Original Article

Overexpression of deubiquitinase (usp 36) in oral squamous cell carcinoma

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Abstract

Aim: Oral cancer is one of the top three types of cancer and is of significant public health importance in India. A common post-translational modification in cells is ubiquitination/deubiquitination, and its dysregulation is closely associated with the development of cancer. Studies on the role of ubiquitination in oral squamous cell carcinoma (OSCC) are lacking. Increased expression of *usp36* has been observed in various types of cancer, and this study aimed to check the gene expression of *usp36* in OSCC patients. In this study, we analyzed the expression of ubiquitin-specific proteases (USPs) 36 in OSCC.

Materials and Methods: A total of 15 OSCC patients at different stages of tumor differentiation and age- and sex-matched controls were recruited for the study. The patients were categorized based on their differentiation patterns. RNA was extracted from the tissues, and *usp36* gene expression was checked in these samples using a quantitative real-time PCR technique.

Results: Our study showed increased expression of *usp36* gene in OSCC patients. The *usp36* mRNA was 231.8 ± 137.94 folds higher in well-differentiated squamous cell carcinoma patients, 38.18 ± 3.77 folds higher in moderately differentiated squamous cell carcinoma patients, and 25.49 ± 7.30 folds higher in poorly differentiated squamous cell carcinoma patients compared to control tissues.

Conclusion: Our study reports, for the first time, an increased gene expression of *usp36* in OSCC tissues.

Keywords: Deubiquitinase, RT PCR, usp 36

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INTRODUCTION

Oral cancer is one of the top three cancers in India and is of significant public health importance. Oral squamous cell carcinoma (OSCC) arising from the oral cavity and lips constitutes one of the most common types of malignancy in the head and neck region. Data from the Global Cancer Observatory (GCO) shows that the annual incidence of OSCC in 2020 was 377,713 cases worldwide, with the highest number recorded in Asia (248,360), followed by Europe (65,279) and North America (27,469).[1] The five-year prevalence of OSCC approached nearly one million (959,248) and followed the same pattern; that is, the highest in Asia, followed by Europe and North America.[2]

OSCC accounts for 84–97% of oral cancer cases. OSCC commonly results from potentially malignant

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lesions or normal epithelium linings.[3] In India, the low-income groups are affected most due to greater/higher consumption of tobacco products and inadequate access to new diagnostic aids, resulting in a delay in reporting oral cancer.[4,5] Because of detection in the late stage, the chances of a cure are very low, almost negative, leaving five-year survival rates of only around 20%.[3] Earlier detection of oral cancer offers the best chance for long-term survival and has the potential to improve treatment outcomes and make healthcare more affordable.[6]

Various conventional clinical techniques, such as physical and histopathological examination, staining, biopsy, and spectroscopic and radiological techniques, are routinely used to detect oral cancer. The diagnosis of cancer in its early stages is a key factor in determining further physical, psychological, and financial losses to the patient. Upon early diagnosis, timely and proper treatment can be initiated that may improve the survival rate up to 90%. With advancements in science and technology, numerous novel techniques have been developed that have advantages compared to the currently practiced conventional diagnostic methodologies.[3] Thus, the identification of a potential prognostic marker is the need of the hour.

In eukaryotic cells, ubiquitination and deubiquitination regulate a number of biological processes by balancing cellular protein degradation.[7] Dysregulation of ubiquitination and deubiquitination contributes to various diseases, including cancer. One of the important roles of DUBs is the regulation of tumor progression. Several reports have suggested that the DUB family members were highly elevated in various cancer cells and tissues in different stages of cancer. These findings suggest that the DUBs could be used as drug targets in cancer therapeutics. Overexpression of USP36, one of the deubiquitinating (DUB) enzymes that belongs to ubiquitin-specific processing proteases (USP), has been observed in various types of human cancers, including breast, lung, and ovarian cancers.[8-10] However, *usp36* expression in OSCC has not been studied till now. The study, therefore, aims to evaluate the expression of the *usp36* gene in OSCC at various stages of differentiation using the RT-PCR technique.

Ethical statements

This study was approved by the Institutional Review Board of our hospital and was carried out in accordance with the Declaration of Helsinki. Written informed consent for the use of tissues for research was obtained from all study recruits at the time of procurement of the specimen.

MATERIALS AND METHODS

Collection of tissue from OSCC patients

As part of a prospective case-control study, biopsy specimens of oral cancerous lesions were obtained from patients diagnosed with OSCC and undergoing surgery at our hospital. The diagnosis of OSCC was based on clinical and histological features and graded according to Broder's four-point classification scale (Grades I to III/IV). OSCC that strongly resembles normal squamous epithelium was graded as low-grade I or well-differentiated. OSCC that includes abnormal mitoses, higher mitotic activity, nuclear pleomorphism, and less keratin was graded as grade II or moderately differentiated, while OSCC that contains immature cells along with typical or atypical mitoses and less or no keratin was graded as high-grade III/IV or poorly differentiated. Tissues of minimal size, typed as early invasive carcinoma, were also grouped under well-differentiated squamous cell carcinoma.

Collection of control tissue samples from donors

Patients undergoing minor oral surgeries were selected as controls for the study. Controls were age- and sex-matched to the patients. The tissues overlying the impacted tooth, which are to be discarded, were taken as control samples for the analysis. The tissues were divided into two parts: one part was fixed in formalin and used for histopathological study, and the other part was stored in RNA® Later solution for gene expression studies.

RNA isolation

Total RNA was isolated from the tissues using a Trisol RNA isolation reagent (Aura Biotechnologies Pvt. Ltd., India). Briefly, 30 mg of tissues were homogenized in 1 ml of Trisol reagent, and subsequently, RNA was extracted according to the manufacturer's protocol. The extracted RNA was quantified and digested using the enzyme DNAse 1 (New England Biolabs, USA) to eliminate DNA contamination. 200 ng of RNA was loaded on an agarose gel to check for the purity and integrity of the RNA.

cDNA conversion

200 ng of DNase (New England Biolabs (NEB), USA) treated total RNA from patient/control tissues were used for cDNA synthesis using the cDNA synthesis kit (Aura Biotechnologies Pvt. Ltd., India). The gene-specific primers were designed for the *usp36* gene and the reference gene *β-actin,* as shown in Table 1.

Quantitative PCR: Method of calculation

200 ng of total RNA was converted into cDNA, and 20 ng of cDNA was used for the qPCR reaction. Real-time PCR

Table 1: Gene selected for expression analysis

Gene name	Gene abbreviation	Primer sequence	Tm (°C)	Product size (bp)
Human ubiquitin specific peptidase 36 (USP36)	Usp36	5' ACT CTC CCA GAC ACC CAC AC 3'	61.12	389
		5' TGG AAC AGT TCG TTT CCT GA 3'	57.01	
Human *β*-actin	*β*-actin	5' CAT CGA GCA CGG CAT CGT CA 3'	63.21	211
		5' TAG CAC AGC CTG GAT AGC AAC 3'	60.13	

was performed with the QuantStudio 5 Real-Time PCR Instrument (ThermoFisher Scientific, USA) using the SYBR Green chemistry. The comparative $2^{(-\Delta Ct)}$ method was used to analyze the result of *usp36* gene relative to the internal control gene (*β-actin*), as described by Pfaffal, 2001.[11] Briefly, the mean Δ Ct values of the controls were determined and used to calculate fold variations within the controls (ΔCt − Mean ΔCt). Fold variations within controls were determined using $2^{(-\Delta Ct)}$. Relative fold variation in patients was determined using the formula

$$\Delta Ct \text{ (patients)} - \text{Mean } \Delta Ct \text{ (controls)} = \Delta\Delta Ct;\ 2^{(-\Delta\Delta Ct)}$$

The dCt values were calculated after normalizing with *β-actin* for every control. The mean dCt was arrived. The fold change of every control was calculated against this mean. Similarly, the fold changes in patients were calculated against this mean of the control.

Statistical analyses

Data were expressed as mean ± SD along with the median and range. The statistical analyses were performed using the program SPSS for Windows, Version 14.0. The Pearson χ^2 test was used to compare the proportions of qualitative variables. The student *t*-test and the Mann–Whitney U tests were used to compare the means of the quantitative variables between two independent groups. A P value < 0.05 was accepted as statistically significant.

RESULTS

Gene expression analyses were done in oral surgical tissues from OSCC patients. Lesion tissues were collected from 10 males (mean age 62.1 ± 9.07 years) and 5 females (mean age 48.6 ± 5.46 years) OSCC patients. Tissue overlying the impaction tooth was used as a control (n = 15). No significant difference was observed between the patients and age- and sex-matched controls.

The details of the 15 patients included in the study and the total RNA yield obtained from their tissues are given in Table 2. Of the 15 cases, 8 were graded as well-differentiated squamous cell carcinoma (WDSCC), 4 as moderately differentiated squamous cell carcinoma (MDSCC), and 3 were poorly differentiated squamous cell carcinoma (PDSCC). No significant change was observed with the age of the patient, and the cancer cell differentiation status.

Primer efficiency

Figure 1 shows the primer efficiency plot of *usp36* primers and *β*-actin primer pairs. Both primer pairs had a slope value of ≤3, and the R^2 value of *usp36* and *β*-actin primers were found to be 0.9991 and 0.9822, respectively.

Since the *usp36* gene is found to be overexpressed in various cancers, we sought to check its expression in oral squamous cell cancers. Based on the extent of differentiation, the samples were classified as well differentiated squamous cell carcinoma (WDSCC), moderately differentiated squamous cell carcinoma (MDSCC), and poorly differentiated squamous cell carcinoma (PDSCC).

We found a 139-fold increase in the usp36 RNA expression among the OSCC cases as compared to controls. [Table 3] On further categorizing them as WDSCC, MDSCC, and PDSCC, statistically significant differences were observed among all three groups, with the expression highest in the WDSCC group, followed by MDSCC and PDSCC as per Mann–Whitney U tests. Figure 2 shows the dot plot of *usp36* gene expression in the different groups.

DISCUSSION

Oral carcinogenesis is a progressive disease where normal epithelium passes through stages starting from dysplasia to finally transforming into invasive phenotypes. OSCC represents the most common form of oral carcinoma. The use of genetic and proteomic approaches in recent years has revealed the molecular pathological picture of oral cancer. There is an active search to identify genetic alterations in oncogenes or tumor suppressor genes, the role of genomic instability and epigenetic modifications, and to generate a gene expression profile in oral oncogenesis.[12] Understanding these genetic changes and gene expression patterns is key to understanding the molecular pathogenesis of oral cancer.

DUBs are able to reverse ubiquitination by detaching ubiquitin from target proteins, and they regulate various cellular functions, including apoptosis, proliferation, and cell survival, by participating in various signaling pathways.

Table 2: Details of OSCC patients and the total RNA yield from tissues

Patient ID	Age	Sex	Differentiation status	Purity (OD_{260}/OD_{280})	RNA concentration (ng/μl)
P1	55	F	MDSCC	1.71	361
P2	40	F	WDSCC	1.78	406
P3	50	F	MDSCC	1.76	437
P4	70	M	PDSCC	1.81	270
P5	70	M	PDSCC	1.77	238
P6	50	F	MDSCC	1.79	343
P7	54	M	PDSCC	1.75	245
P8	73	M	WDSCC	1.78	406
P9	48	F	WDSCC	1.82	317
P10	62	M	MDSCC	1.81	261
P11	69	M	WDSCC	1.73	322
P12	60	M	WDSCC	1.76	437
P13	51	M	WDSCC	1.72	192
P14	68	M	WDSCC	1.76	166
P15	44	M	WDSCC	1.71	254
Mean age±SD (male + female patients)				57.6±10.60 years (40–70 years)	
Total RNA yield (Range)					310.33±86.92 ng/μl (166–437 ng/μl)

WDSCC=Well Differentiated Squamous Cell Carcinoma, MDSCC=Moderately Differentiated Squamous Cell Carcinoma, PDSCC=Poorly Differentiated Squamous Cell Carcinoma

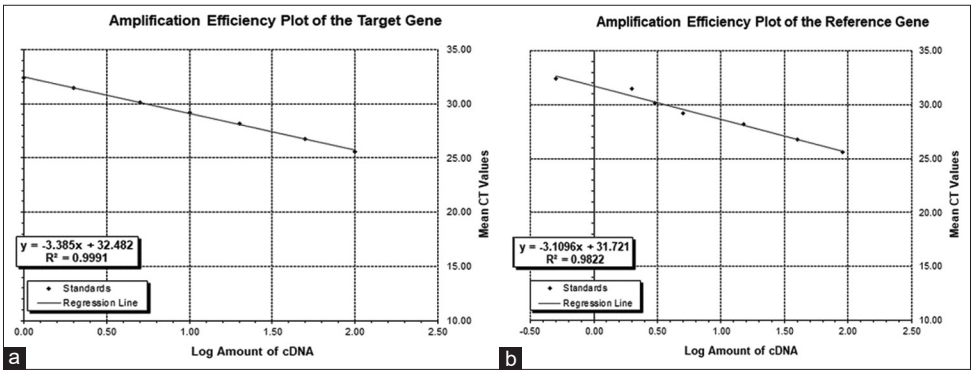

Figure 1: Amplification efficiency plot of (a) *usp36* and (b) *β*-actin primers *Usp36* gene expression in OSCC lesions

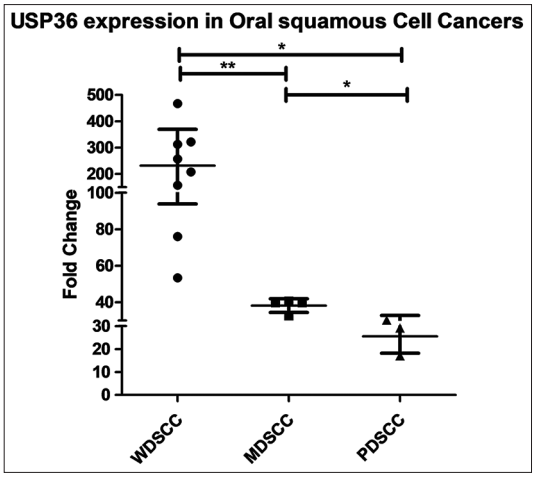

Figure 2: Dot plot showing *usp36* expression in WDSCC, MDSCC, and PDSCC groups. * represent $P < 0.05$, ** $P < 0.01$

All DUBs that play important roles in pathways that are dysregulated in cancer, including DNA repair, cell growth, and apoptosis, are potential drug targets. USP36 belongs to the USP family of DUBs and has a peptide sequence that is rich in proline (P), glutamic acid (E), serine (S), and threonine (T) motifs that are responsible for polyubiquitination.[13] It has been reported that *usp*36 deubiquitinates and stabilizes the transcription factor c-Myc, which is upregulated in cancer.[14] Upregulation of *usp*36 is observed in breast and lung cancer cells.[9,14] Subsequent studies revealed that USP36 is also capable of reducing Histone H2B ubiquitination at the p21 locus, thereby enhancing p21 signaling and regulating cell proliferation.[15] In addition, *usp*36 increases the half-life of superoxide dismutase 2 (SOD2), which is a key mitochondrial antioxidant enzyme.[16]

To the best of our knowledge, so far *usp36* gene expression has not been looked at in oral squamous cell cancers. Hence, in our study, we sought to check the gene expression levels in the tissues of OSCC patients.

Tumor differentiation, defined microscopically by epithelial cell keratinization and keratin pearl formation,[17] is a major prognostic factor in OSCC.[17,18] Poorly differentiated tumors are known to be more aggressive and metastasize earlier, leading to a poor prognosis. The gene *c-myc* is a

Table 3: Gene expression comparison of *usp36* in differentiated cancer cells

Usp36 expression		OSCC	
Fold change w.r.t control tissues		139±141.8	
P		0.0007	
	WDSCC	MDSCC	PDSCC
Fold change w.r.t control tissues	231.80±137.94	38.18±3.77	25.49±7.30
		P	
WDSCC vs. MDSCC		0.0040	
PDSCC vs. WDSCC		0.0121	
PDSCC vs. MDSCC		0.0498	

known oncogene with a central role in almost every aspect of the oncogenic process, orchestrating proliferation, apoptosis, differentiation, and metabolism. Among many mechanisms contributing to the deregulated overexpression of Myc, including transcription, gene amplification, and chromosome translocation, increasing studies have shown that Myc protein stabilization due to impaired Myc degradation pathway plays a key role in cancers.[19-23] Myc stability is tightly controlled by the ubiquitin-proteasome system.[24] The *usp36* is a deubiquitinating factor for the *c-myc* gene along with other genes and has been implicated in many cancer types, prompting us to look for its expression in OSCC. Previous studies have shown that *usp36* can participate in the progression of a variety of cancer types,[9,25] and *usp36* can stabilize the protein stability of the hepatoma promoting protooncogene *c-myc* through dysregulation of its ubiquitination.[26,27] A significant correlation was noted between tumor stage and tumor grade, as increased *usp36* expression was more common in higher tumor stages and grades in hepatocellular carcinoma.[28] Our study showed age or gender did not affect the *usp36* mRNA expression in OSCC. Similar to the study in the HCC, we found overexpression of *usp36* in all OSCC cases. Contrary to what was observed in HCC, we observed increased expression in the WDSCC group compared to the PDSCC group. The expression is not in concurrence with tumor differentiation. A plausible explanation for this could be the unequal distribution of the samples among the three groups or an inherent difference among the hepatocellular or oral cancer tissues that need to be studied. The samples were taken from various sites in the oral cavity, which could also be a reason for the variation in expression. A major limitation of this study is the limited sample size. Further studies with more sample numbers are needed to explore if *usp36* expression plays a contributory role in tumor cell differentiation.

CONCLUSION

Our study reports, for the first time, the increased expression of *usp36*, a deubiquitinating enzyme, in OSCC. Further studies with a larger sample size may provide useful insights into OSCC pathogenesis. All DUBs that play important roles in pathways that are dysregulated in cancer, including DNA repair, cell growth, and apoptosis, are potential drug targets.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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