Original Article

Evaluation of USP22 and Ki-67 expression in oral squamous cell carcinoma: An immunohistochemical study

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Abstract

Background and Aim: USP22 is a positive regulator in tumor growth, its depletion leads to cell cycle arrest at G1 phase. USP22 over expression was positively correlated with proteins involved in proliferation and negatively correlated with tumor suppressor protein tumor supprn. Ki-67 expression is associated with USP22 over expression in oral squamous cell carcinoma (OSCC) and also in cervical and prostate cancers. The aim of this study is to evaluate the expression of USP22 and Ki-67 in OSCC by using an immunohistochemical staining procedure.

Materials and Methods: Immunohistochemistry was used to determine the expression of USP22 protein in 50 archival tissue blocks of histopathologically diagnosed OSCC and 15 normal oral mucosa tissue blocks. The histopathological correlation of USP22 with Ki-67 was done.

Results: Expression of USP22 and Ki-67 was seen in the nuclei of epithelial cells. Statistical analysis of the mean expression of USP22 in OSCC and normal tissue showed a significant difference ($P = 0.000000119$). A significant difference was also observed in Ki-67 between OSCC and normal tissue ($P = 0.00000086$). Correlation test showed a weak correlation ($R = 0.19$) between USP22 and Ki-67 expression of group 1. Similarly, a weak correlation ($R = 0.51$) was observed in group 2.

Conclusion: A statistically significant difference in the expression of USP22 and Ki-67 was observed between normal mucosa and OSCC. It can be used in early diagnosis of OSCC but its use as a prognostic indicator is questionable and should be exemplified with a larger study sample.

Keywords: Ki-67 (Kiel-67), OSCC (Oral squamous cell carcinoma), USP22 (Ubiquitin specific peptidase 22)

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INTRODUCTION

Squamous cell carcinoma of the head and neck is the sixth most prevalent cancer worldwide. Oral squamous cell carcinoma (OSCC) accounts for 40% of all Head and neck squamous cell carcinoma (HNSCC) cases.[1] A novel molecular marker Ubiquitin-Specific Peptidase 22 (USP22) is gaining its importance in the study of cancers.[2] Ubiquitin (Ub) is a small heat shock protein of 8.5 kDa that is expressed ubiquitously in all eukaryotic cells. Post-translational modifications (PTMs) are mechanisms to control the activity of proteins by cells.[3] Ubiquitylation or ubiquitination is one of the PTMs in which the conjugation of ubiquitin at specific lysine (K) or amino-terminal methionine (M1) residues on the target

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proteins occurs. Three different enzymes, ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3), carry out the ubiquitylation process. Deubiquitination is the process of deubiquitinating enzymes (DUBs) cleaving ubiquitin molecules from ubiquitin-conjugated protein substrates,[4] thus regulating their proteasomal degradation, localization, and activity.[5]

The biomarker Ki-67 (Kiel-67) encodes for two protein isoforms with molecular weights of 345 and 395 kDa. Ki-67 is reported to be positively expressed during the cell cycle stages G1, S, G2, and M. Ki-67 levels drop during the anaphase and telophase. Cell proliferation markers play an essential role in the biological behavior of neoplasms. Ki-67 is a sensitive nuclear protein associated with cell proliferation. Due to the fact that it is markedly more heavily expressed in cancerous tissues than in healthy tissues, the Ki-67 index serves as an important diagnostic and prognostic biomarker in OSCC.[6]

Several studies have shown an increased expression of USP22 in various cancers but very few studies are done evaluating the expression of USP22 in OSCC. Theoretically USP22 and Ki-67 are supposed to show positive correlation but this fact is not well proven in literature. This study is aimed to fill the lacunae by evaluating the expression of USP22 and Ki-67 in OSCC.

MATERIALS AND METHODS

Fifty formalin fixed paraffin embedded tissue blocks of histopathologically diagnosed oral squamous cell carcinoma (OSCC) and fifteen tissue blocks of histologically diagnosed normal oral mucosa were retrieved from the archives of the Dept. of oral pathology, St. Joseph Dental College, Eluru. Blocks of patients who received preoperative chemotherapy and radiotherapy and also recurrent OSCC cases were excluded from the study.

3 μr thick sections from each tissue block were taken on positively charged slides using a semiautomatic microtome (Thermo scientific Microm HM 340E, Novel technologies). Slides were incubated for 1 hr at 60ubated for 1nologieantigenicity. Slides were then deparaffinized three times for five minutes each in xylene and then rehydrated in descending grades of alcohol. Antigen retrieval was done using Tris-EDTA buffer in a pressure cooker for 15–20 minutes until pressure was released automatically through 2 whistles. Endogenous peroxidase was blocked by incubating the slides with peroxide blocking reagent for 5 min. After washing with Tris buffer solution (TBS), the sections were incubated separately with USP22 antibody (Diagnostic BioSystems, RMPD004, diluted at 1:200) and Ki-67 ((LOT S279), Diagnostic BioSystems, 6 ml ready to use) for 4 minutes at room temperature. The sections were then treated with Linker 1 (HRP) for 5 minutes at room temperature followed by Linker 2 (Streptavidin biotin) for 5 minutes at room temperature. The reaction products were then visualized using DAB chromogen, incubated for 2 minutes at room temperature, and counterstained with Harris hematoxylin. The slides were then analyzed under a Trinocular Olympus Bx53 Progress CT research microscope.

Microphotographs of all the IHC stained sections under 40× magnification were taken using Prog Res R Capture Pro 2.8.8 JENOPTIK. For each slide, five non-overlapping fields of epithelium with uniform staining were randomly selected for imaging. In each field of epithelium, the total number of cells and the number of positively and negatively stained cells were counted using Image J software. H scores and Ki-67 indices were calculated from the data obtained. Based on IHC staining intensity, the cells of each selected field were categorized into type 1 cells: unstained cells: type 2 cells: bluish brown stain; type 3 cells: faint brown stain; type 4 cells: dark brown stain [Figures 1 and 2].

H score derivation for USP22 expression was done by using the following formula:[7]

H Score = [(1 + intensity of type 1 cells) × total % of type 1 cells) + (1 + intensity of type 2 cells) × total % of type 2 cells) + (1 + intensity of type 3 cells) × total % of type 3 cells) + (1 + intensity of type 4 cells) × total % of type 4 cells)]. Where the score for a The intensity of type 1 cells is 0, the intensity of type 2 cells is 1, the intensity of type 3 cells is 2 and the intensity of type 4 cells is 3. The average H score of 5 fields of each slide was considered as the final H score of that slide.

The ki-67 index was calculated using the following formula:[8]

Ki-67 index = (Total number of positively stained cells/ Total number of cells in the field × 100). The derived data thus obtained was subjected to statistical analysis.

Statistical analysis was done to compare the expression of USP22 and Ki-67 between groups 1 and 2 using an unpaired Student22 and Ki-67 between group analysis. de..and the intensiwas done using Spearman's correlation coefficient analysis.

RESULTS

The mean USP22 and Ki-67 expression values in groups 1 and 2 are given in [Figure 3]. Compared to normal tissues,

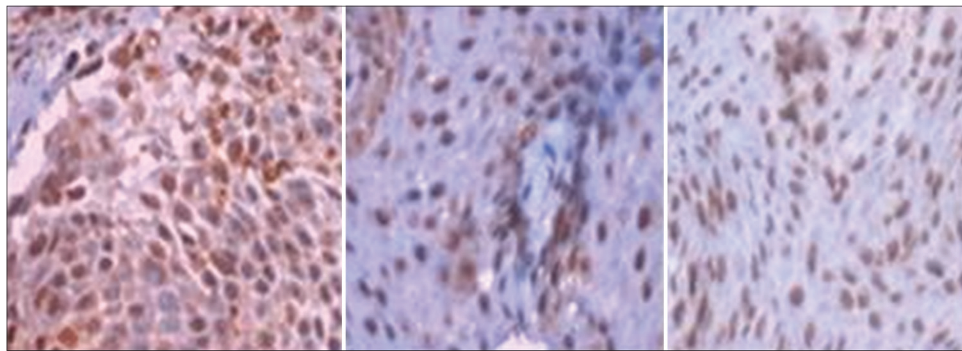

Figure 1: USP22 expression in well, moderate, and poorly differentiated OSCC under 40× magnification (from left to right)

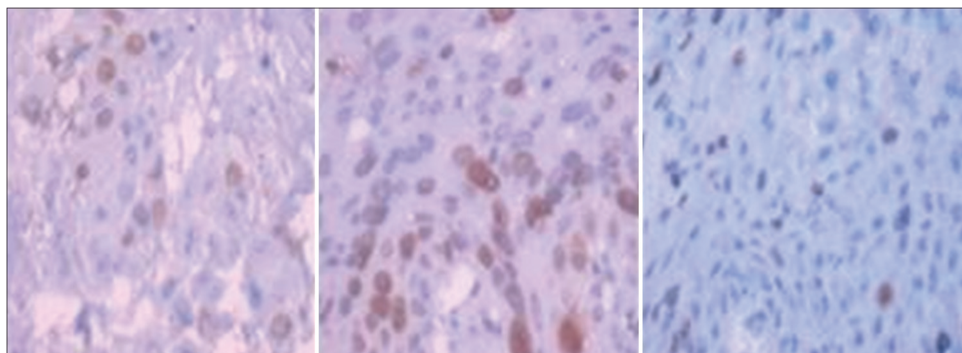

Figure 2: Ki-67 expression in well, moderate, and poorly differentiated OSCC under 40× magnification (from left to right)

a significant increase in USP22 expression was observed in OSCC (*P*- value = 0.000000119) [Table 1]. Compared to normal tissues, a significant increase in the Ki-67 index was observed in OSCC (*P*- value = 0.00000086) [Table 1]. A weak correlation was obtained between the USP22 and Ki-67 indices of both the groups [Table 2, Figures 4 and 5, respectively].

A significantly higher expression of USP22 was observed in moderately differentiated OSCC compared to well-differentiated OSCC whereas other comparisons were insignificant. A significantly higher expression of Ki-67 was observed in moderately differentiated OSCC compared to poorly differentiated and well-differentiated OSCC.

Table 1: Comparison of USP22 and Ki-67 expression between groups 1 and 2

Marker	Group	Mean±SD	P
USP22	Group 1 (OSCC)	331.72±42.73	0.000000119 (S)
	Group 2 (Normal)	245.68±66.24	
Ki-67	Group 1 (OSCC)	57.36±29.53	0.00000086 (S)
	Group 2 (Normal)	14.26±13.57	

S=Significant. Statistical analysis: unpaired *t*-test. Statistically significant if $P<0.05$

Table 2: Correlation of USP22 and Ki-67 expression within group 1 (OSCC) and group 2 (Normal tissue) using Spearman's correlation coefficient test

Spearman's Correlation Test	R	P
Group 1 (OSCC)	0.187803	0.19154
Group 2 (Normal)	0.505809	0.054401

$R=0$—no correlation, $R=1$—perfect association, $R=-1$—perfect negative correlation, R=close to 0—weaker association

DISCUSSION

Cancer is a group of diseases involving abnormal cell growth and having the capacity to invade or metastasize to other body parts.[9] The factors favoring prognosis of OSCC include patient-related factors, tumor-related factors, and treatment-related factors. Among tumor-related factors, molecular markers play a crucial role in determining the prognosis of OSCC. The molecular markers that show prognostic significance in OSCC include cell proliferation markers, protooncogenes and oncogenes, tumor suppressor genes, apoptotic markers, angiogenic markers, cell adhesion markers, cell surface markers, genetic instability markers, enzyme markers, and other miscellaneous markers.[10]

Apart from the regular molecular markers that play an important role in cancer progression and prognosis, USP22, a novel biomarker has been tested for its efficacy and its role as a prognostic indicator in the recent past in a number of cancers like prostate, lung, breast, ovarian, bladder, lymphoma, glioma, mesothelioma, neuroblastoma, acute myeloid leukemia, mantle cell lymphoma with very few

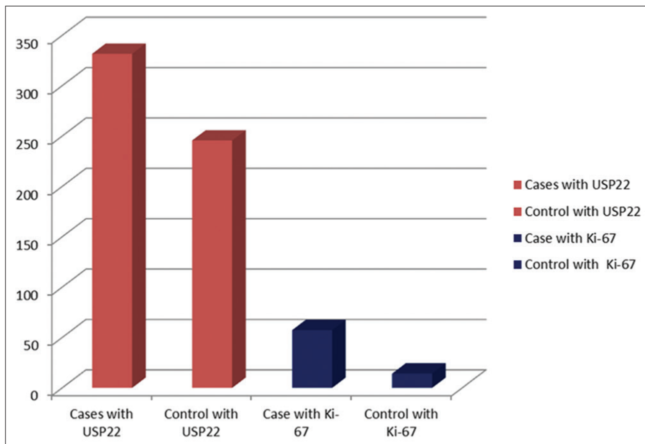

Figure 3: Mean USP22 and Ki-67 expression values in groups 1 and 2

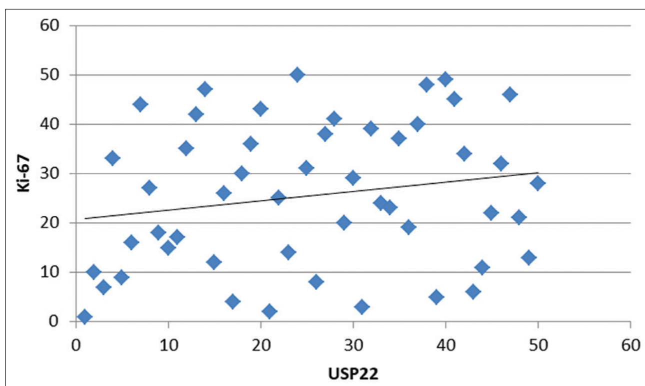

Figure 4: Correlation of USP22 and Ki-67 expression between cases using Spearman correlation coefficient test

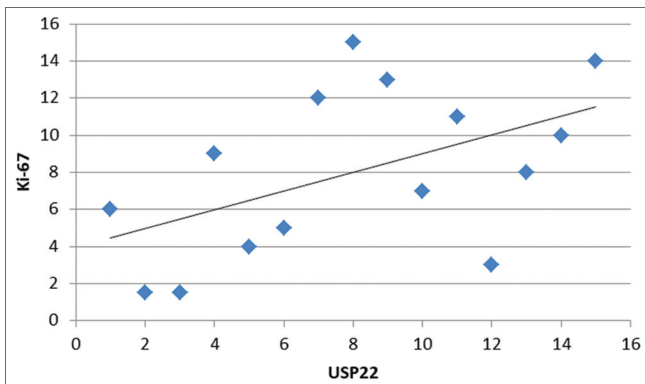

Figure 5: Correlation of USP22 and Ki-67 expression between controls using Spearman correlation coefficient test

exploring OSCC.[11] In the present study a significantly higher expression was observed in OSCC when compared to normal tissues. In concordant to our study, Piao *et al.* also observed a similar increased expression of USP22 from normal to cancerous tissue with a positive expression of 63.32% while our study recorded 93.04% positive expression.[2]

Physiologically, USP22 plays a crucial role in cell protein modifications, cell cycle progression, apoptosis, and cell differentiation. The physiological alterations in USP22 levels are attributed to those circumstances in which there is an increased demand for proteins like physiological growth spurts and cell repair mechanisms. On the other hand, in pathology, the increased USP22 expression may be the effect of regulatory protein that governs USP22 or any oncogenic stimulation in the form of genetic or epigenetic mutations. This genetically altered USP22 is involved in tumor progression because its levels are increased at both mRNA and the protein level.[2] Increased USP22 edits the histone code as part of the mammalian SAGA (Spt-Ada-Gcn5) complex by deubiquitinating H2A and H2B. Deubiquitylation of histones is intimately linked to transcription activation, epigenetic regulation, and cancer.[12]

The over expression of USP22 may augment cancer cell proliferation by repressing p21 and facilitating premature transition through various stages of cell cycle along with the increased capability of FBP1 (Far upstream binding protein). Repression of p21 leads to the proliferation of cyclin-dependent kinases (CDKs) and allows G1/S transition of damaged cells. In pancreatic cancer cell lines a different mechanism in USP22 was found to modulate the lacatenin/Wnt signaling and therefore increase the abundance of FoxM1, a transcription factor that normally represses the expression of p21 and p27. Hence, USP22 overexpression was associated with a reduction in p21 and p27 levels and abundance of Cyclin D1, CDK4, and CDK6, which form a complex that promotes G1 progression.[13]

Tumorigenesis is significantly influenced by crosstalk between USP22 and CCNB1. The overexpression of this CCNB1 promotes unchecked proliferation and unscheduled cell cycle entry in cancer. CCNB1, a member of the cyclin family is one among the 11 death from cancer gene signatures characterized by a surge in malignant potential and metastatic dissemination. Moreover, a positive correlation between USP22 and CCNB1 expression was found in human colon cancers.[14]

The telomere length is maintained by Telomeric repeat-binding factor (TRF1), a USP22 substrate. By controlling TRF1 which maintains telomere length, increased USP22 expression in OSCC may be a key element in the course of the disease.[15]

Evasion of apoptosis is one of the important hall marks of cancer cells. Increased USP22 expression may aid in the development of cancer by preventing apoptosis in cancer cells. USP22 regulates apoptosis by modulating its substrate Sirtuin1 (SIRT1), a member of the sirtuin

family of nicotinamide adenine dinucleotide (NAD+) dependent class III histone deacetylases. The removal of poly-ubiquitin chains from SIRT1 is catalyzed by USP22 to prevent its degradation and increase its abundance. SIRT1 in turn deacetylates TP53 in order to inhibit transcriptional activation of TP53 target genes. USP22/SIRT1/TP53 regulatory pathway prevents DNA-damage-induced apoptosis in embryonic kidney and colorectal cancer cell lines. Upregulation of the USP22/SIRT1/TP53 regulatory pathway was also observed in acute myeloid leukemia stem cells retaining an internal tandem duplication mutation of the tyrosine kinase gene, FMS-like receptor tyrosine kinase-3 (FLT3), which grants resistance to tyrosine kinase inhibitors. Therefore in multiple cancer cell lines, increased USP22 expression attenuates apoptosis.[16]

USP22 has a significant role in coordinating tumor microenvironment. Unrestrained proliferation and anomalies in the microcirculation of solid tumors make cancer cells vulnerable to hypoxic microenvironment.[17] Hypoxia inducible factor (HIF) regulates downstream gene expression and promotes tumor progression under hypoxic conditions. USP22 boosts the stability and transcriptional activity of HIF-1α under hypoxia through deubiquitination and induces upregulation of HIF-1α downstream genes.[16]

"B-celltream geneMoloney murine leukemia virus integration site 1 (BMI1)" is involved in Cancer stem cell (CSC) self-renewal and preservation of CSC stemness. USP22 and BMI1 together form a multiprotein complex acting on their target homologous gene clusters. USP22-BMI1 silences the Hox gene and increases tumor resistance. Preneoplastic lesions such as oral dysplasias and esophageal adenocarcinomas have been shown to have higher BMI1 expression which explains its involvement in the cancerous metamorphosis of Oral epithelial dysplasias (OEDs).[16] Tumor associated inflammation is linked to various stages of Tumorigenesis. COX-2 expression is seen in OSCC and surrounding lymphocytic infiltrate and is also linked to OSCC metastases. The levels of COX-2 are modulated by the ubiquitin/proteasome pathway which targets misfolded or damaged proteins for degradation by the 26S proteasome. Silencing of USP22 downregulates COX-2 and thus inhibits cancer cell proliferation in OSCC by direct interaction leading to modulation of stability and activity of COX-2 through controlling of its ubiquitination status.[18]

Cellular myelocytomatosis oncogene (C-MYC) is one of the main agents promoting tumor formation. It has been established that USP22 is required for c-MYC transcriptional activity, through a direct mechanism where USP22 deubiquitylates c-MYC, causing its stabilization or activation, or indirectly through the recruitment of other transcriptional machinery, or by the removal of ubiquitin from histones at c-MYC target genes.[19]

Thus USP22 as a deubiquitinating protein has its regulatory effect on a number of proteins that modulate cell cycle and hence its enhanced expression in OSCC would unquestionably compound cancer progression compromising prognosis and treatment outcome.

A significant increase in Ki-67 index was seen in OSCC when compared to normal tissues. Analogous to our study, an increased expression of Ki-67 from normal epithelium to OED and from oral epithelial dysplasia to OSCC was observed in a study conducted by Takkem *et al*.[20] As Ki-67 is a proliferative marker, the cancerous cells show an increased proliferative index which is the reason for increased Ki-67 expression in OSCC when compared to normal mucosa. Similar to our findings, Birajdar *et al.* observed that only the basal and parabasal layers of normal epithelium expressed the Ki-67.[21]

In OSCC the expression of ki-67 was also seen in the superficial layers. The present study also showed similar type of expression in OSCC cases where the Ki-67 expression was observed in superficial layers also while in group 2 the Ki-67 expression was limited to basal and parabasal layers. Ki-67 is not expressed in cells showing an arrest in the cell cycle, that is, G0 phase and starts to be expressed in the S-phase, progressively increasing through S and G2 phases which reaches a plateau at mitosis.[22] Ki-67 either directly or indirectly modulates the normal cell cycle regulatory proteins like p53 and p21. The p53 suppresses gene transcription in Sp-1 binding site of Ki-67 promotor region.[23]

The ki-67 modulates the action of p21 indirectly through the alteration of p53-p21-Rb signaling pathway. A notable target of p53 is the gene CDKN1A, which codes for p21. P53 causes the transcription of p21/CDKN1a by interacting with sites in the promoter.[24]

CONCLUSION

The current study showed a statistically significant differential expression of USP22 in normal epithelium and OSCC and a similar difference was observed with Ki-67. Despite its intriguing significance in the etiology, diagnosis, prognosis, and even treatment of several malignancies, there is a critical need for thorough research addressing USP22 involvement in OSCC. Limited research has enabled

the current study to be chosen to assess the function of USP22 in OSCC. The study's limitations include the small sample size and the scarcity of poorly differentiated OSCC cases. The results of the study evolve USP22 as a useful diagnostic marker in OSCC. Although USP22 is overexpressed in OSCC, its usefulness as a prognostic indicator is questionable. To assess the accurate and precise prognostic value of USP22 in OSCC, there is a need for conducting the study on a larger sample including all grades of OSCC and with long-term follow-up for assessing overall survival rate and disease-free survival rate.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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