ORIGINAL ARTICLE

Immunohistochemical expression of budding uninhibited by benzimidazole related 1 in leukoplakia and oral squamous cell carcinoma

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ABSTRACT

Background: Budding uninhibited by benzimidazole related 1 (BUBR1) is an important protein in the mitotic spindle assembly checkpoint. Alterations in expression of BUBR1 have been reported in many premalignant and malignant lesions. **Aim:** To compare the expression of BUBR1 with respect to the normal mucosa and degree of dysplasia in oral leukoplakia (OL) and also with respect to different histopathological grades of oral squamous cell carcinoma (OSCC). **Materials and Methods:** Neutral buffered formalin-fixed and paraffin-embedded biopsy specimens 30 each of normal, OL and OSCC tissue were included in this study. The expression of BUBR1 was detected using immunohistochemistry (IHC). The scores obtained were subjected to ANOVA test. **Results:** Significant correlation was found in immunostaining between normal, dysplasia and OSCC groups with a *P* value of 0.00001. The expression of BUBR1 was significant when compared with different degrees of dysplasia and in different histopathological grades of OSCC with a *P* value of 0.00001. **Conclusion:** Higher IHC scores were obtained with increased histopathological grades of OL and OSCC suggesting its role as a prognostic indicator.

Key words: Budding uninhibited by benzimidazole related 1, oral leukoplakia, oral squamous cell carcinoma

INTRODUCTION

Over 100 years ago, Fleming introduced the term "Mitosis," explained the cell division and transmission of chromosomes at each cell division.[1] Ubiquitin-mediated degradation of proteins at the exact time will mediate the proper transition from one stage to the next stage in mitosis. Anaphase and mitotic exit inhibitors (Securin and cyclin B) must not be degraded until chromosomes have achieved bipolar attachment to the spindle.[2]

Eukaryotic cells have checkpoints, which monitor their entry into the next stage of the cell cycle. G_2-M checkpoint and mitotic checkpoint are the two major checkpoints which controls the onset of mitosis and mitotic progression.[3] Cells that do not satisfy the checkpoint often die or exit mitosis into the next G_1 as single tetraploid cells.[4] Mitotic checkpoint/spindle assembly checkpoint monitors the proper assembly of the mitotic spindle and blocks the onset of anaphase unless all of the chromosomes are stably attached to "Kinetochore."[5]

The core components of the spindle assembly checkpoint (SAC) were originally identified in the budding yeast "*Saccharomyces Cerevisiae*" and include the budding uninhibited by benzimidazole (BUB) proteins. BUB1; BUB3; the mitotic arrest deficient (Mad) proteins Mad1, Mad2 Mad3; and Mps1 are required for spindle pole body duplication in yeast and was also shown to be essential for spindle checkpoint function.[4]

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BUB related 1 (BUBR1) also called BUB1B, MAD3 play an important crucial role in SAC. Although they share a similar domain organization, BUB1 and BUBR1 (MAD3) are paralogs and have distinct roles in the SAC function.[6] BUBR1 encodes a protein of 120 kDa, the amino acid sequences of which resemble both MAD3 and BUB1 of budding yeast.[3] The hBUB1B gene, which encodes BUBR1, is located on human chromosome 15q14-21, which is a region with a high incidence of loss of heterozygosity associated with several tumors.[7] The hBUB1B gene contains a C-terminal serine-threonine protein kinase domain that is highly homologous to BUB1 protein.[8]

BUBR1 mitotic-checkpoint protein monitors proper attachment of microtubules to kinetochores and links regulation of chromosome-spindle attachment to mitotic checkpoint signaling. Thus, disruption of BUBR1 activity results in a loss of checkpoint control, chromosomal instability caused by premature anaphase and/or the early onset of tumorigenesis.[9]

BUBR1 plays an important role in regulating natural ageing and its expression has been found to be declining in mouse tissues.[7] It is also involved in several cellular processes which include apoptosis, megakaryopoiesis and DNA damage.[10] Up-regulation of BUBR1 was found in carcinomas of the lung, colon, stomach, breast, bladder, kidney, esophagus, thyroid, ovary, salivary duct, liver and in the head and neck region.[11] BUBR1 is also postulated to be a possible predictive marker for tumor recurrence as it was also found to be associated with tumor progression and the tumor recurrence in case of oral squamous cell carcinoma (OSCC).[12] They found that the overexpression of BUBR1 might be a new tumor marker for predicting a more aggressive biological behavior of solid human neoplasms.[11]

This study was undertaken to evaluate and compare the expression of BUBR1 in different histological grades of oral leukoplakia (OL) and OSCC.

MATERIALS AND METHODS

Neutral buffered formalin-fixed and paraffin-embedded biopsy specimens 30 each of normal (n = 30), OL (n = 30) and OSCC (n = 30) were retrieved from the Department of Oral pathology and Microbiology, for the purpose of this study and compared with that of normal subjects. All the cases of OL were categorized according to histological grading of World Health Organization criteria [Table 1] and cases of OSCC were graded histopathologically using Broder's (1927) grading system [Table 2].

All the sections obtained were immunostained for BUBR1 (Genxbio Health Sciences, Delhi, India, AT1320a) using the avidin-biotin technique. Specimens were counterstained with Mayer's hematoxylin, dehydrated and mounted. Negative controls were obtained by omitting the primary antibody.

Table 1: Number of cases in each grade of dysplasia

Histological grades	Number of cases
Mild dysplasia	13
Moderate dysplasia	10
Severe dysplasia	7

Table 2: Number of cases in each grade of oral squamous cell carcinoma

Histological grades	Number of cases
Grade I	12
Grade II	10
Grade III	8
Grade IV	0

The presence of brown colored end product at the site of target antigen was indicative of positive immunoreactivity. The negative control tissue demonstrated the absence of staining. Normal oral mucosal tissue was taken as negative control [Figure 1a] and spleen was taken as positive control [Figure 1b] with each batch of staining. The evaluation of study cases was graded as positive or negative.

To enumerate the BUBR1 stained slides, 300 cells were examined manually in at least five areas and a mean percentage of positive-stained cells were determined. Then, each sample was assigned to one of the following staining scores: 0 (<10%), 1 (10–25%), 2 (26–50%), 3 (51–75%), 4 (76–90%) and 5 (91–100%). All these observations were carried out by two observers to eliminate inter-observer bias. The results were analyzed statistically using an ANOVA test.

RESULTS

Among 30 cases of dysplasia, the number of cases in mild dysplasia were 13 (43.33%), moderate dysplasia were 10 (33.33%) and severe dysplasia were 7 (23.33%). Similarly in carcinoma group, out of 30 cases in carcinoma, the number of cases in Grade I were 12 cases (40%), Grade II were 10 cases (33.33%), Grade III were 8 cases (26.67%) and Grade IV were 0 cases (0%).

In dysplasia group, the scoring was found to be 0 in 3 cases (10%), 1 in 11 cases (36.67%), 2 in 7 cases (23.33%), 3 in 2 cases (6.66%), 4 in 2 cases (6.66%) and 5 in 5 cases (16.66%). Similarly in carcinoma group, of total 30 cases, the scoring was found to be 0 in 1 case (3.33%), 1 in 7 cases (23.33%), 2 in 7 cases (23.33%), 3 in 7 cases (23.33%), 4 in 2 cases (6.66%) and 5 in 6 cases (20%). In normal group, of 30 cases, the scoring was found to be 0 in all 30 cases (100%). When a comparison was made with respect to staining scores between normal, dysplasia and carcinoma groups, the results were found to be statistically significant with a P = 0.00001 [Table 3 and Figure 2].

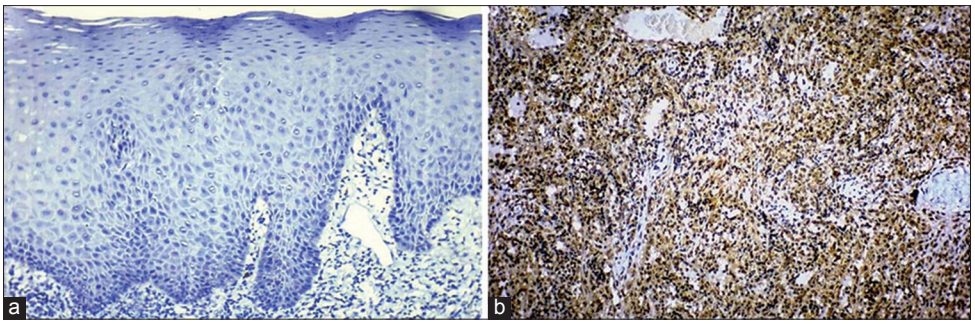

Figure 1: Photomicrograph of normal oral mucosa that was used as a negative control (a) and spleen as a positive control (b) for BUBR1 expression (IHC stain, ×100)

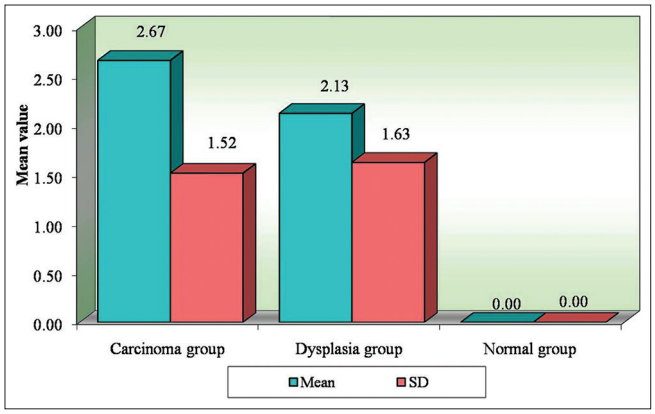

Figure 2: Comparison of three groups with respect to staining intensity scores

Of 13 cases of mild dysplasia [Figure 3a and b], the staining score was found to be 0 in 3 cases (23.07%), 1 in 10 cases (76.9%). In 10 cases of moderate dysplasia [Figure 3c and d], the staining score was found to be 1 in 1 case (10%), 2 in 7 cases (70%) and 3 in 2 cases (20%). In 7 cases of severe dysplasia [Figure 3e and f], the staining score was found to be 4 in 2 cases (28.57%) and 5 in 5 cases (71.43%). A statistically significant difference was observed between various histopathological grades of dysplasia with respect to immunohistochemistry (IHC) scores with a $P = 0.00001$ [Table 4 and Figure 4].

Of 12 Grade I cases [Figure 5a and b] of OSCC, the staining score was observed to be 0 in 1 case (8.33%), 1 in 7 cases (58.33%), 2 in 4 cases (33.33%). In 10 cases of Grade II [Figure 5c and d], the staining score was 2 in 3 cases (30%) and 3 in 7 cases (70%). In 8 cases of Grade III [Figure 5e and f], the staining score was found to be 4 in 2 cases (25%), 5 in 6 cases (75%). A statistically significant difference was observed between various histological grades of OSCC with respect to IHC scores with a $P = 0.00001$ [Table 5 and Figure 6].

DISCUSSION

Aneuploidy is a very early event in the progression of cancer. Various factors appear to play a role in aneuploidy which includes sister chromatid cohesion, abnormal kinetochore

Table 3: Comparison of normal, dysplasia and carcinoma groups for budding uninhibited by benzimidazole related 1 expression with respect to the staining intensity scores

Groups	Mean	SD	Median	Sum of ranks
Carcinoma group	2.67	1.52	2.50	1900.5
Dysplasia group	2.13	1.63	2.00	1669.5
Normal group	0.00	0.00	0.00	525
H			56.8911	
P			0.00001*	

*Significant. Kruskal–Wallis ANOVA test, P=0.00001. SD: Standard deviation

Table 4: Comparison of various histological grades of dysplasia (mild, moderate, severe) for budding uninhibited by benzimidazole related 1 expression with respect to staining intensity scores

Histopathology grading	Mean	SD	Sum of ranks
Mild dysplasia	0.77	0.44	96.00
Moderate dysplasia	2.10	0.57	180.00
Severe dysplasia	4.71	0.49	189.00
H		25.5131	
P		0.00001*	
Mild dysplasia versus moderate dysplasia		*P*=0.0001*	
Mild dysplasia versus severe dysplasia		*P*=0.0003*	
Moderate dysplasia versus severe dysplasia		*P*=0.0006*	

*Significant. Kruskal–Wallis ANOVA test, P=0.00001. SD: Standard deviation

Table 5: Comparison of various histological Grades (I, II, III) of oral squamous cell carcinoma with respect to staining intensity scores

Histopathology grading	Mean	SD	Sum of ranks
Grade I	1.25	0.62	84.00
Grade II	2.70	0.48	169.00
Grade III	4.75	0.46	212.00
H		25.0680	
P		0.00001*	
Grade I versus Grade II		*P*=0.0003*	
Grade I versus Grade III		*P*=0.0002*	
Grade II versus Grade III		*P*=0.0001*	

*Significant. Kruskal–Wallis ANOVA test, P=0.00001. SD: Standard deviation

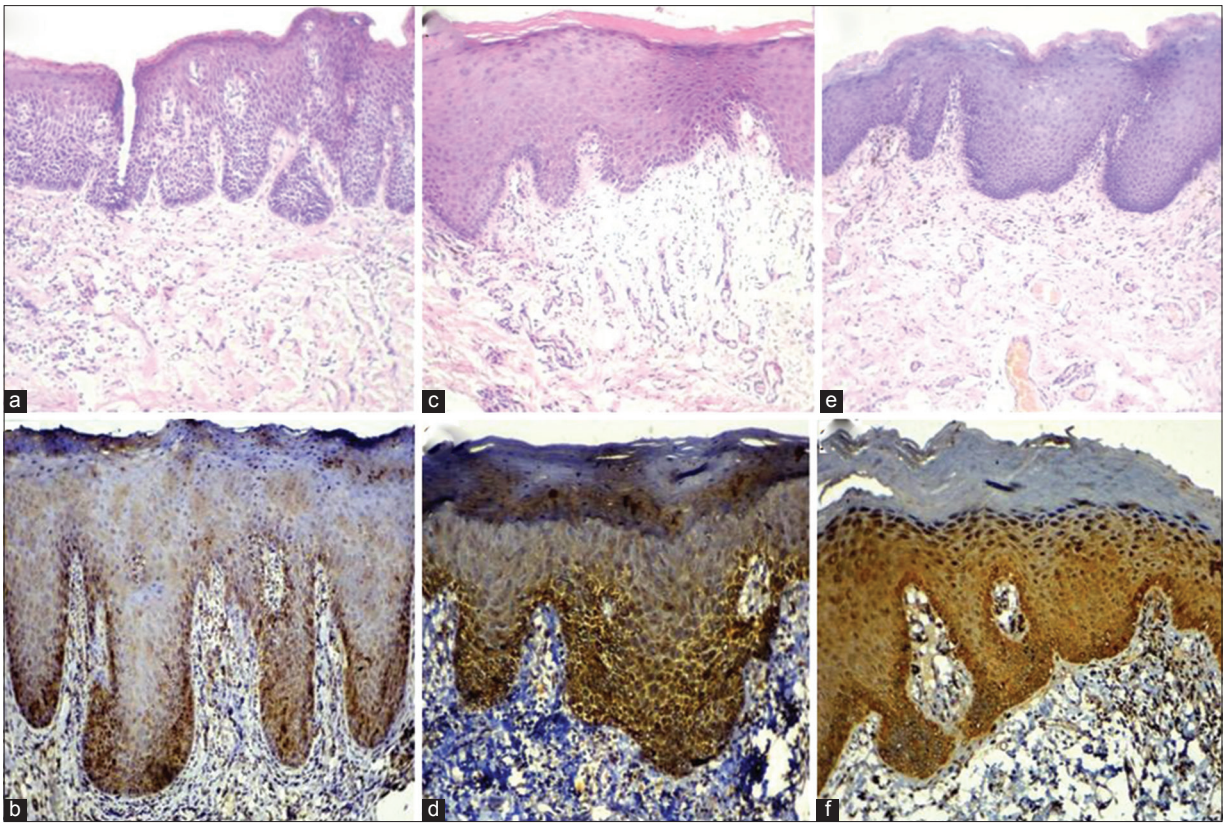

Figure 3: Photomicrograph of H&E and corresponding immunohistochemical BUBR1 stained slides of mild dysplasia: (a) H&E stain, x100 and (b) IHC stain, x100; moderate dysplasia: (c) H&E stain, x100 and (d) IHC stain, x200; and severe dysplasia: (e) H&E stain, x100 and (f) IHC stain, x200

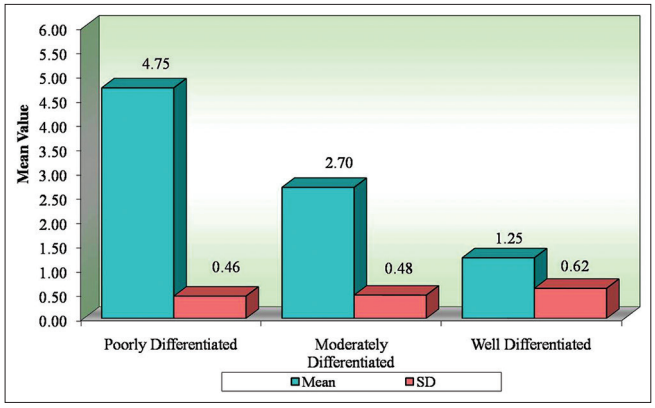

Figure 4: Comparison of histopathological grading in dysplasia with staining intensity scores

structure, mitotic checkpoint dysfunction or centrosome abnormalities.[13] Impaired SAC function has also been suggested to be one of the common causes of aneuploidy in human cancers.[14]

The SAC is essential for proper segregation of chromosomes during mitotic cell division.[11] Depletion or inactivation of several checkpoints of SAC machinery has been shown to result in the loss of checkpoint control. Among these components, BURB1 is an important protein in the mitotic SAC machinery, which protects the cell from chromosome missegregation and aneuploidy during mitosis.[15]

BUBR1 protein also known as BUB1B by Human genome organization[14] or Mad[4,6] has a critical role in regulating SAC machinery, using 3 independent mechanisms, it acts as a diffusible inhibitor, it facilitates catalysis at the kinetochore and it is a protein required for chromosome alignment during metaphase.[16] Mechanism behind the chromosomal alignment in the metaphase plate was that the mitotic checkpoint proteins disassociate from anaphase promoting complex/cyclosome thus triggering the destruction of securin and cyclin B. Separase, a protease (inhibited by securin binding and cyclin B/cdk1 mediated phosphorylation) then cleaves the kleisin subunit of cohesion, thereby allows sister chromatid disjunction and anaphase onset.[17,18]

hBUB1B is expressed in various human tissues with a high mitotic index, such as fetal tissues, but not in differentiated tissues. Thus, the hBUB1B gene expression is undetectable in normal tissues.[7] Wang *et al*. demonstrated that BUBR1 is an essential gene and its absence results in death during embryonic development. For normal mammalian development, haploinsufficiency of this gene results in splenomegaly as well as extramedullary megakaryopoiesis in the spleen.[19]

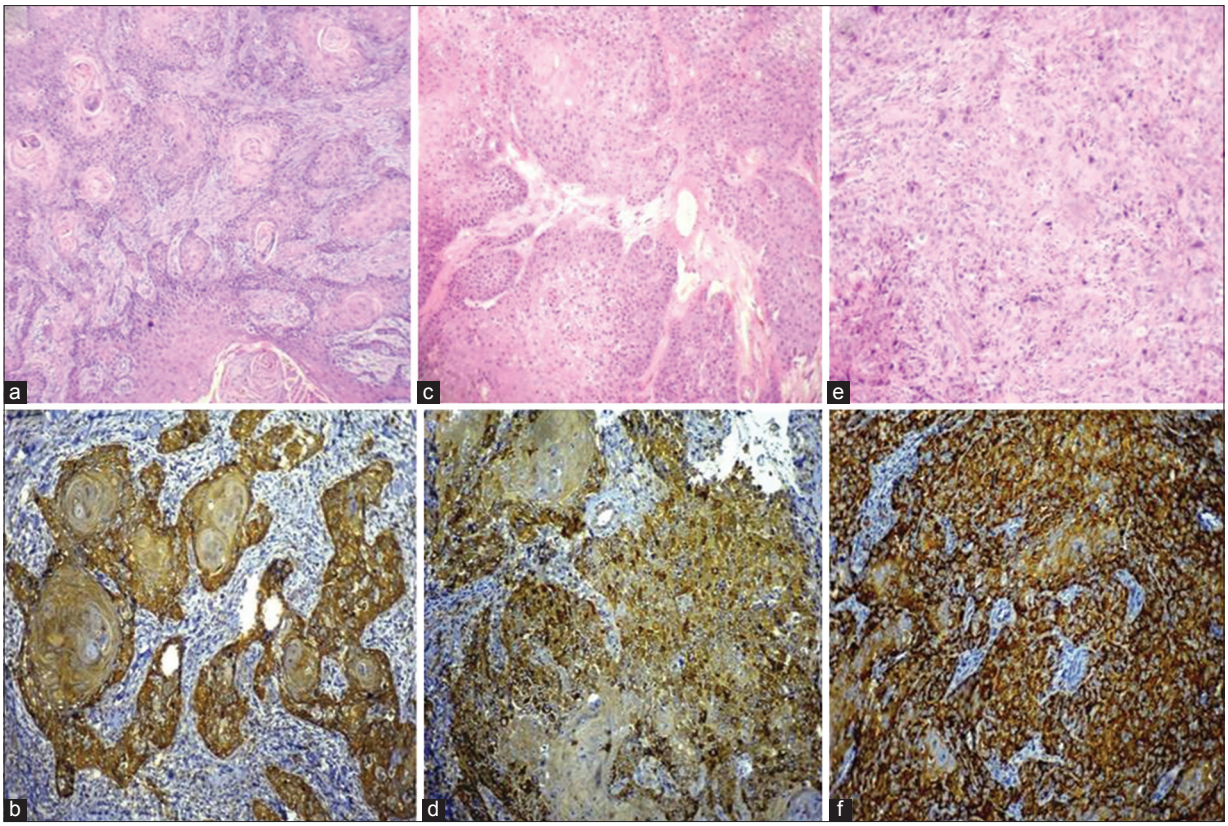

Figure 5: Photomicrograph showing H&E and immunohistochemical BUBR1 expression in oral squamous cell carcinoma Grade I: (a) H&E stain, x100 and (b) IHC stain, x100; Grade II: (c) H&E stain, x100 and (d) IHC stain, x100; Grade III (e) H&E stain, x100 and (f) IHC stain, x100

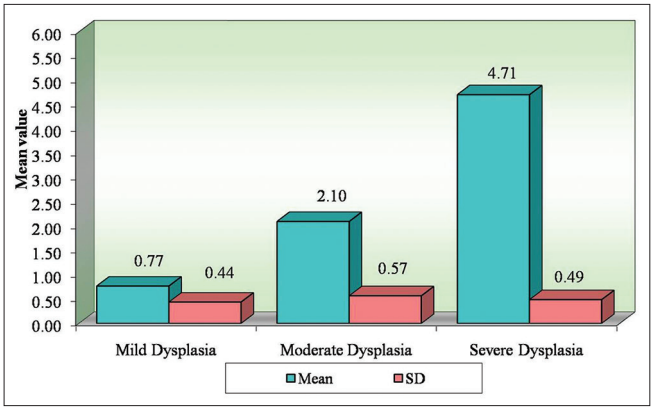

Figure 6: Comparison of histopathological grading in carcinoma with staining intensity scores

Overexpression of BUBR1 has been observed in human cancer cells during G2/M phase of the cell cycle.[7] This aberrant expression plays a significant role in cancer initiation and progression.[13] Its expression is considered as a marker for poor survival in certain types of human cancer.[6] Mutation of hBUB1B gene appears to be a rare event in human malignancy supporting the view that BUBR1 overexpression is as a result of up-regulation of the normal gene.[7] Yamamoto *et al*.[20] and Burum-Auensen *et al*.[21] observed BUBR1 overexpression in bladder cancer and ulcerative colitis-associated colorectal cancer which was correlated with higher histological grade, advanced pathological stage, tumor recurrence and disease progression. The results of this study were in accordance with the above studies.

Among the head and neck cancers, BUBR1 was expressed in potentially malignant disorders which include lichen planus, oral submucous fibrosis and verrucous hyperplasia.[7] BUBR1 expressions were also found to be expressed in OSCC,[7,11,15] leukoplakia[7] and malignant salivary gland tumors.[15]

As the other solid tumors, squamous cell carcinoma of the oral cavity often exhibits chromosomal instability leading to aneuploidy.[11] The mechanism responsible for this chromosomal instability in OSCC are largely unknown, but it is thought to be due to the dysregulated expression of the components of the mitotic spindle-associated protein complex.[7]

Overexpression in premalignant lesions suggests an early event during step-wise malignant transformation and in head and neck cancers reflects the aggressiveness of these tumors. BUBR1 is considered as a biomarker for human oral squamous cell carcinogenesis.[7]

In this study, an attempt was made to evaluate the expression of BUBR1 immunohistochemically with respect to different

histopathological grades of oral dysplasia and OSCC. The results of this study showed an increased expression of BUBR1 with respect to varying histological grades of oral epithelial dysplasia and OSCC. BURB1 was found to be overexpressed as the grade of OSCC progressed from well to poor differentiation.

Hsieh *et al*.[7] found that overexpression of BUBR1 protein was not only observed in potentially malignant disorders but also in squamous cell carcinoma of the oral mucosa and suggested that this overexpression is associated with centrosome amplification and finally suggested that BUBR1 protein is one of the contributing factors involved in the pathogenesis of OSCC. The results of the above study were in accordance with this study which showed an overexpression in both dysplasia and OSCC cases.

In this study, the results were found to be statistically significant with respect to the expression between normal, dysplasia and carcinoma groups and also with respect to different histopathological grades of dysplasia which was consistent with the study conducted by Hsieh *et al*.,[7] who suggested that this upward extension of BUBR1 staining pattern in dysplasias reflects the severity of oral dysplasia.

Increased expression of BUBR1 in OSCC have been noticed in studies conducted by Lira *et al*.,[15] and Hsieh *et al*.,[7] which were in accordance with this study showing statistically significant difference between different histological grades of OSCC. In addition, Lira *et al*. observed that human papillomavirus was also more prevalent in carcinoma cases with high BUBR1 expression and showed a significantly shorter survival.[15]

In this study, overexpression of BUBR1 was seen with respect to higher histological grade of OSCC which were in contrary to the study conducted by Rizzardi *et al*.,[11] who analyzed the expression of BUBR1 in 49 cases of OSCC by IHC and compared the findings with clinicopathologic parameters, proliferative activity and DNA ploidy. Theyfound that the overexpression of BUBR1 is associated with less advanced pathologic stage and showed longer survival periods but shorter recurrence-free survival than those without it.

Mostly the overexpression of BUBR1 has been related not only in genomic complexity, chromosomal instability, DNA aneuploidy, p53 expression, high cell proliferation but also to the more advanced pathologic stage, higher histological grade, the presence of metastasis and to a poor prognosis.[11] These findings were consistent with the studies conducted by Hsieh *et al*.[7] and Lira *et al*.[15] The results in this study were also in accordance with the above-mentioned studies. Therefore, BUBR1 expression may be used as a significant prognostic marker in oral epithelial dysplasia as well as in OSCC.

CONCLUSION

The following conclusions were drawn from the study:

- The results suggest statistical significance in the expression of BUBR1 in normal subjects, patients with OL and OSCC
- Expression of BUBR1 varied with different histopathological grades of epithelial dysplasia and OSCC
- Higher IHC scores were obtained with increasing grades of dysplasia and OSCC suggesting its role as a prognostic indicator
- Comparison of staining scores between different histological grades of dysplasia and OSCC were found to be statistically significant.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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