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Full Length Research Paper

Analysis of differentially expressed proteins in the exfoliated cells of normal and squamous cell carcinoma of the uterine cervix to define candidate markers for cervical cancer

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Abstract

Cervical cancer continues to take extra ordinary toll on the lives of women in India, which has been attributed to the lack of systematically organized population screening programmes. Cost effective screening tools are yet to be identified for low resource countries. Proteomic studies constitute a reliable way to determine the biological alterations induced by HPV infection. Aim of the study was to identify the differentially expressed proteins in the exfoliated cells of Squamous Cell Carcinoma. Protein extracted from exfoliated squamous cells of normal smears and squamous carcinoma cells was analyzed by using Liquid Chromatography-Mass spectrometry method and the DNA was subjected for multiplex PCR for HPV status. The malignant samples were positive for oncogenic HPV. Proteome profiling revealed unique expression of 104 proteins in SCC while 20 proteins were found up-regulated and 64 proteins were down regulated. Up-regulated proteins include different isoforms of cytokeratin, ras related proteins, annexin, endoplasmin, vimentin, gelsolin, actin and heats hock proteins etc which can be used to define novel markers of cervical intra epithelial neoplasia.

Key words: Cervical cancer, proteomics, squamous cell carcinoma, human papilloma virus, liquid chromatography mass spectrometry.

INTRODUCTION

Cancer of the uterine cervix is one of the most common malignancies worldwide and it has the second highest incidence in India and other developing countries.

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Screening for cervical cancer is one of the most prevalent and successful public health measures for prevention of both the incidence of invasive cervical cancer and disease related mortality (Pollack et al., 2006). The cervical epithelium undergoes a spectrum of epithelial abnormalities before progressing to advanced disease. These abnormalities are classified as dysplasia-low to high grade/squamous intra epithelial lesions.

Identification and treatment of these premalignant lesions has been proved to prevent the occurrence of cervical cancer. The significance of Pap smear test for identification of cervical intra epithelial lesions is well established and a rapid reduction in the incidence of cervical cancer has been achieved in many of the developed countries like Finland, Sweden, and Denmark by the introduction of systematically organized population screening programme using Pap smear test. Persistent infection with high risk Human Papilloma Virus (HPV) has been identified as an etiological factor in most of the cases (Munoz et al., 2003; ZUR Hausen, 2002). Among the 15 high-risk (oncogenic) types of HPV, type16 and 18 are reported to be responsible for about 70% of all cervical cancers. So HPV DNA test has been advised along with Pap smear screening. It is also now established that oncogenic HPV infection alone is not sufficient for tumor development (Matos et al., 2005; Delvenne et al., 2007). Other factors like prolonged use of oral contraceptives, smoking habits and multiple sexual partners could play important role in the persistence of HPV infection and progression to cervical cancer (Tjalma et al., 2005). The recent development of vaccines against oncogenic HPV is reported to have the potential to reduce the incidence of cervical dysplasia and cancer (Fernandez et al., 2010) and prophylactic vaccination has been initiated in some of the developed countries. HPV genome test either as primary screening method or in conjunction with Pap smear cannot be implemented in low resource countries due to the lack of adequate financial resource and technology. Moreover 80-90% of HPV infection is transient and the cytological abnormalities caused are temporary only. So Pap smear based screening remains as the best method to preselect women at high risk. However, none of the above screening methods have been implemented in India and other similar countries and cervical cancer continues to take extra ordinary toll on the lives of women in these countries. In India, organized population screening programmes using Pap smear could not be implemented due to the lack of trained personnel and adequate financial resources to screen all eligible women in the community. However, sporadic screening programmes are available in selected areas where, all women with cytological abnormalities are being treated or followed up with repeat Pap smear examination, even though their lesions are likely to revert to normal. An average of 6000 women is being screened annually in the early cancer detection programmes of the Regional Cancer Centre Thiruvananthapuram, Kerala. About 15% of these cases are being diagnosed to have low-grade squamous intra epithelial lesion (LSIL/Atypical Squamous cells of Undetermined significance (ASCUS) /Atypical glandular cells of undetermined significance (AG-US) and these cases are further evaluated by colposcopy followed by biopsy and treated by cryosurgery or leep, if the abnormality is persistent one. About 80% of the preselected women are being treated unnecessarily causing a heavy over use of resources. It is because of our inability to correctly assess the malignant potential of the preselected lesions. If a marker protein can be validated to identify high risk cervical intraepithelial lesions, treatment and further follow up can be limited to them alone (Nijhuis et al., 2006). Also it can be tried as a stand-alone test for screening for cervical cancer in low resource countries. Furthermore, the marker can be tried by incorporating it along with the conventional pap staining technique which may make the identification of abnormal cells easier than the conventional method. The combined smear technique will pave way for the development of more reliable software for automation of Pap smear screening, which can bring about revolutionary changes in the prevention and control of cervical cancer. Earlier, we have suggested visual inspection plus symptom history as a pre selection criterion for identifying high risk women in low resources setting (Sujathan et al., 1994). A semi-automated computerized Pap smear analysis system was also been developed by our team (Deepak et al., 2015). Proteome based cervical cancer markers are yet to be reported even though a few studies on proteomics of cervical cancer and pre cancer tissues appeared in the literature (Peng, 2001, Su-Mi et al., 2006). A previous study of protein profiling on vaginal fluids reported a series of sixteen candidate marker proteins discriminating samples from healthy and HPV-infected women (Geert Van Raemdonck et al., 2014). As the vaginal fluid contain a collection of different type of epithelial and non epithelial cells from the whole of female genital tract, the sensitivity and specificity of the proteins may not be sufficient enough to consider it as a candidate marker for screening purpose. Differentially expressed proteins of cells specifically from the cervical epithelium should be compared with that of its malignant counterpart. The aim of the present study was to compare the protein expression profiles of cervical cancer cells with that of normal cervical cells specifically from the ectocervical scrapings in order to identify the differentially expressed proteins associated with cervical cancer so that a cost effective marker for malignancy can be defined. We have standardized a technique to separate the cells from non-cellular components and high quality protein was extracted from the cells separated from pap smears. The differentially expressed proteins were identified in the exfoliated cells of squamous cell carcinoma by liquid chromatography mass spectrometry method and compared the same with that of normal cells. Also we have attempted to elucidate their possible biological

function and their role in different molecular pathways which in our knowledge is the first report of proteome profiling on cells seperated from Pap smear samples.

MATERIALS AND METHODS

The samples for the study were obtained from 1850 women attending the population screening programme organized by the Regional cancer centre over a period of two years. It includes 650 symptomatic women who have attended the gynecology clinic of Women &Children hospital, Thycaudu, Thiruvananthapuram with some gynecological complaints. The study was approved by the Institute Review Board as well as the Human Ethical Committee of RCC (HECNo.36/2008). Informed consents were obtained from all participants. The Study material included cervical smears and tissues samples ranging from negative for intraepithelial lesion (NILM) to invasive squamous cell carcinoma. Cervical scrape smears were obtained from all participants by using Ayers spatula, which were fixed in 95% ethanol and processed in the classical pap staining method. These smears were diagnosed according to the Bethesda system of Pap smear reporting. After one week, a repeat smear from five women with normal cytology and matching age and menstrual status were invited and a second sample of cells was obtained from the ectocervix for proteome analysis. The cells were collected in vials containing TBS (pH 8). A second sample of cells from women with invasive squamous cell carcinoma were obtained in TBS and kept at -80C. Colposcopy biopsies were obtained from these women on the same day and processed in the surgical pathology division of RCC.

Protein extraction

Pooled cells of five samples suspended on the buffer were vortexed for two minutes at 1000 rpm. The cells were washed in TBS repeatedly to remove mucus and other debris. The cell button was then vortexed with density gradient solution supplied by Sure Path Liquid Based Cytology system. Then the samples were concentrated at 5000 rpm for five minutes at 4^0C and the separated cells were washed in TBS and finally made in to a cell pellet at 8000 rpm for 10 minutes at 4^0C. These cells were transferred into sterile eppendorf tubes. One smear from each sample was prepared by the liquid based cytology method and stained with classical pap staining method to assess the cellularity of the sample. The cell pellets were re-suspended in 50-100 µLs of RapiGest reagent (waters) and were incubated for 45 minutes-1 hour in ice. The re-suspended cells were then homogenized thoroughly using a homogenizer and were subjected to 3 cycles of freeze-thawing using liquid nitrogen. The samples were then centrifuged at 14000rpm for 20 minutes at 4^0C. The supernatant was collected and stored at -80^0C.

LC-MS/MS analysis

Protein estimation of each sample was performed using Bradford method and equal amounts from each protein samples were subjected to in-solution trypsin digestion to make peptides and the extracted peptides were analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS) using synapt G2 HDMS (waters) connected online through nano ACQUITY UPLC (waters). Protein identification and expression analysis of the Post-Ms Data were performed by Protein Lynx Global Server (PLGS, Waters) using NCBI Reference Sequence Database for Human. High through put protein profile and expression data were further subjected to bioinformatics analysis using DAVID (Database for annotation, visualization and integrated discovery) for biochemical pathway.

HPV analysis

HPV identification of all the samples selected for the study was done on cervical scrape cells by a multiplex PCR method using a HPV DNA analysis Kit supplied by Bangalore Genei. An amplification product of size varying between 230-270 bp is indicative of an infection with oncogenic HPV. This Kit detects oncogenic HPV types 16,18,31,33,35,45,52 and 58. A portion of the cells were used for DNA extraction. Genomic DNA was extracted from cervical scrape cells by following the protocol provided in the kit. PCR reactions were carried out as per the instruction of the manufacturer with a 25µl reaction amplifying for 35 cycles with annealing temperature 58^0C for 45 seconds. Positive and negative controls were included in each reaction set. The amplicons were visualized in 2% agarose gel electrophoresis.

RESULTS

The mean age of the study group of normal samples was 35years and that of malignancy was 58 years. The smears obtained from the pooled cells prepared after suspending in density gradient solution showed mainly squamous cells in a clean background with a predominance of intermediate type of squamous cells in cytologically normal samples (figure 1) and malignant cells in samples of Squamous cell carcinoma (figure 2). The mean protein concentration of cells of selected samples was 6mg/l. In mass spectrometry profiling, a

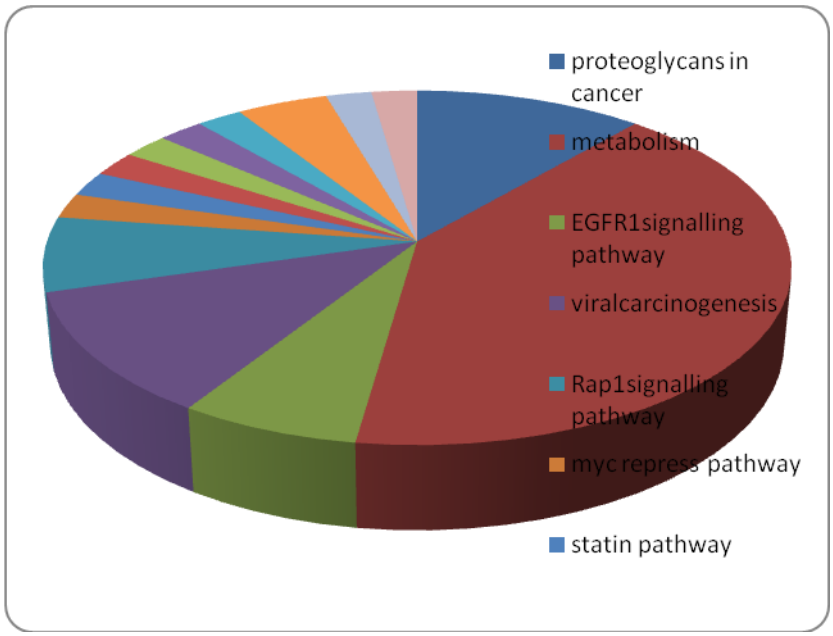

Figure 1. Proteins significantly associated with altered biological pathways.

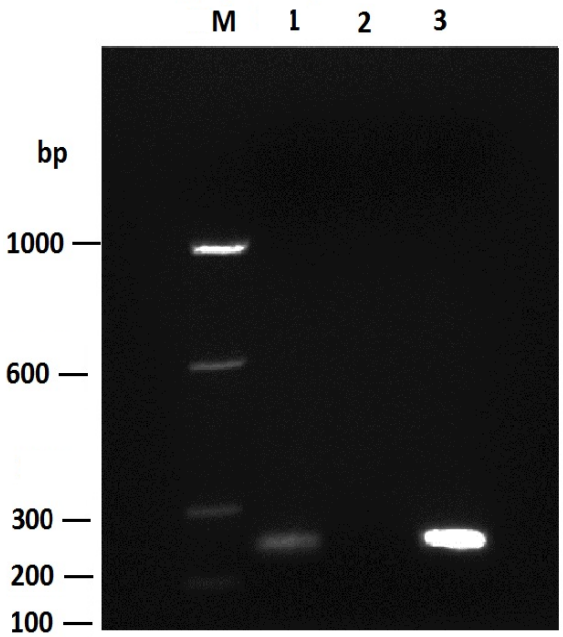

Figure 2. PCR amplified product of HPV DNA analysed on 2% agarose gel.
Lane M: DNA molecular weight marker(Ready to use)
Lane 1: sample positive for oncogenic HPV
Lane 2: Negative control
Lane 3: positive control

total of 56 unique proteins were identified in samples with normal cytology (table 1). These include both cytoplasmic and nuclear proteins. Seventeen different isoforms of keratin were found uniquely expressed in the normal

Sl.No.	Accession	Description	Gene Symbol
1	XP_005277141.1	PREDICTED 40S ribosomal protein S9 isoform X1 Homo sapiens	RPS9
2	NP_853515.2	keratin type I cytoskeletal 27 Homo sapiens	KRT27
3	NP_998776.1	phosphate carrier protein mitochondrial isoform b precursor Homo sapiens	SLC25A3
4	NP_057204.2	E3 ubiquitin protein ligase RLIM Homo sapiens	RLIM
5	NP_000214.1	keratin type I cytoskeletal 12 Homo sapiens	KRT12
6	NP_060003.2	myosin 4 Homo sapiens	MYH4
7	NP_001093582.1	myosin 2 Homo sapiens	MYH2
8	NP_817089.1	cadherin like protein 26 isoform a precursor Homo sapiens	CDH26
9	NP_005043.1	ras related C3 botulinum toxin substrate 3 Homo sapiens	RAC3
10	NP_001613.2	alpha 2 HS glycoprotein preproprotein Homo sapiens	AHSG
11	XP_006714775.1	PREDICTED calpastatin isoform X17 Homo sapiens	CAST
12	XP_006726591.1	PREDICTED mucin 5AC Homo sapiens	-
13	NP_001005209.1	transmembrane protein 198 Homo sapiens	TMEM198
14	NP_001010858.2	E3 ubiquitin protein ligase RNF187 Homo sapiens	RNF187
15	NP_001186757.1	cornifin A Homo sapiens	SPRR1A
16	NP_001243731.1	40S ribosomal protein S3 isoform 1 Homo sapiens	RPS3
17	NP_001135779.1	general transcription factor IIH subunit 1 Homo sapiens	GTF2H1
18	XP_005251119.1	PREDICTED 14 3 3 protein zeta delta isoform X3 Homo sapiens	YWHAZ
19	NP_005134.1	haptoglobin isoform 1 preproprotein Homo sapiens	HP
20	NP_005321.1	hemoglobin subunit epsilon Homo sapiens	HBE1
21	NP_003281.1	tropomyosin alpha 4 chain isoform 2 Homo sapiens	TPM4
22	XP_006710008.1	PREDICTED mucin 5AC partial Homo sapiens	-
23	NP_008876.3	small proline rich protein 2D Homo sapiens	SPRR2D
24	NP_899196.1	E3 ubiquitin protein ligase RLIM Homo sapiens	RLIM
25	XP_006720215.1	PREDICTED myosin 6 isoform X4 Homo sapiens	-
26	NP_005979.1	small proline rich protein 2A Homo sapiens	SPRR2A
27	NP_005971.1	protein S100 P Homo sapiens	S100P
28	NP_006816.2	cytoskeleton associated protein 4 Homo sapiens	CKAP4
29	XP_006718761.1	PREDICTED calpain 1 catalytic subunit isoform X1 Homo sapiens	CAPN1
30	NP_003520.1	histone H3 1 Homo sapiens	HIST1H3A
31	NP_057406.2	ras related protein Rab 14 Homo sapiens	RAB14
32	XP_005266224.1	PREDICTED Golgin subfamily A member 3 isoform X6 Homo sapiens	GOLGA3
33	NP_001185797.1	calpain 1 catalytic subunit Homo sapiens	CAPN1
34	NP_001121178.1	alpha 1 antitrypsin precursor Homo sapiens	SERPINA1
35	XP_006712347.1	PREDICTED transmembrane protein 198 isoform X1 Homo sapiens	-
36	NP_065801.1	exportin 5 Homo sapiens	XPO5
37	NP_001231867.1	thioredoxin isoform 2 Homo sapiens	TXN
38	NP_001002236.1	alpha 1 antitrypsin precursor Homo sapiens	SERPINA1

Table 1. Unique proteins identified in normal samples.

Table 1 Cont.

39	NP_001620.2	arachidonate 5 lipoxygenase activating protein isoform 1 Homo sapiens	ALOX5AP
40	XP_005271356.1	PREDICTED RNA 3 terminal phosphate cyclase isoform X2 Homo sapiens	RTCA
41	NP_004300.1	rho GDP dissociation inhibitor 1 isoform a Homo sapiens	ARHGDIA
42	NP_001159923.1	kininogen 1 isoform 3 precursor Homo sapiens	KNG1
43	NP_001138791.1	plastin 1 Homo sapiens	PLS1
44	NP_113665.3	protein yippee like 3 isoform 1 Homo sapiens	YPEL3
45	NP_009057.1	transitional endoplasmic reticulum ATPase Homo sapiens	VCP
46	XP_005264525.1	PREDICTED ras related protein Rab 1A isoform X1 Homo sapiens	RAB1A
47	XP_005269291.1	PREDICTED DNA helicase B isoform X1 Homo sapiens	HELB
48	NP_001142.2	ADP ATP translocase 1 Homo sapiens	SLC25A4
49	XP_005258006.1	PREDICTED fructose bisphosphate aldolase C isoform X3 Homo sapiens	ALDOC
50	NP_001165783.1	plastin 1 Homo sapiens	PLS1
51	XP_006716418.1	PREDICTED integrator complex subunit 10 isoform X5 Homo sapiens	INTS10
52	XP_005259339.1	PREDICTED alpha actinin 4 isoform X2 Homo sapiens	ACTN4
53	NP_068582.2	cadherin like protein 26 isoform b Homo sapiens	CDH26
54	NP_004628.4	ras related protein Rab 7a Homo sapiens	RAB7A
55	NP_003524.1	histone H3 1 Homo sapiens	HIST1H3I
56	NP_002626.1	phosphate carrier protein mitochondrial isoform b precursor Homo sapiens	SLC25A3

squamous cells. The others were proteins involved in immunity and defense, protein metabolism and modification and other cellular processes such as cell signaling, cell adhesion and cell cycle. One hundred and four unique proteins observed in samples of SCC. Functional analysis of these proteins revealed that ankyrins, decorin, receptor type tyrosine, and Ras related proteins have roles in activities such as cell motility activation, proliferation, cell-cell contact and cell growth. 26 proteins were found up regulated and 61 were found down regulated in SCC. The most abundant proteins found both in normal samples and malignancy was different isoforms of cytokeratin, Ras related proteins, vimentin, s100, tubulin, histone proteins etc. (6.619-2.248). The up-regulated proteins with their accession number and gene symbols are shown in (table 2). The expression levels of endoplasmin (HS P90 B1) vimentin (VIM), tubulin (TUBA1A), peripherin (PRPH), and cytokeratin (KRT7) were found higher compared with other up-regulated proteins. Among the upregulated proteins, keratin type 1 cytoskeletal (9&10), vimentin, desmin, lamin isoform, tubulin alpha 1 chain isoform2 and actin cytoplasmic 2 were cytoskeletal proteins. The down regulated proteins are shown in (table 3). Annexin, one of the membrane localized proteins was found down regulated in these samples. Analysis of the functional association of uniquely expressed proteins revealed that HLA class I histocompatibility antigen alpha chain G precursor, predicted 1433 protein epsilon isoform XI homosapiens, complement C3 precursor and gelsolin are associated with viral oncogenesis, whereas ankyrin 3 isoform3, decorin isoform a pre protein, Ras related protein Rab I A isoform 2, receptor type tyrosine protein phosphatase c isoform c isoform 2 precursor, and peroxidoxin1 etc. were found associated with cell proliferation. Proteins decorin isoform a preproprotein, thioredoxin domain containing protein 5 isoform 3, gelsolin isoform, mothers against deca penta pelgic homolog 3 isoform2, and coronin IA etc. were found associated with apoptosis. Pathway analysis of all the differentially expressed protein in SCC (figure 3) showed that many of the proteins have association with different molecular signalling pathways, which includes , RAB35 (cAMP signaling pathway) DCN (TGF-beta signaling pathway), KRT7 (EGFR1 signaling pathway), COL6A3 (PI3K-Akt signaling pathway), LGALSS1 (Myc repress pathway), RAPGEF1(Rap1 signaling pathway), PFN1(GI3 signaling pathway), GFAP(nuclear signaling by ERBB4, Rap1 signaling pathway), and KRT7(EGFR1 signaling). The proteins found associated with other malignancies were different isoforms of Protein S 100, histone proteins, glycophorin, annexin, ankyrin and heat shock proteins (gastric cancer, prostate cancer, breast cancer, pancreatic cancer, lung cancer etc.) and peroxido-

Sl No	Accession	Description	Genesymbol
1	NP_009005.1	coronin 1A Homo sapiens	CORO1A
2	NP_000055.2	complement C3 precursor Homo sapiens	C3
3	NP_003371.2	vimentin Homo sapiens	VIM
4	NP_001918.3	desmin Homo sapiens	DES
5	NP_005563.1	lamin isoform C Homo sapiens	LMNA
6	NP_000217.2	keratin type I cytoskeletal 9 Homo sapiens	KRT9
7	NP_005315.1	histone H3 3 Homo sapiens	H3F3B
8	NP_001257329.1	tubulin alpha 1A chain isoform 2 Homo sapiens	TUBA1A
9	NP_001002857.1	annexin A2 isoform 2 Homo sapiens	ANXA2
10	NP_001605.1	actin cytoplasmic 2 Homo sapiens	ACTG1
11	NP_005013.1	profilin 1 Homo sapiens	PFN1
12	XP_006717563.1	PREDICTED vimentin isoform X1 Homo sapiens	VIM
13	NP_001129487.1	annexin A2 isoform 2 Homo sapiens	ANXA2
14	NP_003290.1	endoplasmin precursor Homo sapiens	HSP90B1
15	NP_006253.2	peripherin Homo sapiens	PRPH
16	NP_001054.1	serotransferrin precursor Homo sapiens	TF
17	NP_001186883.1	actin cytoplasmic 2 Homo sapiens	ACTG1
18	NP_000412.3	keratin type I cytoskeletal 10 Homo sapiens	KRT10
19	NP_000217.2	keratin type I cytoskeletal 9 Homo sapiens	KRT9
20	NP_003290.1	endoplasmin precursor Homo sapiens	HSP90B1

Table 2. Upregulated proteins in SCC.

xin, vimentin, endoplasmin, mothers against deca pentalgic homolog were involved in the regulation of tumour formation. The proteins ankyrin, lumican, decorin, and gelsolin involved in the regulation of various processes in the carcinogenesis were adhesion, angiogenesis, metastasis, and oncogenesis. HPV analysis of the samples revealed that all the samples of SCC were positive for oncogenic HPV (figure 4).

DISCUSSION

The cervical epithelium of women in the childbearing age group is always under the influence of ovarian hormones. The cellular pattern varies in accordance with the age and menstrual status. Also it is difficult to separate the cervical epithelial cells from the mucus and other inflammatory non-epithelial cells. So there are only limited reports currently available on proteome profile on cervical epithelium and the available ones are either on tissues or cervical fluid; both of them contain several cellular and non-cellular components in addition to the epithelial components. Janet S Rader has suggested a protocol for extracting both high-quality mRNA and protein from cervical swabs in an aqueous RNA-preservation agent called RNA *later* (Janet S. Rader et al., 2008). Using this method, they have characterized proteins that are present in cervical swabs. The proteins obtained may not be a true representation of the cervical epithelium because the sample they used may contain all epithelial and non epithelial cells from the cervix. We could separate the squamous epithelial cells without many cellular and non-cellular components. Kristin in a similar type of study have found an average of 153 unique proteins in normal smears, which they designated as “Normal Pap test core proteome” (Kristin etal. 2014). Where as we have found only 56 unique proteins in normal smears. Kristin has isolated proteins from the whole cell contents remaining after preparing Pap smears, which may contain endocervical cells as well as non epithelial cells in addition to the ectocervical epithelium, which may be the reason for the higher

	Accession	Description	Gene Symbol
1	XP_005256841.1	PREDICTED 14 3 3 protein epsilon isoform X1 Homo sapiens	YWHAE
2	NP_006133.1	14 3 3 protein sigma Homo sapiens	SFN
3	NP_005175.2	calmodulin Homo sapiens	CALM3
4	NP_001139698.1	keratin type II cytoskeletal 72 isoform 2 Homo sapiens	KRT72
5	NP_000055.2	complement C3 precursor Homo sapiens	C3
6	NP_005546.2	keratin type II cytoskeletal 6B Homo sapiens	KRT6B
7	NP_000691.1	annexin A1 Homo sapiens	ANXA1
8	NP_001734.1	calmodulin Homo sapiens	CALM2
9	XP_005271597.1	PREDICTED apolipoprotein A I isoform X2 Homo sapiens	APOA1
10	NP_000415.2	keratin type II cytoskeletal 5 Homo sapiens	KRT5
11	NP_005337.2	heat shock 70 kDa protein 1A 1B Homo sapiens	HSPA1B
12	NP_002264.1	keratin type II cytoskeletal 8 isoform 2 Homo sapiens	KRT8
13	NP_055439.1	protein S100 A6 Homo sapiens	S100A6
14	NP_001243222.1	keratin type II cytoskeletal 8 isoform 2 Homo sapiens	KRT8
15	NP_001531.1	heat shock protein beta 1 Homo sapiens	HSPB1
16	NP_006588.1	heat shock cognate 71 kDa protein isoform 1 Homo sapiens	HSPA8
17	NP_001161078.1	ras related protein Rab 35 isoform 2 Homo sapiens	RAB35
18	NP_001902.1	cathepsin G preproprotein Homo sapiens	CTSG
19	NP_694881.1	heat shock cognate 71 kDa protein isoform 2 Homo sapiens	HSPA8
20	NP_001258901.1	heat shock protein HSP 90 beta isoform c Homo sapiens	HSP90AB1
21	NP_001243225.1	immunoglobulin lambda like polypeptide 5 isoform 2 Homo sapiens	IGLL5
22	NP_002146.2	heat shock 70 kDa protein 6 Homo sapiens	HSPA6
23	NP_005013.1	profilin 1 Homo sapiens	PFN1
24	NP_775109.2	keratin type II cytoskeletal 6C Homo sapiens	KRT6C
25	NP_005176.1	calmodulin like protein 3 Homo sapiens	CALML3
26	NP_005547.3	keratin type II cytoskeletal 7 Homo sapiens	KRT7
27	NP_002298.1	galectin 7 Homo sapiens	LGALS7
28	NP_001119574.1	haptoglobin isoform 2 preproprotein Homo sapiens	HP
29	NP_524147.2	myosin light polypeptide 6 isoform 2 Homo sapiens	MYL6
30	NP_002955.2	protein S100 A8 Homo sapiens	S100A8
31	NP_001035972.1	galectin 7 Homo sapiens	LGALS7B
32	NP_002265.2	keratin type I cytoskeletal 13 isoform b Homo sapiens	KRT13
33	XP_005257402.1	PREDICTED keratin type I cytoskeletal 15 isoform X1 Homo sapiens	KRT15
34	NP_000241.1	myeloperoxidase precursor Homo sapiens	MPO
35	NP_005545.1	keratin type II cytoskeletal 6A Homo sapiens	KRT6A
36	XP_005271596.1	PREDICTED apolipoprotein A I isoform X1 Homo sapiens	APOA1
38	NP_001054.1	serotransferrin precursor Homo sapiens	TF

Table 3. Down regulated proteins in SCC.

Table 3 Cont.

39	NP_005611.1	protein S100 A11 Homo sapiens	S100A11	
41	NP_002956.1	protein S100 A9 Homo sapiens	S100A9	
42	NP_001393.1	elongation factor 1 alpha 1 Homo sapiens	EEF1A1	
43	NP_005339.3	heat shock protein HSP 90 alpha isoform 2 Homo sapiens	HSP90AA1	
44	XP_005251118.1	PREDICTED 14 3 3 protein zeta delta isoform X2 Homo sapiens	YWHAZ	
45	NP_002266.2	keratin type I cytoskeletal 15 Homo sapiens	KRT15	
46	NP_000030.1	apolipoprotein A I preproprotein Homo sapiens	APOA1	
47	NP_000691.1	annexin A1 Homo sapiens	ANXA1	
48	XP_005271597.1	PREDICTED apolipoprotein A I isoform X2 Homo sapiens	KRT14	
49	NP_000415.2	keratin type II cytoskeletal 5 Homo sapiens	KRT36	
51	NP_000517.2	keratin type I cytoskeletal 14 Homo sapiens	ENO1	
52	NP_001243222.1	keratin type II cytoskeletal 8 isoform 2 Homo sapiens	YWHAB	
53	NP_001531.1	heat shock protein beta 1 Homo sapiens	KRT4	
54	NP_001902.1	cathepsin G preproprotein Homo sapiens	CALM1	
55	XP_005257819.1	PREDICTED keratin type I cuticular Ha6 isoform X1 Homo sapiens	KRT36	
56	NP_002146.2	heat shock 70 kDa protein 6 Homo sapiens	HSPA6	
57	NP_002955.2	protein S100 A8 Homo sapiens	S100A8	
58	NP_002271.3	keratin type I cuticular Ha5 Homo sapiens	KRT35	
59	XP_005271596.1	PREDICTED apolipoprotein A I isoform X1 Homo sapiens	APOA1	
60	NP_003395.1	14 3 3 protein beta alpha Homo sapiens	YWHAB	
61	NP_005611.1	protein S100 A11 Homo sapiens	S100A11	
62	NP_002263.3	keratin type II cytoskeletal 4 Homo sapiens	KRT4	
63	XP_006720321.1	PREDICTED calmodulin isoform X1 Homo sapiens	CALM1	
64	NP_002956.1	protein S100 A9 Homo sapiens	S100A9	

number of proteins in their samples. The uniquely expressed proteins we observed were found to have functional roles in molecular activities of cell including protein metabolism, carbohydrate metabolism, molecular chaperone, transcription, energy metabolism, RNA metabolism, tumour suppression, signal transduction, cell cycle regulation etc which correlates with similar studies. (Liebler, 2002; Roepstorff, 1997; Godovac-Zimmermann, 2001). Cytoskeletal proteins were the major group of up-regulated and uniquely expressed proteins in SCC with an abundance of different isoforms of cytokeratins, which supports the finding of Su-Mi Bae on tissue samples using two dimensional gel analysis (Su-Mi Bae, 2006). Cytokeratin is one of the major filament proteins in epithelial cells; their primary function is to protect epithelial cells from stress damage that may leads to cell death. Modification at the amino and carboxyl terminals of cytokeratin (phosphorylation, glycosylation and Trans glutamination) occurs during the oncogenic transformation of normal cells (Coulombe, 2002). These modification events lead to increase filament solubility. Tumour necrosis and cell lysis in carcinoma may release cytokeratin fragments into biological fluids including blood, urine and fluid in the abdomen and lung from ascites and pleural effusion, respectively by an unknown mechanism (Uenishi et al., 2006). However, the release of cytokeratin fragments is associated with the proteolytic degradation of cytokeratin during cell death, abnormal mitosis and spill over from cells undergoing proliferation and apoptosis (Oshima, 2002). A previous study has shown that cytokeratin levels are relatively low in healthy subjects and elevation is associated with apoptotic resist-

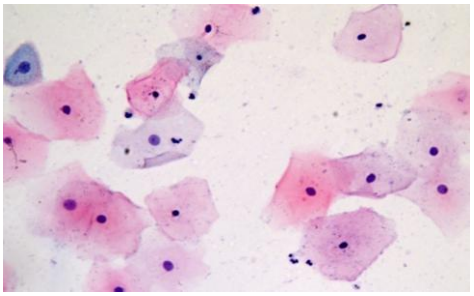

Figure 3. Normal cytology.

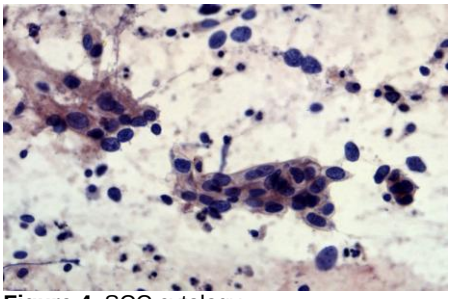

Figure 4. SCC cytology.

ance and malignant progression (Yuan et al., 1998). The different isoforms of cytokeratin in the samples of squamous cell carcinoma may be due to release of cytokeratin in invasive tumour cells. The presence of vimentin may be due to the expression of epithelial mesenchymal transition associated genes during invasion of the tumor. Functional role of Annexin is linked to anti-inflammatory activity but alteration of this gene has been detected in multiple tumours and involved in integrated pancreatic cancer and integrated breast cancer pathway (NP_000691.1). Among the Unique proteins observed in squamous cell carcinoma cells, ankyrins, Ras related proteins, decorin, receptor type thyrosine protein phosphatase, heat shock proteins, HLA class histocompatibility antigen, lumican, galectin, and gelsolin have certain role in the oncogenic transformation of which, ankyrin, lumican and decorin are in the proteoglycans family. The proteoglycans in the tumor micro environment contribute to various biological properties associated with malignancy like proliferation, adhesion, angiogenesis and metastasis. Decorin and Lumican are Leucin rich proteoglycans (XP_006719333.1) that can function as tumor repressors and regulate the signaling pathway by interacting with their core proteins and their multiple receptors. Among the different isoforms of Heat shock proteins (HSPA1B, HSPB1, HSPA8, HSPA6, HSP90AA1, HSPE1), HSPE1 is uniquely present and others were seen down regulated in SCC samples, (table 3) but a recent study in cervical biopsy samples have shown that heat shock protein 27(HSP 27) is one of the members of the small HSP family that function as a molecular chaperone in protein translocation, folding and overlapping , and have an essential role in tumor formation, both by promoting autonomous cell proliferation and by inhibiting apoptotic pathways such as the activation of caspase (Kamradt et al., 2002) and NF-kappa B (Kammanadiminti, 2006). Up regulation of HSP27 is commonly found in breast (Oesterreich et al., 1993) and ovarian cancer (Langdon et al., 1995). Proteins that are related to viral carcinogenesis uniquely expressed in our samples of malignancy were HLA class 1 histocompatibility antigen A and gelsolin isoform b (NP_001229687.1, NP_937895.1). There is a strong association between high risk HPV infection and the development of cervical cancer. Through the expression of many potent oncoproteins, these tumor viruses promote an aberrant cell proliferation through modulating signaling pathways in cell and escape from cellular defense mechanism such as blocking apoptosis and these oncoproteins disrupt the pathway necessary for maintaining host genome integrity that leads to malignant transformation.

The proteins obtained in invasive carcinoma samples seemed to be associated with various molecular pathways, namely TGF-beta signaling pathway (decorin), EPO signaling pathway (collagen alpha 3VI chain), PI3kAkt signaling pathway (receptor type thyrosine protein phosphatase c isoform), cAMP signaling pathway (dematin) and RAP signaling pathway (Ras related protein). Among these pathways, TGF- beta signaling pathway and RAP signaling path ways are involved in many cellular processes including cell growth, differentiation, apoptosis, homeostasis and other cellular functions. Regulation of TGF-beta signaling might be tightly linked to tumor progression, since TGF-beta is a potent growth inhibitor in most cell types (Miyazono, 2015). The PI3k-Akt signaling pathway is an intracellular signaling pathway important in regulating the cell cycle, so it is directly related to cellular quiescence, proliferation, cancer and longevity and this pathway is activated in a variety of different human cancers, and inhibitors of this pathway are under active development as anti-cancer therapeutics (Kwok Kin Wong et al., 2010). Another protein identified was Galectin-1, as uniquely expressed in SCC, which act as an autosome negative growth factor that regulate cell proliferation, very recently it was reported that these proteins can stimulate tumour angiogenesis and it was previously found in the vasculature of many human tumors including colon, head and neck, lung, and prostate etc. (Thijssen et al., 2010; Jung et al., 2007). Protein named rap guanine nucleotide exchange factor- 1 isoform and mothers against decape-

ntapelgic homolog 3 isoform 2 were reported to have some role in Rap 1 signaling pathway and pleury potency of stem cells regulating pathway (NP_001138574.1). Several proteins that were differentially expressed in LC/MS analysis of the cervical scrape smear from the squamous cell carcinoma patients have been recently reported to be expressed in other malignancies (galectin1&7,vimentin, protein S100), (Yao et al., 2007; Gebhardt et al., 2006). The other proteins identified in carcinoma samples were glyceraldehydes phosphate dehydrogenase, phospho glycerate mutase, enolase, triosphosphate isomerase, L-lactate dehydrogenase, ribosomal proteins, histone proteins etc. Similar studies reported some association for this group of proteins with the incoming nutrients for energy production and macromolecular biosynthesis to support cell growth and DNA Replication in the rapidly proliferating cells. This increase in cell metabolism has been described in cancer cells, which exhibit much high intake of glucose than normal cells as well as increased rates of glycolysis lactate production in presence of oxygen (Warburg, 1956; Ferguson, 2008; Resendis-Antonio et al., 2010; Vander et al., 2009). Squamous cell carcinoma antigen -2 (SCCA2) has been reported in cervical cancer tissues as a differentially expressed protein in a study of the cervical cancer tissues (Su-Mi Bae, 2006), which we couldn't find in the squamous carcinoma cells. Some of the previously established inhibitory proteins and DNA replication licensing proteins in Squamous cell carcinoma tissues were not identified in the current study which may be due to their low abundance.

CONCLUSION

The current study has identified a technique to separate the squamous epithelial cells without other cellular and non-cellular components often present in Pap smear and could reproducibly extract protein from the separated cervical cells. We got a very complex mixture of proteins in the scrape smears from patients diagnosed as SCC compared with normal samples and identified some tumor associated proteins, including HLA class 1 Histocompatibility antigen A, gelsolin isoform b, Galectin 1&7, ankyrin, protein S100 and cytokeratin KRT9, which can be tried to define candidate markers of cervical cancer and precancerous lesions with malignant potential. However, these finding needs to be further verified in epithelial cells separated from cervical scrape smears without the presence of macrophages and other non-epithelial cells. Also the proteomic results have to be compared with gene expression pattern of the same samples to identify overlapping patterns of expression.

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