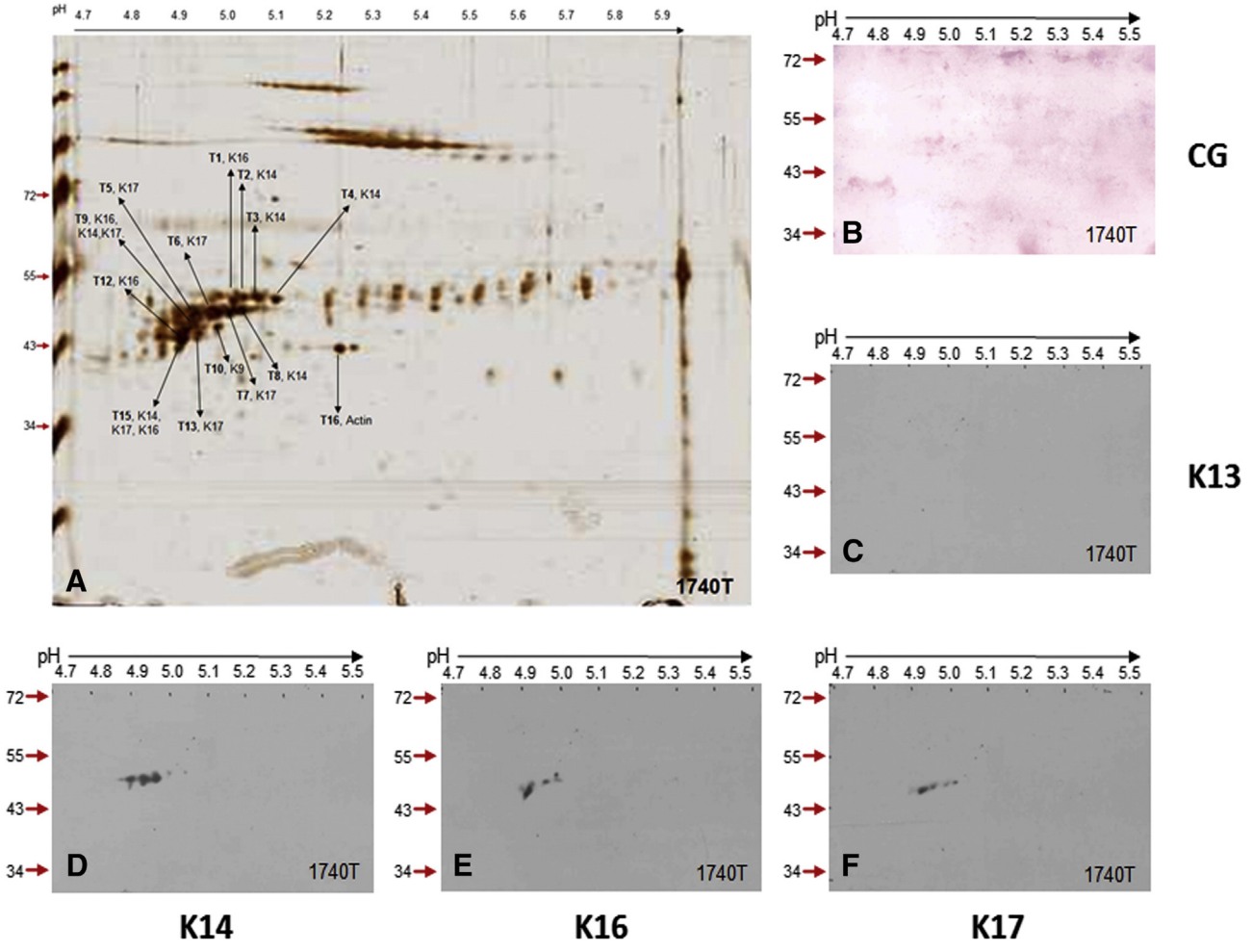

Fig. 9 – Validation of keratin identities obtained by 2DE–MS using western blotting with antibodies specific for K13, K14, K16 and K17 for sample 1740 T. Figures show the profiles obtained for sample 1740 T. (A) 2DE-silver stained gel profile and the location of the keratins. (B) Colloidal stained 2DE blot to show the position of the resolved proteins. (C) Autograph of the blot stained with antibody to K13, (D) K14, (E) K16, and (F) K17. The pH range used for IEF is given above the gels and blots. On the silver gel profile, the spot number (TX) and the identity of the keratin (KX) as per mass spectrometry data in Table 3S is shown. The 2DE blot was cut beyond pH 5.5 to minimize antibody requirements.

is obtained. Histologically, the cut margin tissues may appear similar to the normal tissues but their keratin signature clearly distinguishes if they are similar to the keratin signature for tumor tissue or not. The observed expression patterns can be of clinical significance so as to determine the surgical margins that are molecularly transformed and should be resected during surgery.

3.3. *Immunostaining of 2DE blots for O-GlcNAc*

There is a very early report of glycosylation of K13 in the human keratinocytes cultured from juvenile foreskin on irradiated 3T3 feeder layers and A431 cells derived from epidermoid carcinomas of the vulva [74]. To evaluate the status in tissue samples, western blots from enriched keratins resolved by 2DE were probed with antibody to O-GlcNAc. It is seen in Fig. 11 that this antibody recognizes the same series of spots identified as K13 in the gels shown in Fig. 2A and D.

3.4. *Localization of the keratins on normal, cut margin and tumor tissues by immunohistochemistry*

Fig. 12 shows the sections from normal, cut margin and tumor tissues for the sample 1737 stained with antibodies to K4, K13, K14, K16 and K17. The patterns for these keratins in samples 1657, 1687, 1736, 1740, 1741 and 1751 are shown in Figs. 8S to 13S. It is seen that K4 and K13 are mainly seen in the normal or cut margin tissue sections while K14, K16 and K17 are seen in the tumor samples.

4. Discussion

Keratins have been used as biomarkers for diagnosis and prognosis in various cancers [19]. In most studies of head & neck cancer the expression of a few specific keratins has been evaluated by IHC as described in Section 1.1. Aberrant expression of the simple epithelial K8/18 has been reported in the literature for the stratified non-keratinized epithelium of oral cancer [20,21,29,33–37,40–43]. Sakamoto et al. have reported that in OSCC cases elevated levels of Keratin 8 and 18 were exceptional and their expression levels were lower than other keratins [75]. In earlier studies from our laboratory we have reported the need of mass spectrometry to confirm antibody based findings [38]. This was with reference to the use of an antibody (clone CY90) to K18 which was shown to cross react with K13 irrespective of presence or absence of K18. These observations necessitate caution in accepting the reported presence of K18 in head & neck cancers with this antibody. In the present study, the expression of keratins in a subsite of the oral cavity, the gingivo buccal complex has been investigated by mass spectrometry, 2DE, western blotting, silver staining and immunohistochemistry. This study also reports the presence and glycosylation of K13 in normal epithelium, absence of K4/K13 and increased expression of K14, K16 and K17 in the cancer of the gingivo buccal complex. The mixed expression pattern seen in cut margins shows the continuum of expression from normal to transformed state and validates the observations. K8/18 were not identified by mass spectrometry in the samples evaluated in this study.

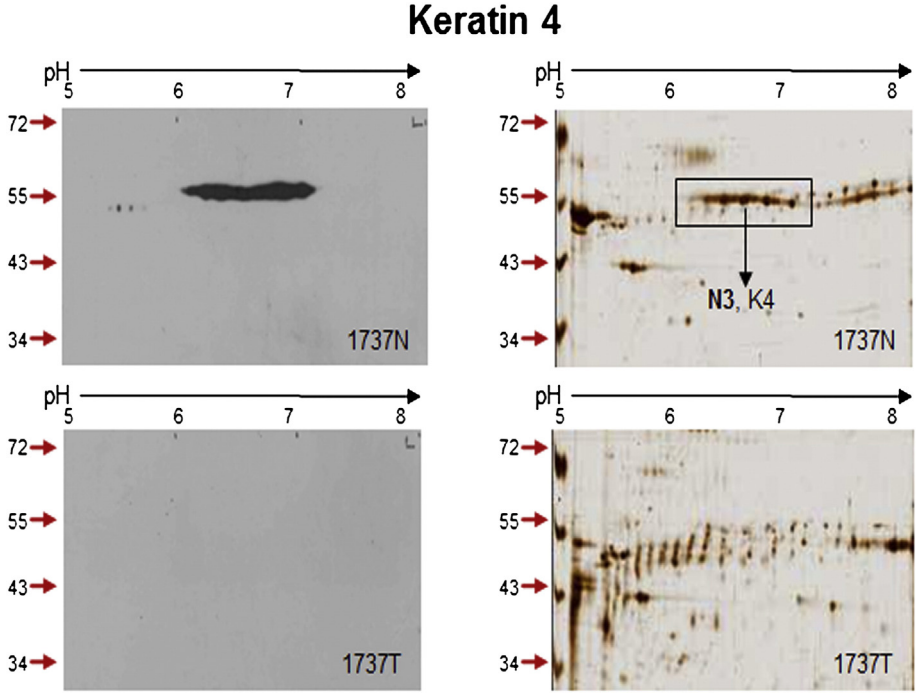

Fig. 10 – Profiles of enriched keratin preparations from samples 1737 N and 1737 T resolved by 2DE on a 17 cm IPG strip of pH range 5–8 and stained for K4 after western blotting. Figure shows autographic profile of the blot stained with antibody to keratin 4 for normal and tumor samples and the position of K4 on silver stained gel as obtained by mass spectrometry. Mass spectrometry data is given in Table 3S. The pH range used for IEF is shown above the gels and blots. The identity of the keratin and spot number is as per Table 3S indicated. The pH range used for IEF is shown above the gels and blots.

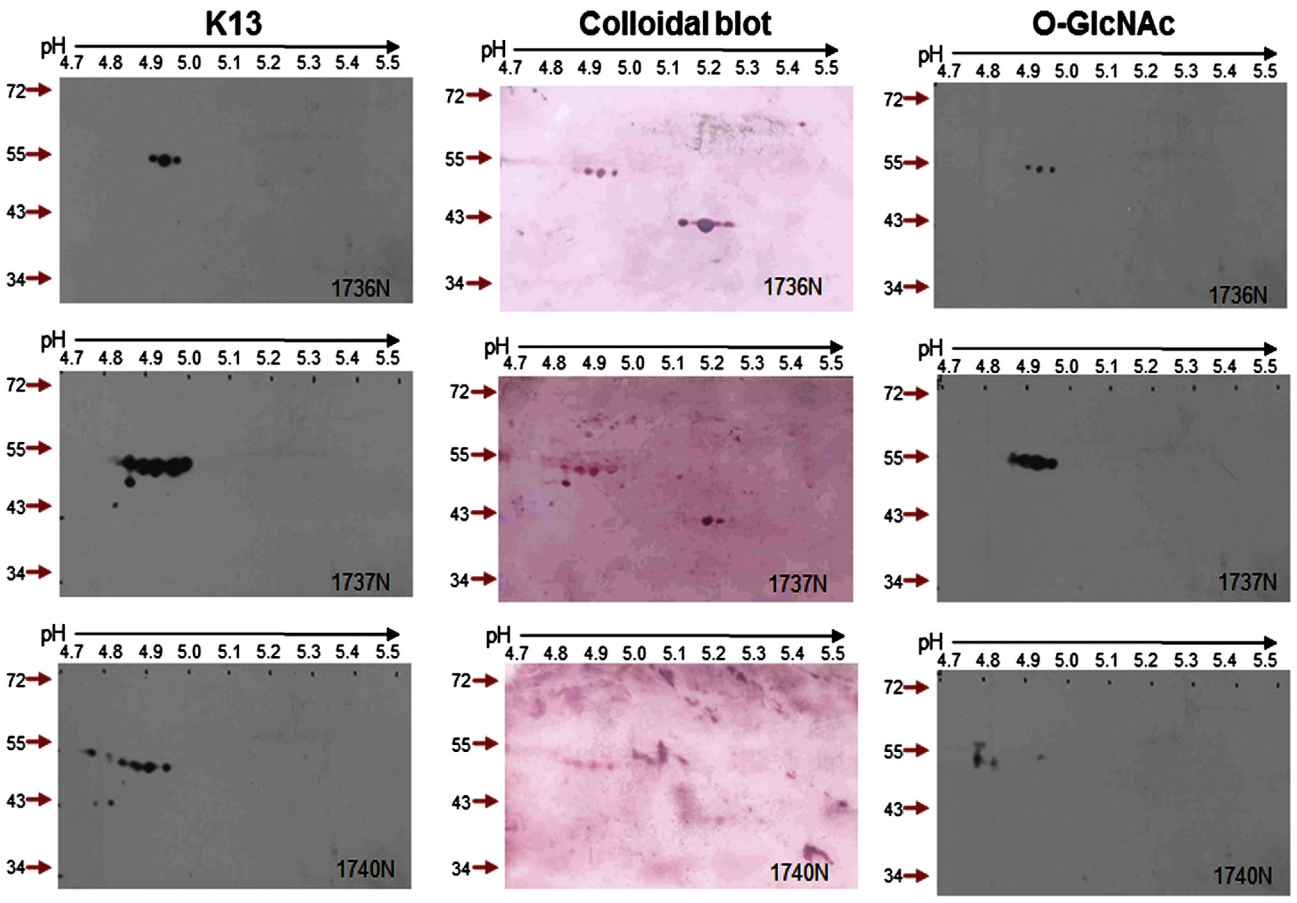

Fig. 11 – 2DE autographic profiles of western blots stained with antibody to Keratin 13 and O-GlcNAc. Enriched keratin preparations from samples 1736 N, 1737 N and 1740 N were resolved by 2DE and transferred to PVDF membrane. The blots were stained with the specific antibodies and the autographic profiles show the position of the signals obtained with each antibody. The blots were finally stained with colloidal gold. The autographic signals overlap with the corresponding position of the protein on the colloidal gold stained blots. The pH range used for IEF is shown above and the molecular weight of the standards used is indicated on the left side of the blots.

K4/13 are reported to be expressed in the suprabasal layer of stratified squamous epithelium [76]. Literature reports show that in some squamous cell carcinomas they are lost and in some they are focally and variably expressed such as in head and neck carcinoma and show increased expression in poorly differentiated tumors [17,77]. K13 is also focally expressed in transitional cell carcinomas of the urinary tract [17,77]. In our study K4/13 are also seen in the suprabasal layer and majority of the normal epithelia express these keratins. Mouse K4 plays a key a role in maintaining epithelial cell integrity [78]. Point mutations in K4/13 have been associated with the autosomal dominant disorder, white sponge nevus [79,80]. Although, their role is not very clear in the human tissues, they may have a role in maintaining the tissue architecture.

K5/14 are strongly expressed in undifferentiated basal cell layers of stratified epithelium containing stem cells and down regulated in the differentiating suprabasal levels [81,82]. They bundle up as tonofilaments and are attached to desmosomes and hemidesmosomes [17]. Most squamous cell carcinomas strongly express these keratins while little, focal or no staining is seen in adenocarcinomas [14,77,83–85]. The increased expression could contribute to the disorganised arrangement of the epithelial cells in transformed tissues.

K6/16 are constitutive keratins of stratified epithelia and are often expressed as a pair. They are expressed in mucosal tissue where it is in a state of high proliferation [83,86,87]. There are three very homologous isoforms of K6, K6a, K6b, and K6c which are encoded by separate genes [86]. K6 is expressed in the suprabasal layers of non-keratinizing squamous epithelium [17]. They are involved in mechanical scaffolding and migration and re-epithelialization [18]. K6/16 are strongly expressed in squamous cell carcinoma of several sites [77,83]. The increased expression could contribute to the invasiveness of the cancers.

K17 is absent in non-keratinizing stratified squamous epithelia and reported to be expressed in squamous cell carcinomas [17,83]. K17 is reported to influence cell growth and size by regulating protein synthesis [2]. K8/18 are components of simple epithelia. They are involved in structural and mechanical properties of the cell as well as they have a role in cell cycle and apoptosis [17,18,88]. They are reported to be aberrantly expressed in squamous cell carcinomas of various

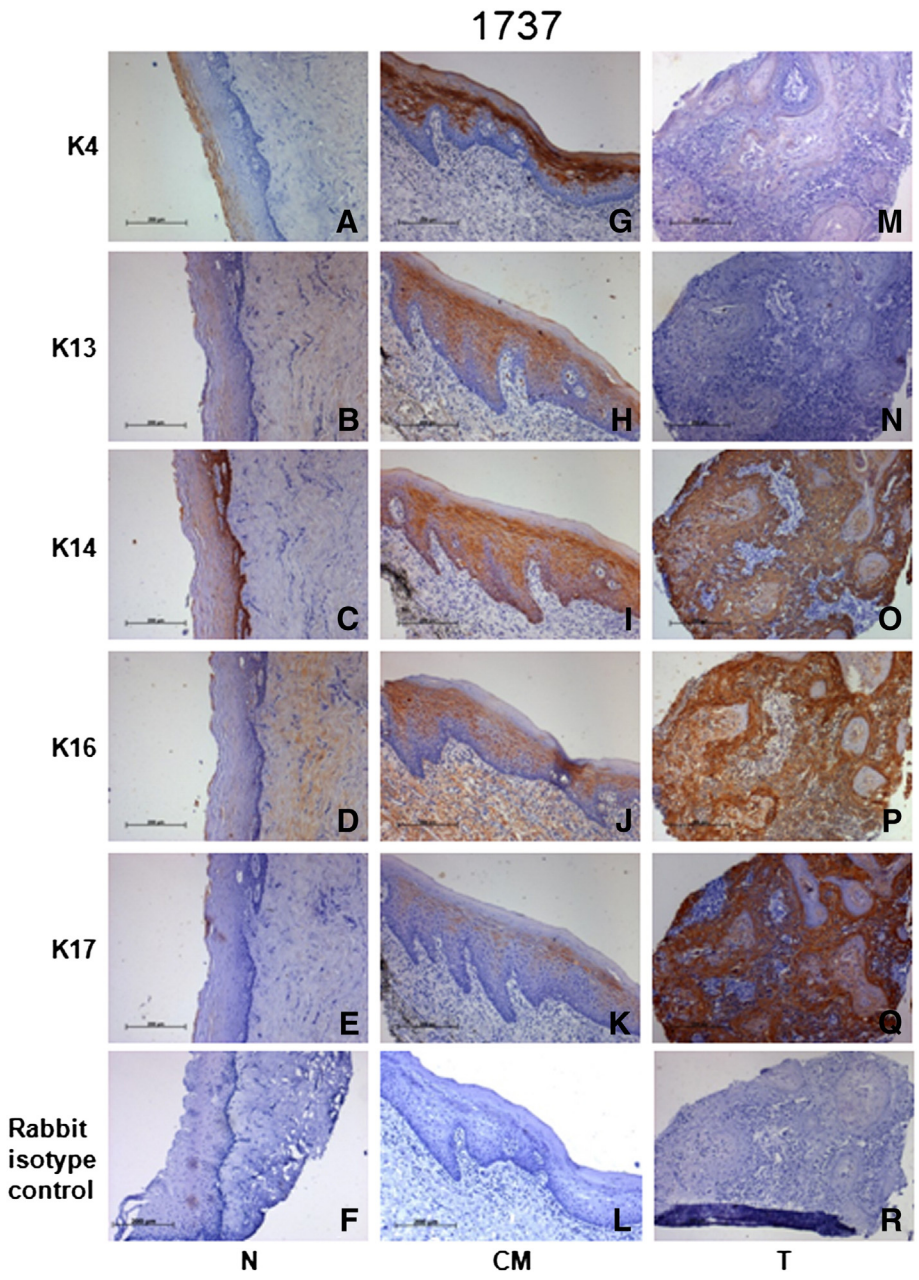

Fig. 12 – Immunostained profiles of keratin expression on tissue sections from sample set 1737. The first column shows normal (N) tissue sections (A to F), the second column sections from the cut margin (CM), (G to L) and the third column sections from the tumor tissue (M to R) from sample 1737. The sections were immunostained with antibodies to keratin K4 (A, G, M), K13 (B, H, N), K14 (C, I, O), K16 (D, J, P), K17 (E, K, Q) and rabbit isotype as a negative control (F, L, R). The images in each row correspond to the pattern for each keratin and control antibody written on the left side. The magnification used is indicated on the panels.

tissues including that of the head and neck [83,89]. This study did not identify K8/18 by mass spectrometry in either normal or GBC cancer tissues.

In summary the keratin signature obtained by mass spectrometry and validated by 2DE, silver staining, western blotting and IHC provides markers to distinguish the normal tissue from the molecularly transformed area. The early disappearance of K13 and appearance of K14, K16, and K17 as seen in the cut margins, indicates that the histologically normal tissue has already acquired molecular characteristics of the tumor. As cut margins are used to identify limits of surgical resection, evaluation of the keratin profile could be used to advantage by the clinician to monitor and/or reduce recurrence in patients. Studies to understand the mechanisms regulating the expression of these keratins will provide clues to the transformation process. The combination of K14, 16 and K17 could be tested as markers for identification of micro-metastasis in the cervical lymph nodes.

5. Conclusion

This study has identified K4, K13, K14, K16 and K17 which can distinguish between normal and tumor tissues and has potential as biomarkers in cancer of the gingivo-buccal complex.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2013.07.011.

Author contribution

The research work was conceptualised by SZ. The appropriate tissue samples were identified and provided by AD and PC following surgical resection. The histopathological analysis was done by AP and SK. The immunohistochemistry was coordinated by SM and she also assisted in western blotting. RG contributed to the evaluation and discussion of the data. AF performed the experimental work, analysed and interpreted the results and wrote the manuscript under the guidance of SZ.

Conflicts of interest

All authors state no conflicts of interest.

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