Evaluation of Vimentin as a Potential Poor Prognostic Indicator and Salivary Biomarker for Oral Cancers and Pre-Cancers by Mass Spectrometry Based Proteomics

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Abstract

Background : Oral tongue squamous cell carcinoma (OTSCC) is an aggressive cancer with high morbidity and mortality rates, despite multimodality management. There are currently no clinically relevant molecular markers to help identify patients at a higher risk of recurrence and failure.

Methods: 2D-DIGE coupled with tandem mass spectrometry was performed on tissues obtained from early staged OTSCC along with its paired apparently adjacent normal tissue samples (n=10). Top upregulated protein was validated using another independent set of tissue samples by Immunohistochemistry (n=346), comprising of retrospective early stage OTSCC (n=150) and prospective series of oral pre-cancers, normal and oral cancers (n=195). For further validation of protein expression, saliva samples collected from Oral Cancer and pre-cancer samples were analysed by ELISA (n=80).

Results: We found vimentin, the mesenchymal protein to be the most upregulated protein in tongue tumour tissues compared to adjacent apparent normal tissues. Vimentin was found to be significantly overexpressed in oral pre-cancers along with cancers compared to normal tissues.

Conclusion: Vimentin detection in saliva can be useful diagnostic test to detect oral precancers that may have malignant potential needing closer follow up. Salivary ELISA for vimentin can additionally be useful for disease monitoring in oral cancers.

Background

Oral Tongue Squamous cell carcinoma (OTSCC) represents a major portion of oral cavity cancers, especially in India. Studies show a sharp increase in the incidence of tongue cancers in India[1]. Our earlier studies on south Indian patient population have shown that early staged tongue cancers (T1 and T2) constitute nearly 45% of all OTSCCs[2]. Studies have shown that OTSCC occurs at a younger age than cancers occurring in other subsites of oral cavity[3]. Despite being detected at an early stage about 40% of patients still die of the disease and need tailored treatment. Depth of invasion, tumour grade, perineural invasion are some of the factors indicating an aggressive phenotype but till date there are no relevant molecular markers indicating the high- risk tumours.

Proteomics helps to study the complete protein complements of the cell, which is a promising approach for the identification of novel protein biomarkers. These proteins can be used as key targets for therapeutic intervention and also as promising candidates for early detection of cancers[4, 5]. We have some recent studies showing the preliminary application of proteomics for the identification of biomarkers for OSCC [6, 7]. Comparison of protein expression profiles between OSCC and normal cell lines or tissues has revealed replicable and significant changes in the expression levels of number of proteins, including some metabolic enzymes, modulators of signal transduction pathways, and oncoproteins[8]. In the current study, we have performed 2D DIGE based proteomic profiling coupled with mass spectrometry approach and have validated the expression of top upregulated protein Vimentin, eventually to explore the prognostic stratification of early staged OTSCC. We have additionally studied the vimentin expression in Oral precancers and cancers comparing with normal tissues. Salivary ELISA for vimentin has been attempted to evaluate the secretion of vimentin in samples from normal healthy volunteers comparing with saliva from patients presenting with oral leukoplakia, OSMF and Oral squamous cell carcinoma.

Materials And Methods

Patients and tissue specimens

All research involving human participants had been approved by the authors' Institutional Review Board (IRB) and all clinical investigations had been conducted according to the principles expressed in the Declaration of Helsinki. A written informed consent was obtained from all the participants and the content of the informed consent was approved by the respective Institutional Research Board namely, Cancer Institute WIA; Protocol 1 HNCOG (Cancer Institute, Women India Association; Protocol 1 Head and Neck Co- operative Oncology Group) SBDCECM105/13/58 (Sree Balaji Dental College and hospital Ethical Committee Meeting reference number 105/13/158) and the Department of Oral medicine and Radiology, Sree Ramachandra Dental College and hospital from June 2018 till January 2019. Institutional Ethics Committee approval (IEC No.CSP/17/AUG/60/239) and Ethics approval from Department of Oral and maxillofacial pathology, Saveetha Dental College and hospital from December 2017 to August 2019 (SRB/SDMDS11/170MP/01) was obtained before the commencement of the study.

Patient tissue samples

Histologically apparently normal adjacent tongue tissues along with paired early staged OTSCC tumour tissues (n=10) were obtained from patients presenting with OTSCC and undergoing surgery. Formalin fixed paraffin embedded samples from buccal leukoplakia (n=50), oral cancers (n=71) were obtained from Sree Balaji Dental College and hospital and formalin fixed tissues from oral submucous fibrosis samples (n=32) and normal buccal mucosa tissues (n=42) were obtained during third molar extraction were collected from Sree Ramachandra Dental College and Hospital. This was an independent cohort (n=195) for validation studies. Saliva samples (n=80) were collected from oral cancer patients (n=45), patients with oral potentially malignant lesions (n=15) and normal (n=20) obtained from Saveetha Dental College and Hospital and Sree Ramachandra Dental College and Hospital. Additionally, formalin fixed paraffin embedded sections from retrospective series of exclusively early staged tongue cancer patients [T1 and T2] (n=150) were obtained from Cancer Institute WIA who had been treated between 1995 to 200 for validation studies of the findings with the complete treatment follow up. All the FFPE sections (n=345) used for the study were histologically examined by oral pathologist VS and PR.

Patient Saliva samples

ELISA was done for saliva samples (n=80) collected from Oral cancer patients, patients presenting with oral potentially malignant lesions and absolute normal volunteers. The study participants were requested to refrain from drinking, eating, chewing tobacco or smoking 1 hour prior to the collection of saliva. After obtaining the informed consent of the patient, 0.5 to 1 ml of whole unstimulated saliva was collected by passive expectoration and patients were asked to spit into a 50-mL sterile tube containing 10µL of proteinase inhibitor (Proteinase inhibitor cocktail P2714 Sigma Aldrich). The saliva samples were transferred to 1.5-mL sterile microtubes and centrifuged for 3 minutes at 13,000 rpm. Supernatants, separated from the cellular phase, were immediately aliquoted and stored at -80^{o}C within 60 minutes after saliva collection.

Proteomics - Proteins Labelling with CyDyes

Pooled OTSCC tumour and pooled adjacent uninvolved tissues were used for the proteomic profiling. Lysine labelling protocol, (minimal labelling) used in this study is described before[9]. The processed tissue proteins were labelled individually with dyes Cy3 and Cy5 while the pooled tissue proteins prepared by mixing equal aliquot of protein from all samples in an experimental set up were labelled with Cy2. The final volume for all preparations was adjusted to a total of 340 uL with rehydration buffer (7 M urea, 2 M thiourea, 1% IPG buffer, 50 mM DTT, 4% CHAPS, and a trace amount of bromophenol blue). A reciprocal labelling experiment was also performed.

Proteomics – 2D gel electrophoresis

Two-dimensional gel electrophoresis of CyDye labeled proteins was done as described before[10, 11] with the following modifications. Eighteen cm IPG strips of pH 4–7 (GE Healthcare, Uppsala, Sweden) was employed in the first dimension. Labelled proteins were focused for a total of 80,000 Vhs at a constant temperature (20^{o}C) under linear voltage ramp after an active IPG rehydration at 30 V in a IPGPhor III (GE Healthcare, Uppsala, Sweden) apparatus. Following IEF, each IPG strip was placed in the equilibration buffer containing 2% DTT first followed by incubation in another buffer in which the DTT was replaced by 2.5% iodoacetamide. The second dimension PAGE (12.5%) was carried out in an EttanDaltSix systems (GE Healthcare, Uppsala, Sweden) at 1 W/gel for 1 hr and 13 W/ gel for 5 hr. All experimental procedures were performed in dim light or in the dark.

Protein Visualisation and DeCyder Image analysis

The protocols for the protein visualization and image analysis using DeCyder has been mentioned previously[12]. Briefly, after second dimension electrophoresis, the gels were scanned with Typhoon FLA 9500 Variable Mode Imager (GE Healthcare, Uppsala, Sweden). Cy2, Cy3 & Cy5 images were captured using the settings recommended by the manufacturer. A DeCyder differential in-gel analysis (DIA) module was used for image analysis between samples within the same gel while a DeCyder biological variation analysis (BVA) module was performed for pairwise image analysis among multiple gels. Student's t-test and ANOVA were used to compare the average spot volume and differences of protein abundance for all detectable spots between the tumor and normal groups. Reciprocal dye labelling was performed to normalize bias in labelling.

Protein Identification and Mass Spectrometry (MS)

Pooled tongue tissue proteins (250 µg) were separated on 18 cm IPG strips of pH 4-7 in the first dimension. First and second dimension electrophoresis were done as given under 2D DIGE method. The second-dimension gels were stained with colloidal coomassie blue G-250 and gel spots from this preparative gel were excised manually for in-gel trypsin digestion and LC-MS/MS was performed. Extracted peptides were dried under vacuum for 90 min and stored at 4 °C. Zip tip purified peptides were analysed using nano-RPLC (Thermo

Scientific, USA) coupled with an Orbitrap Elite Mass spectrometer (Thermo Scientific, USA). Peptides were ionized by positive mode electrospray with an ion spray voltage of 1.9 kV. The MS data were acquired in positive ion mode over mass range m/z 350–4000 Da using Xcalibur software (version 2.2.SP1.48) (Thermo Scientific USA). MS data were analysed using Proteome Discoverer software v.1.4 (Thermo Scientific) using Sequest algorithm with database downloaded from Uniprot as described earlier[12]. The combined list of official gene symbols corresponding to the identified proteins was used for input. We used STRING (www.string.db.org)[13] network construction.

Immunohistochemistry (IHC)

The IHC detection methods was as mentioned previously[14]. Briefly, IHC for vimentin was performed on 5 µm sections of FFPE tissues. The sections were deparaffinized in xylene and rehydrated in absolute ethanol. Antigen retrieval was done with 0.05M citrate Buffer (pH 9) in pressure cooker for 20 minutes. Endogenous peroxidase activity was blocked by incubation in 0.03% hydrogen peroxide in distilled water for 10 minutes and then washed with phosphate buffered saline (PBS). Sections were counterstained with hematoxylin, dehydrated, and mounted in DPX. Positive controls and negative controls were included appropriately where primary anti-body was replaced with 2% BSA in negative control. Immunostaining of the sections was reviewed with the corresponding haematoxylin and eosin-stained sections.

IHC Scoring

Immunohistochemical scoring for the target was done as described earlier[15]. Briefly, the percentage grade of stained tumor cells was scored as 0, negative; 1, <10%; 2, 11–50%; 3, 51–80%; or 4, >80% positive cells and the intensity of stain was scored as 0, negative; 1, weak; 2, moderate; or 3, strong. The immunoreactive (IR) score was calculated as a product of the percentage grade and intensity score with the IR score ranged from 0 to 12. The immunoreactivity was divided into three groups on the basis of the final score: negative immunoreactivity was defined as a total score of 0, low immunoreactivity was defined as a total score of 1–4, and high immunoreactivity was defined as a total score >4. The immunostaining of the tumor invasive front was evaluated using the same method as mentioned for tumor areas.

ELISA in Saliva Samples

The RayBio® Human Vimentin ELISA (Enzyme-Linked Immunosorbent Assay) kit is used to quantify the expression of vimentin in saliva samples (n=80), of which saliva samples from patients with oral cancer (n=45), patients with oral potential pre-malignant lesions (n=20) and healthy volunteers (n=15) were used. This is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human Vimentin in saliva samples. This assay employs an antibody specific for human Vimentin coated on a 96-well plate. Standards and samples are pipetted into the wells and Vimentin present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human Vimentin antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of Vimentin bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm. The standard graph was plotted with the vimentin standard protein provided in the kit. Using the standard graph the protein concentration were extrapolated for the unknown OD values obtained from saliva samples obtained from patients and healthy volunteers.

Statistical analysis

The relative levels of stained protein spots compared with the internal standard spots were analyzed by DeCyder Difference In-gel Analysis (DIA) and DeCyder Biological Variation Analysis (BVA) software modules (GE Healthcare). Student's *t*-test was used to calculate statistically significant differences between 2 groups in relative abundance of individual protein spots among the groups in 2D-DIGE. $P<0.05$ was considered statistically significant. Other statistical analysis was done using SPSS (IBM Corporation version 16).

Results

Quantitative proteomics using 2D DIGE and Mass Spectrometry in OTSCC shows vimentin as the most upregulated differentially expressed protein in early staged tongue cancer

Comparative proteomic analysis of pooled tongue tissue samples obtained from OTSCC patients compared to pooled adjacent apparent normal samples are shown in Fig. 1. More than 95 protein pairs were obtained in the image analysis platform, among which

45 were upregulated in tumour samples compared with adjacent normal protein samples. Out of the differentially expressed proteins, top 10 differentially expressed spots were taken for mass spectrometry. Table 1 describes the protein identification details for the differentially regulated proteins with the accession number matched in the database, mass spec probability score, percentage of sequence coverage match. Table 2 describes the average fold ratio with ANOVA value for differentially regulated proteins. The top 10 differentially regulated proteins have been listed in Table 2, in which eight proteins were significantly upregulated and two proteins were significantly downregulated in tumour samples. Quantitative 2D-DIGE proteomic approach coupled with tandem mass spectrometry identified, Vimentin as the topmost upregulated protein in OTSCC (Fig. 1a).

Table 1
List of differentially regulated proteins identified using Mass spectrometry

Spot ID	Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
1	B0YJC4	Vimentin OS = Homo sapiens GN = VIM PE = 3 SV = 1 - [B0YJC4_HUMAN]	76.06	51.97	31	12	28	52	431	49.6	5.25
2	P60174	Triosephosphate isomerase OS = Homo sapiens GN = TPI1 PE = 1 SV = 3 - [TPIS_HUMAN]	52.41	49.30	3	2	13	29	286	30.8	5.92
3	P30041	Peroxiredoxin-6 OS = Homo sapiens GN = PRDX6 PE = 1 SV = 3 - [PRDX6_HUMAN]	49.05	51.79	3	9	13	31	224	25.0	6.38
4	P30084	Enoyl-CoA hydratase, mitochondrial OS = Homo sapiens GN = ECHS1 PE = 1 SV = 4 - [ECHM_HUMAN]	63.59	45.86	1	9	15	39	290	31.4	8.07
5	P52565	Rho GDP-dissociation inhibitor 1 OS = Homo sapiens N = ARHGDIA PE = 1 SV = 3 - [GDIR1_HUMAN]	135.67	57.35	8	10	20	138	204	23.2	5.11
6	Q32Q12	Nucleoside diphosphate kinase OS = Homo sapiens GN = NME1-NME2 PE = 2 SV = 1 - [Q32Q12_HUMAN]	43.60	47.60	9	4	9	21	292	32.6	8.48
7	P02671	Fibrinogen alpha chain OS = Homo sapiens GN = FGA PE = 1 SV = 2 - [FIBA_HUMAN]	42.42	32.68	4	5	22	40	866	94.9	6.01
8	Q5I6Y6	Lamin A/C transcript variant 1 OS = Homo sapiens GN = LMNA PE = 2 SV = 1 - [Q5I6Y6_HUMAN]	29.77	20.18	9	6	11	17	664	74.0	7.18
9	P02144	Myoglobin OS = Homo sapiens GN = MB PE = 1 SV = 2 - [MYG_HUMAN]	81.93	68.83	8	8	16	50	154	17.2	7.68

Spot ID	Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
10	P24844	Myosin regulatory light polypeptide 9 OS = Homo sapiens GN = MYL9 PE = 1 SV = 4 - [MYL9_HUMAN]	51.33	54.65	9	7	9	25	172	19.8	4.92

Table 2
List of differentially expressed proteins with regulation status, fold ratio and ANOVA

Spot ID	Gene ID	Regulation status in Tumor	Average fold ratio	ANOVA
1	VIM	UP	4.89	0.00064
2	TPI1	Up	3.75	0.00047
3	PRDX6	Up	3.6	0.0041
4	ECHS1	Up	3.31	0.0007
5	ARHGDIA	Up	2.89	0.00096
6	NME2	Up	2.13	0.0025
7	FGA	Up	1.98	0.0045
8	LMNA	UP	1.59	0.046
9	MB	Down	-1.5	0.021
10	MYL9	Down	-1.92	0.0032

Figure 1b shows the highly upregulated protein vimentin marked in a box region across both the gels. The 3D view and the graphical log value of vimentin protein spot expression in 2D DIGE gel was analyzed and represented in Fig. 1c along with the magnified 2D gel view. The average ratio of upregulation of vimentin was 4.89 folds higher compared to the normal counterpart. The one-way ANOVA p value was found to be 0.00064 (Fig. 1d). The log standardized abundance was significantly high compared to the normal tissue protein (Fig. 1e).

Functional classification of the identified Vimentin protein and biological network analysis reveals its significance in tumorigenesis

The STRING[13] cluster analysis revealed that Vimentin forms a strong protein interaction with other partners, comprising of three major networks; first cluster is with most of the Small Nuclear Ribonucleo Proteins (SNRP) proteins; second cluster with different members of tropomyosin and third cluster with many caspases (Fig. 2). All these protein networks are known to have a key role in different tumorigenesis pathways; thus, this interaction analysis describes the significance of the identified Vimentin protein's regulation in tumorigenesis process.

Validation of Vimentin Expression to evaluate the role in early staged OTSCC

To validate the expression of Vimentin and its role in OTSCC, we undertook a retrospective cohort of exclusively early staged (T1 and T2) OTSCC patients (n = 150) treated as per the decision of the multispeciality board between 1995 to 2007. The clinicopathological features of early staged OTSCCs analysed based on vimentin protein expression is shown in Table 3. Median age of the cohort was 55 years and median OS was 74 months and DFS was 22 months. The pattern of vimentin expression was predominantly cytoplasmic. Positive immunoreactivity for Vimentin was identified in 58 (38.6%) patients and Vimentin was negative in 92 (61.31%) patients.

Table 3
Clinicopathological features of early stage OTSCC (with follow-up after treatment) analysed based on Vimentin protein expression

Clinical Parameters	N 150	Vimentin Negative (n = 92)	Vimentin Positive (n = 58)	P value
Age				
< 55 years	82	45 (54.9)	37 (45.1)	
> 55 years	68	47 (69.1)	21 (30.9)	
Sex				
Male	102	65 (63.7)	37 (36.3)	
Female	48	27 (56.2)	21 (43.8)	
Site				
Lateral border	132	77 (58.3)	55 (41.7)	
Tip	2	0	2 (100)	P = 0.012
Dorsum	5	5 (100)	0	
Ventral Aspect	11	10 (90.9)	1 (9.1)	
Stage				
Stage 1	56	42 (75)	14 (25)	P = 0.008
Stage 2	94	50 (53.2)	44 (46.8)	
Tumor Size				
0–2 cm	58	43 (74.1)	15 (25.9)	
2.1-3 cm	89	46 (51.7)	43 (48.3)	P = 0.009
> 3 cm	3	3 (100)	0	
Pattern				
Exophytic	42	22 (52.4)	20 (47.6)	
Infiltrating	88	52 (59.1)	36 (40.9)	P = 0.014
Ulcerated	20	18 (90)	2 (10)	
Grade				
WDSCC	116	73 (62.9)	43 (37.1)	
Mod to poorly	27	14 (51.8)	13 (48.1)	
Tobacco Habits				
Chewer	44	21 (47.7)	23 (52.3)	
Smoker	27	19 (70.4)	8 (29.6)	
Chewer + Smoker	17	12(70.6)	5 (29.4)	
Non User	62	40 (64.5)	22 (35.5)	
Alcohol (Yes)	21	13 (61.9)	8 (38.1)	
Upfront Management				
Observation	73	51 (69.9)	22 (30.1)	
RND	33	17 (51.5)	16 (48.5)	

Clinical Parameters	N 150	Vimentin Negative (n = 92)	Vimentin Positive (n = 58)	P value
Rad to Neck	24	24 (54.5)	20 (45.5)	
Failure Pattern				
No Evidence of Disease	65	46 (70.8)	19 (29.2)	
Local Recurrence	27	14 (51.9)	13 (48.1)	P = 0.028
Nodal Recurrence	18	14 (77.8)	4 (22.2)	
Locoregional Recurrence	39	18 (46.2)	21 (53.8)	
Distant Metastasis	1	0	1 (100)	
Treatment Outcome				
No Evidence of Disease (NED)	65	46 (70.8)	19 (29.2)	P = 0.038
Failure	85	46 (54.1)	39 (45.9)	
Survival				
Alive - NED	84	49 (58.3)	34 (40.4)	
Alive with Disease	4	4 (100)	-	
Dead	63	39 (61.9)	24 (38.1)	

Numbers in the brackets indicate percentages

Vimentin expression was significantly correlated to higher clinical stage and increasing tumour size in early staged OTSCC

Among the vimentin positive tumours, 46.8 % (44/94) belonged to stage 2 compared to 25% (14/56) in stage 1 which was significant statistically ($p = 0.008$; $\chi^2 = 7.038$). Among the vimentin positive tumours, 48.3% (43/89) had a tumour size > 2 cm compared to 25.9% (15/58) of vimentin positive immune-expression in tumours of size less than 2 cm which was significant statistically ($p = 0.009$; $\chi^2 = 9.394$). There was decreased vimentin positivity identified in tongue tumours that was ulcerated 10% (2/20) compared to the exophytic and infiltrating ($p = 0.014$; $\chi^2 = 8.536$).

Vimentin expression was significantly correlated with failure of treatment, pattern of recurrence and poor progression free survival in early staged OTSCC

The patients with showing strong and positive immunoexpression of vimentin had a higher failure rates [45.9% (39/85) vs 29.2% (19/65)] which was statistically significant, ($p = 0.038$; $\chi^2 = 4.306$) and the data was represented in Table 3. Positive immunoexpression of vimentin was significantly associated with locoregional recurrence [53.8% (21/39) vs 29.2% (19/65)] patients showing no evidence of disease which was significant statistically ($p = 0.028$; $\chi^2 = 10.892$).

Vimentin expression at Invasive front of the tumours indicated failure of treatment and locoregional recurrence in early staged OTSCC

Vimentin at ITF was positive in 47.6% (50/105) of patients whose tumours showed an invasive tumour front. Among the patients who showed locoregional recurrence with ITF in the tumours, 68% (17/25) showed a positive vimentin at ITF compared to 32% (8/25) patients whose tumours ITF had negative immune-expression as shown in Table 4.

Table 4
Vimentin expression at Invasive tumour front (ITF) vs pattern of failure in early staged OTSCC with treatment follow up

Vimentin Status	No Evidence of Disease (NED)	Local Recurrence	Nodal Recurrence	Locoregional recurrence
Negative expression at ITF (n = 55)	28 (50.9)	9 (16.4)	10 (18.2)	8 (32)
Positive expression at ITF (n = 50)	20 (40)	10 (20)	3 (16)	17 (68)

P = 0.002; $\chi 2$ = 14. 792 (numbers in brackets denote percentages)

Patients undergoing modified neck dissection for neck management had the best survival among the early staged OTSCC

Patients whose tumours had positive vimentin expression had an decreased DFS compared to the patients whose tumours had negative vimentin expression and this association was statistically significant (Log Rank = 4.068 ; p = 0.044) (Fig. 3a). The patients in this cohort were subjected to upfront neck management, namely neck node observation (n = 73), modified radical neck dissection [MRND] (n = 33) and radiation to neck (n = 44). Kaplan Meier survival curves of DFS based on upfront neck management showed that patients who underwent MRND having a better survival (p = 0.006; log rank = 10.094) compared to patients given radiation to neck. (Fig. 3b)

Vimentin expression correlates with severity of dysplasia in oral pre-cancers and indicates malignant potential

Based on the findings of the retrospective study, we undertook another cohort of patients (n = 196) with oral leukoplakia (n = 50), OSMF(n = 32) and invasive cancers involving buccal cavity (to ascertain if Vimentin can useful in oral cancer inclusive of both sites oral tongue and buccal cavity. This cohort had 82 oral precancers comprising of leukoplakia (n = 50) and OSMF (n = 32) along with normals (n = 42) and Cancers of buccal cavity (n = 72). Table 5 shows the demographic details of subjects and patients who presented with oral pre-cancers at different stages and oral cancers presenting to the dental clinic. The median age of this group was 45 years.

Table 5
Vimentin Expression in Oral Pre-cancers and Oral Buccal cancers along with Normal

Clinical Parameters	N 195	Vimentin Negative (n = 144)	Vimentin Positive (n = 51)	P value
Age				
< 45 years	**93**	81 (87.1)	12 (12.9)	P = 0.000
> 45 years	**102**	63 (61.8)	39 (38.2)	$\chi 2$ = **16.163**
Sex				
Male	139	107 (77)	32 (23)	
Female	56	37 (66.1)	19 (33.9)	
Diagnosis				
Normal	42	42 (100)	0	
Mild Dysplasia	24	22 (91.7)	2 (8.3)	
Moderate Dysplasia	13	10 (76.9)	3 (23.1)	
Severe Dysplasia	13	11 (84.6)	2 (5.4)	P = 0.000
OSMF	32	31 (96.8)	1 (3.2)	$\chi 2$ = **77.037**
WDSCC	40	17 (42.5)	23 (57.5)	
MDSCC	29	9(31)	20 (69)	
Verrucous	3	3 (100)	0	
Habits				
Pan	96	70 (72.9)	26 (27.1)	
Betel Quid	35	21 (60)	14 (40)	P = 0.045
Sharp tooth	64	53 (82.8)	11 (17.2)	$\chi 2$ = **6.181**

Numbers in the brackets indicate percentages

Vimentin expression was analysed by IHC in totally 195 samples, of which 144 samples (73.9%) showed the vimentin negative expression and 51 samples (26%) showed vimentin positive expression in IHC analysis. The median age of this cohort was 45 years and age had a significant association with the vimentin positive expression ($p = 0.000$; $\chi^2 = 16.163$). The immuno-expression pattern of vimentin is represented in Table 5 describing the positive expression of vimentin having a significant association ($p = 0.000$: $\chi^2 = 68.524$) with oral precancers and cancers compared to apparent normals. Interestingly, vimentin was significantly correlated to the de-differentiated state of the oral precancers with oral cancers ($p = 0.000$; $\chi^2 = 77.037$). Vimentin is a significant biomarker for oral pre-cancers that may have an aggressive potential to turn into malignancy. Figure 4 shows the IHC based patterns of vimentin expression in Oral cancers.

Vimentin secretion is significantly elevated in saliva samples obtained from Oral Cancer and Oral pre-cancer patients compared to healthy volunteers

Since tissue availability in oral cancers and pre-cancers involve invasive procedures as a biopsy, we wanted to evaluate if vimentin could be detected in saliva, as saliva can serve as a non-invasive medium for early detection and also for disease monitoring. Vimentin secretion in saliva could be detected as significantly high in Oral cancer and pre-cancer patients. The concentration obtained in salivary ELISA for Vimentin detection range from 2.3–6.4 ng/ml for healthy volunteer samples, for precancer samples, the concentration ranges from 4.5–16.8 ng/ml and for cancer samples, the concentration ranges from 4.8–127 ng/ml. The fold increase between healthy volunteer sample and precancer samples were statistically significant. The differential expression was analysed using SPSS software and observed to be statistically significant and the data was represented in Fig. 5a. The diagnostic potential of the underlying

pathological implication could be detected by ROC curve analysis showing AUC = 0.8 which had a high statistical significance (Fig. 5b & 5c).

Discussion

Vimentin is a well-known mesenchymal protein acting as a scaffold for signalling proteins that are important for cancer cell invasion[16] wound healing, tissue repair[17] tissue ageing and apoptosis[18, 19]. This study describes the role of vimentin that emerged as the most upregulated protein in OTSCC by quantitative proteomics. We have validated the expression of vimentin in early staged OTSCC inferring its role as a poor prognostic indicator. The role of vimentin was further evaluated in oral precancers and in saliva indicating that vimentin can be a very useful marker in oral cancer for determining prognosis and can be used for early detection of the disease in saliva and Oral precancer tissues. Vimentin can be good biomarker because none of the normal buccal mucosa tissues expressed vimentin as expression of vimentin is indicative of mesenchymal transition. The current study showed aberrant vimentin expression in oral premalignant lesions and oral cancers.

Previous study done as a meta-analysis of differentially expressed genes in OTSCCs has shown the comprehensive expression profiling of genes identifying the role of extracellular matrix with EMT based deregulation in OTSCC showing the role of tumour microenvironment in OTSCC with a number of extracellular matrix (ECM) components playing a crucial role in patient prognosis[14].

As the first step, we evaluated the global proteomic profiles in early staged oral tongue cancer samples by 2D DIGE followed by mass spectrometry analysis. 2D-DIGE technique enables direct comparison of protein profile between tumor and normal samples on the same single 2D gel, thus reducing technical variability which could affect the expression pattern of proteins. Recent studies have used the conventional 2D electrophoresis[7] and identified a panel of 12 proteins in tongue cancer, but absence of validations in normal tissues in the same gels can possibly lead to biased conclusions owing gel-to gel variations. To overcome this, we attempted 2D DIGE based proteomic discovery in the current study and have shown that proteins involved in cytoskeletal remodelling that are involved in the process of tumorigenesis. Proteomics approach can give rise to several markers, as shown in the current study but these biomarkers need to be chosen as per their clinical relevance based on validation studies Our studies has shown additional 9 top differentially regulated proteins that can be subsequently validated in oral cancer tissue and saliva samples. Along with vimentin, we also obtained laminin A/C transcript, myoglobin as significant markers by proteomics approach and they are well known stromal components playing important role in ECM modulation. Vimentin has been evaluated as a useful marker for aggressive pathology and poor prognosis in tongue cancers, its utility can be explored in patients to assess patients who are more likely to fail treatment, despite being early staged.

To validate this finding, as a second step, we evaluated the vimentin expression in retrospective series of exclusively early staged OTSCC. Early staged OTSCC need a biomarker to identify the patients who are more likely to fail despite being in T1 or T2 stage. The aggressive nature of OTSCC is reflected by the increased rates of local recurrence, occult node and distant metastasis. Though several histological features like extracapsular spread, perineural invasion, and presence of lymphovascular emboli are adverse factors, there is still an unanswered need of objective molecular markers that can be useful to identify patients needing further attention.

This study showed that upfront neck management was an important factor to predict event free survival. Patients who underwent modified neck dissection had the best overall survival among the early staged OTSCC showing the importance of neck dissection in OTSCC. This result was in agreement to the previous randomised controlled trial showing elective neck dissection showed higher rates of overall and disease-free survival in early staged OTSCC[20]. We did not find a significant correlation with overall survival and vimentin expression unlike the disease-free survival which is similar to previous reports[21]. Positive vimentin expression was found to be associated with increased stage, increased size of the tumour, increased treatment failures, increased locoregional recurrence and poorer disease-free survival. Earlier studies have identified vimentin over-expression to be a poor prognostic indicator in OTSCC by univariate analysis[21–24]. Our study confirms the earlier findings. It has been suggested that cancer cells present in the invasive tumour front (ITF) are more aggressive in terms of their metastatic potential[25]. Our study emphasises that expression of vimentin assessed at ITF can indicate the EMT switch which is known to be associated with increased motility and invasiveness. We found a significant association with locoregional recurrence and vimentin expression at ITF. Vimentin at ITF and tumour sites has been shown to be strongly correlated to aggressive phenotype contributing to poor prognosis[26]. Aberrant expression of vimentin has been incriminated in various epithelial cancers[27–32] including OSCC[33]. Vimentin has been shown as a predictive biomarker for tumor growth and metastasis, although its understanding is limited in OTSCC prognosis[34, 35].

As a third step, we wanted to evaluate the significance of vimentin in oral pre-malignant lesions that would comprise of leukoplakia, OSMF as well as buccal cancers. We wanted to evaluate if Vimentin can be a biomarker for oral cancers, comprising of both the major

sites buccal as well as tongue. We found a significant association of vimentin expression in oral precancers and cancers. Increased age was correlated to the vimentin expression mainly because of higher incidence of cancers in subjects with increasing age. Most of the potentially malignant disorders are asymptomatic and treatment can be of three types namely close observation, surgical excision/ablation and medical treatment. There is a lack of standardised diagnostic criteria in visual inspection of oral cavity to identify potentially malignant lesions that may eventually progress. Previous studies have shown Vimentin expression in lesions of leukoplakia and submucous fibrosis could be an early event in tobacco and areca nut associated tumorigenesis process.

As the fourth step, we have evaluated vimentin expression in saliva as a non-invasive means and have shown that vimentin can be a good marker for both early detection and disease monitoring in oral cancers. Our current study confirms this finding with a higher vimentin expression in saliva samples using ELISA method with a significant diagnostic potential for identifying patients with poor prognosis. This can be validated in higher number of samples of oral pre-cancers and cancers for early detection and disease monitoring. To our knowledge this is the first study evaluating vimentin in saliva from our country. Vimentin has been shown to be secreted by distinct population of vascular endothelial cells and activated macrophages and can accumulate in the blood previously[36, 37].

Vimentin secretion has been shown to be induced by pro-inflammatory cytokines TNF-α, and LPS suggesting that vimentin secretion is an inflammatory response[38]. We found that vimentin secretion was indeed very elevated in certain oral precancer samples that they could be an inflammatory response and more prone for malignant transformation in future.

A recent report reviewing all the promising biomarkers identified in tongue cancers have shown vimentin as one of the strongest bio-marker with significant relevance as a marker with clinical utility[39] proving that it is an important marker of OTSCC which innately shows a higher propensity to metastasize confirming our reports. As per our findings Vimentin in pre-cancers can help identify patients most likely to progress to malignancy as is an useful early detection marker. In recent days, the non-invasive nature of saliva and its significant relationship with plasma levels made saliva an very attractive diagnostic tool[40].Up to our knowledge, this is the first report to describe the status of vimentin expression in saliva samples obtained from precancer, cancer patients with oral squamous cell carcinoma. Oral cancer is a very common cancer in Indian population and there were very few studies aimed to identify the biomarkers with validation study. We have identified the candidate proteins altered in our Indian population and validated them with both IHC and ELISA analysis.

Conclusion

In conclusion, 2D DIGE coupled with tandem mass spectrometry was found useful to identify differentially expressed proteins in OTSCC tissues. All the quantitative tissue proteomics-based markers identified in current study needs validation in OTSCC tissues as a prospective study with larger numbers of samples. The current study has been pursued for vimentin, a well characterised EMT marker. It was found clinically relevant to prognosticate early OTSCC patients most likely to fail treatment, requiring specific tailored treatment. Vimentin was also useful as an early detection biomarker of precancers in oral cavity. In addition, the vimentin protein expression has been validated in saliva samples obtained from precancer and cancer patients and found to be significantly upregulated when compared to normal samples, proving its role as a useful biomarker for early detection and disease monitoring.

Declarations

Ethics approval and consent to participate

For all the samples used in this study, the ethics approval had been obtained and the details were enclosed in the methodology section. The consent to participate in the study was obtained from all the patients.

Consent for publication

Not Applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest Statement

All authors disclose that there is no conflict of Interest.

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AUTHOR CONTRIBUTIONS

CONTRIBUTION	
STUDY CONCEPT	AS, VR
STUDY DESIGN	AS, VR
DATA ACQUISITION	AS, TSV, VS, HK, CVD
QUALITY CONTROL OF ALGORITHMS	AS, VS, PR, AK
DATA ANALYSIS AND INTERPRETATION	AS, TSV, VR
STATISTICAL ANALYSIS	AS , TSV, VR
MANUSCRIPT PREPARATION	AS, AK, VR
MANUSCRIPT EDITING	AS, AK, VR
MANUSCRIPT REVIEW	AS, VS, PR, AK, TSV, HK, CVD,VR

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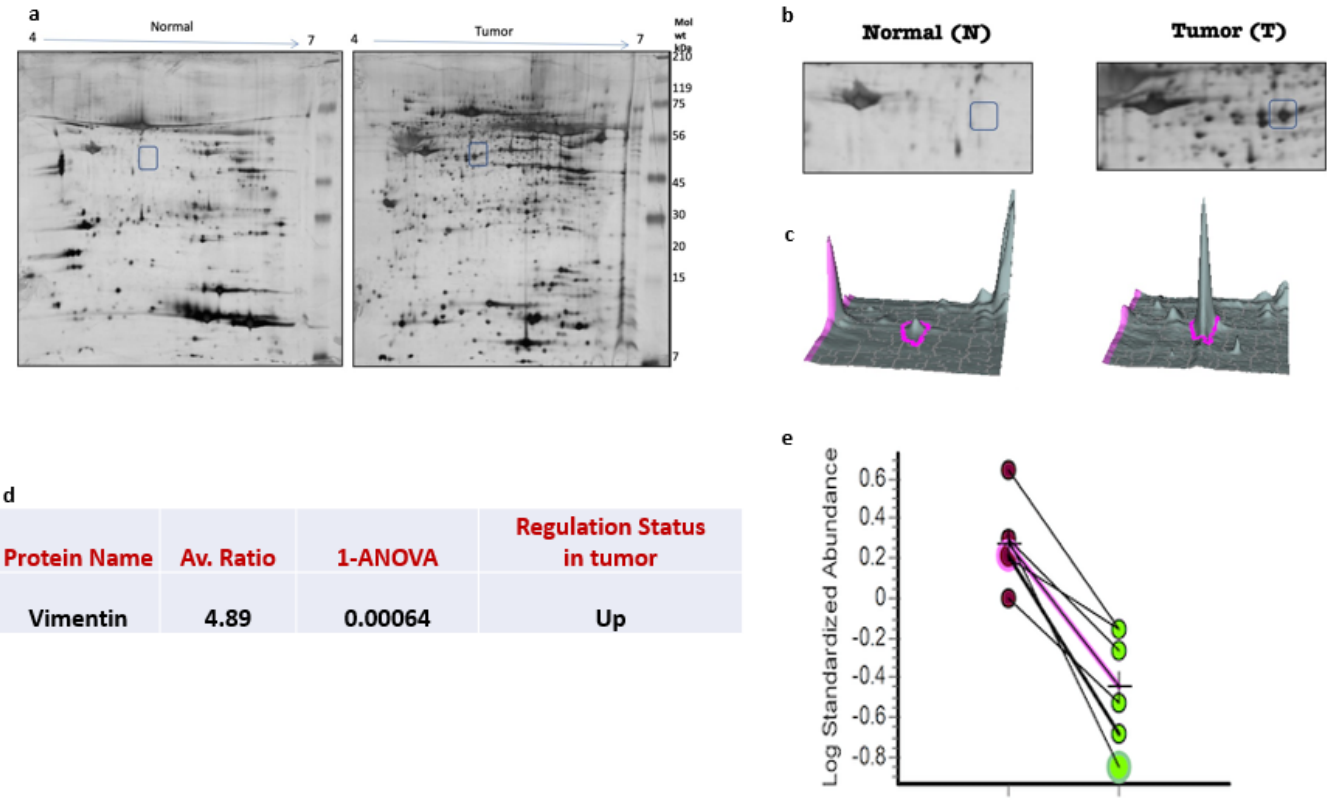
Figures



d

Protein Name	Av. Ratio	1-ANOVA	Regulation Status in tumor
Vimentin	4.89	0.00064	Up

Figure 1

Representative 2DE gels of Normal and Tumor sample, 3D view, graphical representation and fold ratio analysis for differentially regulated proteins

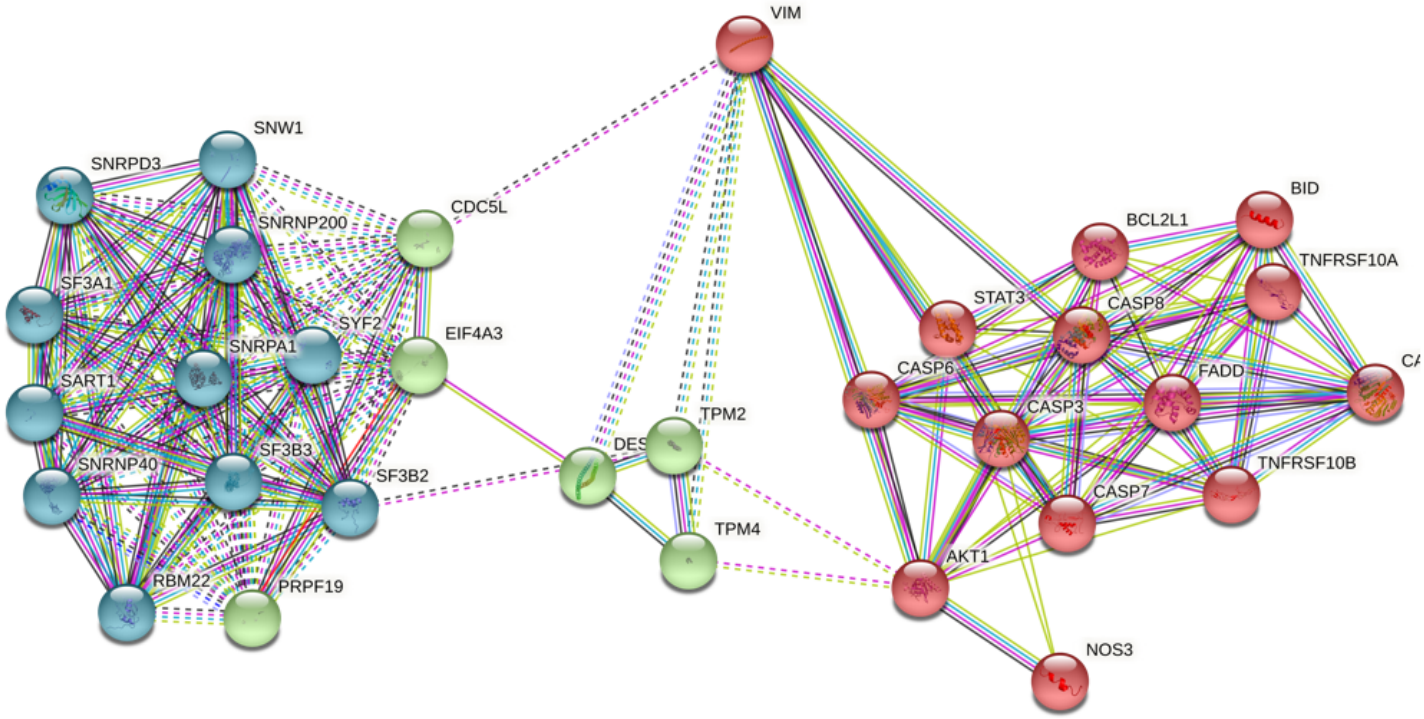


Figure 2

Cluster Analysis for the differentially regulated protein Vimentin using STRING database

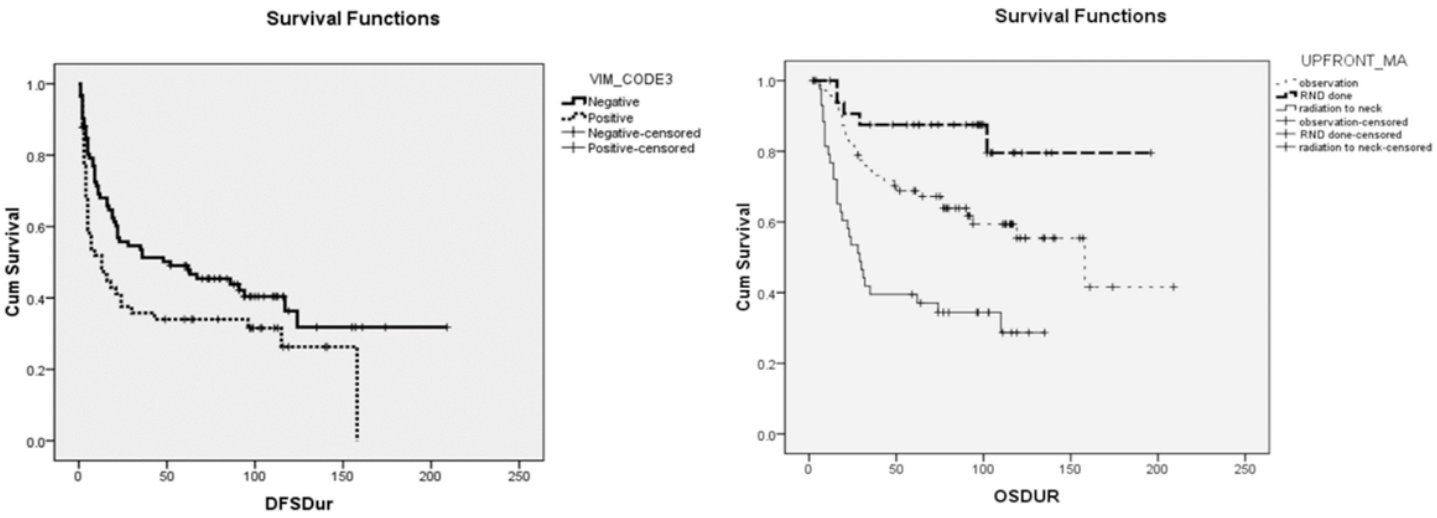


Figure 3

a : Kaplan Meier curves showing survival fractions in Patients with vimentin expression pattern b : Kaplan Meier curves showing survival fractions in Patients undergoing the different types of upfront neck management

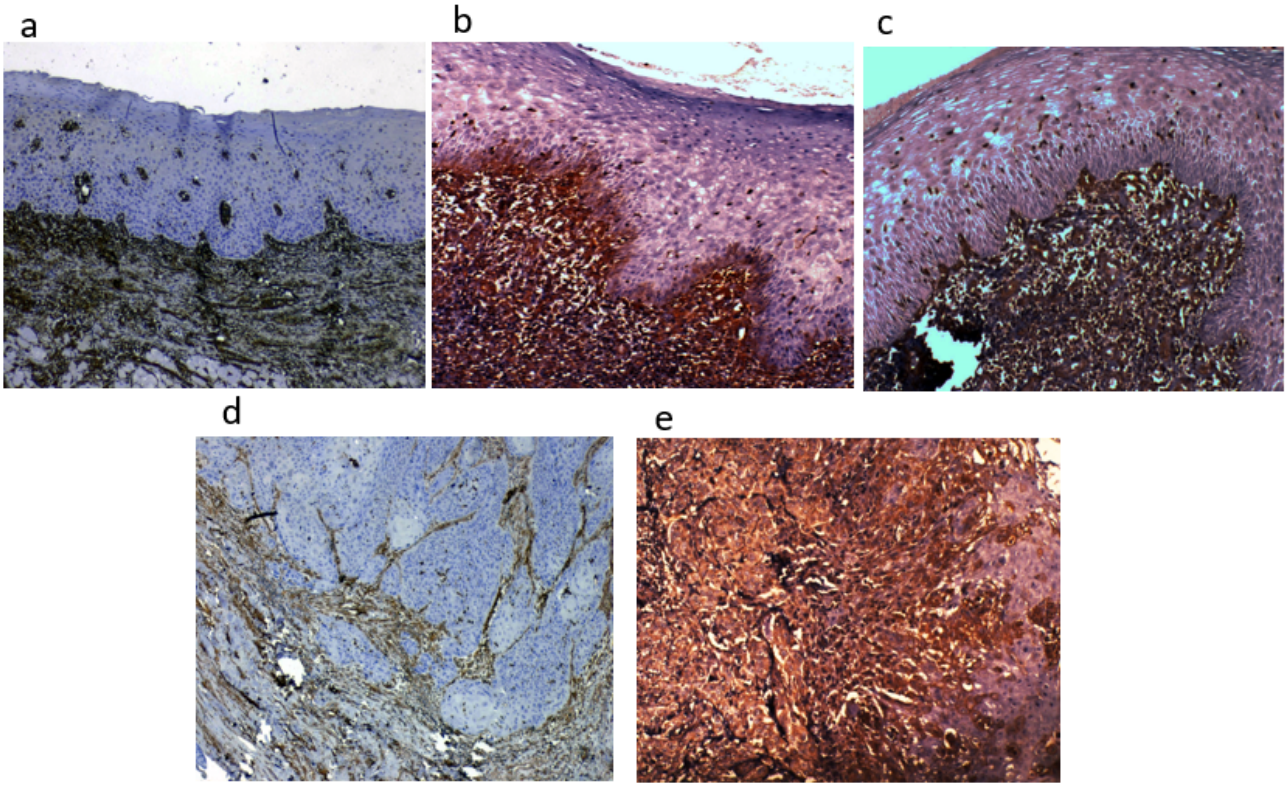
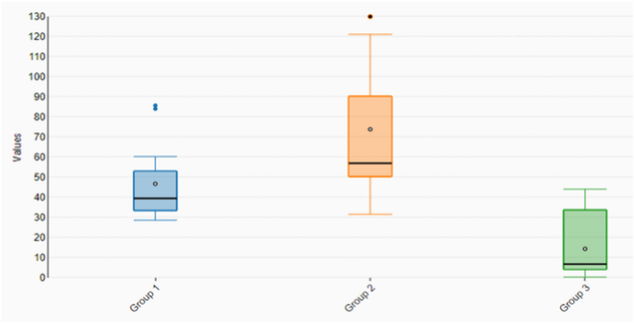


Figure 4

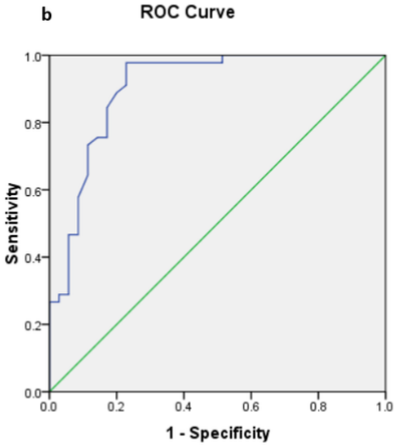
IHC analysis for Vimentin protein expression

a



DATA SUMMARY								
Groups	N	Min	Q1	Median	Q3	Max	Mean	SD
Group 1 - OSMF	15	29	33.47	39.51	53.055	85.76	46.76	18.08
Group 2 - Cancer	45	32	50.35	57.01	90.35	289.93	73.93	43.91
Group 3 - Normal	21	0	3.26	6.18	33.26	44.1	13.72	15.43

b



c

Area	Std.Error	Asymptotic Significance	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
0.905	0.036	0.000	0.834	0.976

Figure 5

a: Scatter plot representation for Vimentin ELISA with data summary values b & 5c: ROC curve, AUC analysis for Vimentin ELISA