Original Article

Evaluating the expression of GLUT-1 in oral leukoplakia

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Abstract

Aim: The aim of the present study is to analyze the role of GLUT-1 in detection of early alterations occurring in oral leukoplakia. This study was to evaluate the expression of GLUT-1 in normal oral epithelium, the expression of GLUT-1 levels in the tissue samples of oral leukoplakia and to statistically compare the expression of GLUT-1 in normal epithelium and oral leukoplakia.

Materials and Methods: The study sample comprised formalin-fixed and paraffin-embedded tissue specimens from 23 cases of histopathologically diagnosed oral leukoplakia and formalin-fixed paraffin-embedded tissue specimens from 10 cases of normal oral mucosa. Sections were mounted on glass slide coated with Aminopropyltriethoxysilane (APES; Sigma chemical co., USA) and processed for subsequent immunohistochemical study to demonstrate GLUT-1.

Results: GLUT-1 expression in normal oral mucosa revealed weak positivity in all 10 cases (100%). The oral leukoplakia cases showed immunopositivity in all 23 cases (100%) of which 10 cases (39.14%) demonstrated focal positivity and 13 cases (60.86%) of diffuse positivity. The results were compared statistically using ANOVA test was significant at $P = 0.002$.

Conclusion: The present study shows expression of GLUT-1 in leukoplakia may be used as a reliable marker to identify the high risk group for malignant transformation.

Keywords: GLUT-1, leukoplakia, malignant, precancer

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INTRODUCTION

The term "potentially malignant disorders" (PMD) was recommended to refer precancer as it conveys that not all lesions described under this term may transform into cancer.[1,2] Critically evaluating all definitions proposed so far for oral leukoplakia, the working group agreed that the term leukoplakia should be used to recognize white plaques of questionable risk having excluded (other) known diseases or disorders that carry no risk of cancer. Oral leukoplakia is a common lesion of the oral mucosa, which is defined as a white patch or plaque that cannot be characterized clinically or pathologically as any other disease.[3] The percentage of leukoplakia that progress to invasive squamous cell carcinoma is accepted to be directly related to the severity of the dysplastic changes,

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and it ranges from 5% to 43%.[4] Many authors relate dysplastic alterations to a higher tendency of malignant transformations. However, due to the subjectivity of dysplasia analysis and the possibility of nondysplastic leukoplakia to develop into malignancy, there has been a need to develop other criteria, more specifically to predict the malignant potential of these lesions. In spite of emerging new biomarkers, the epithelial dysplasia is the most reliable indicator for predicting the potential malignant transformation of oral premalignant lesions.[5]

Identification of high-risk oral premalignant lesions and intervention at premalignant stages could constitute one of the keys in reducing the mortality, morbidity and expense of treatment associated with oral squamous cell carcinoma (OSCC).[6] Cellular energy metabolism is one of the main processes that is affected during the transition from normal to cancer cells. In particular, glucose metabolism is very often altered in tumor cells.[7] Glycolysis is a catabolic process that converts one molecule of glucose to two pyruvates with the production of two adenine triphosphate (ATP) and two reduced nicotinamide adenine dinucleotide molecules. Pyruvate in the presence of oxygen undergoes oxidation to form CO_2 and H_2O in the oxidative phosphorylation pathway. Thus in turn resulting in the production of approximately 36 molecules of ATP. Alternatively, in the absence of oxygen, pyruvate is transformed into lactic acid in the anaerobic glycolysis pathway. However, conversion of glucose to lactic acid can occur in the presence of oxygen, and this process is known as the "Warburg effect or aerobic glycolysis."[8] Most cancer cells produce large amounts of lactate regardless of the availability of oxygen. This increased aerobic glycolysis is considered by some investigators as the 7th hallmark of cancer (the others, initially proposed by Hanahan and Weinberg, being limitless replicative potential, self-sufficiency in growth signals, resistance to apoptosis, insensitivity to antigrowth signals, sustained angiogenesis and tissue invasion and metastasis). Glucose and its storage form glycogen are the most important sources of energy for oxidative metabolism in mammalian cells. The entry of glucose into cells occurs by a saturable and stereospecific nonenergy-dependent process of facilitated diffusion through specific transmembrane glucose transporter proteins (GLUTs). The gene family of these proteins is comprised of different members, which are structurally related and exhibit considerable homology in their primary sequences.[9,10] GLUT-1, which is one of the 14 members of the mammalian facilitative glucose transporter family, is not detectable in a large proportion of cells from normal tissues, except for erythrocytes, germinal cells from the testis, renal tubules, perineum of the peripheral nerves and endothelial cells in the blood–brain barrier vessels and yet appears to be expressed aberrantly in many cancers.

Several studies have shown a close relationship between GLUT-1 expression, tumor development and unfavorable prognosis of several tumors.[11-17] The level of GLUT-1 expression might be an appropriate marker to analyze hypoxia and glucose metabolism.[18,19] Several authors have performed liable number of studies to evaluate the employment of GLUT-1 as biomarker in many tumors such as colon cancer, breast cancer, lung cancer, thyroid cancer and cervical cancer.[20-26] However, not many studies have been performed to evaluate expression of GLUT-1 in dysplastic epithelium and OSCC. Hence, the present study was undertaken to evaluate the expression of GLUT-1 in normal oral mucosa and oral leukoplakia and also to analyze whether GLUT-1expression can be considered as an early diagnostic marker for OSCC.

MATERIALS AND METHODS

Tissue sample

The study sample comprised of formalin-fixed, paraffin-embedded tissue specimens from 23 cases of histopathologically diagnosed oral leukoplakia classified according to Barnes *et al*., classification is compared with formalin-fixed, paraffin-embedded tissue specimens from 10 cases of normal oral mucosa [Tables 1 and 2, Figures 1 and 2].

Methodology

Three to four serial sections of 3–5 μm thickness were made from paraffin-embedded tissue blocks. First two sections were mounted on egg albumin-coated slide and stained with hematoxylin and eosin to confirm the diagnosis and to check for respective areas.

Immunohistochemistry

Two sections were mounted on glass slide coated with Aminopropyltriethoxysilane (APES; Sigma chemical co., USA) and processed for subsequent immunohistochemical study to demonstrate GLUT-1. In brief, first peroxide block was done for 5 min with PolyExcel hydrogen peroxide to block any endogenous peroxidase activity. Then, the tissue sections are washed with phosphate buffer saline for 10 min and covered in protein block and incubated for 10 min to avoid cross reaction. Thereafter, the tissue sections were covered with GLUT-1 primary antibody (PathnSitu Biotechnologies PVT. LTD. India) (optimally diluted to a ratio of 1:50 in PBS) and incubated for 30 min. The tissue sections were washed with phosphate buffer saline for 10 min and were incubated with PolyExcel Target Binder for 10 min. After

wash with phosphate buffer saline for 10 min, the tissue sections were covered with PolyExcel PolyHRP and incubated for 10 min. The excess PolyExcel PolyHRP was removed by thorough rinse with phosphate buffer saline for 10 min. Next, the tissue sections were covered with StunnDAB working solution (1 drop of Stunn DAB chromogen in 1 ml Stunn DAB buffer) and incubated it for 5 min; the excess chromogen is removed by thorough wash in distilled water for 10 min. The tissue sections were counterstained with Mayer's Hematoxylin for appropriate time followed by bluing in tap water for 10 min. Then, the slides were dehydrated through graded alcohols, dried and cleared with xylene and mounted in DPX. The immunostained slides were observed for positivity under 4X/10X/40X magnifications under light microscope.

Interpretation of staining

The presence of brown-colored end product in the epithelium at the site of target antigen in the form of membranous staining was considered as positive immunoreactivity of GLUT-1. The expression in the epithelium of normal mucosa and oral leukoplakia with respect to the quality of staining that is intensity of expression was also observed. Photomicrographs were recorded from 3 representative areas under high-power objective (×40) in an orderly manner.

The intensity of staining and percentage of GLUT-1 positive cells were evaluated in an area of 100 μm × 100 μm in the epithelium of normal individuals and oral leukoplakia and was evaluated based on Remmele *et al*., 1987 criteria.

- Percentage of GLUT-1 positive cells was calculated using the formula

$$\frac{\text{No. of GLUT} - 1 \text{ positive cells in all three fields}}{\text{Total of no. of cells in all three fields}} \times 100$$

Scores were awarded based on scale of 0 point to 4 points (0 points – no cells with positive reaction; 1 points – up to 10% positive cells; 2 points – 11%–50% positive cells; 3 points – 51%–80% positive cells; 4 points – over 80% positive cells).

Intensity of the reaction color was awarded on scale of 0 points to 3 points (0 points – no reaction color; 1 points – reaction color of low intensity; 2 points – moderate intense reaction color; 3 points – intense reaction color).

The final score represented a product of the values of percentage of positive cells and intensity of reaction color with the score ranging from 0 to 12 points. 0 points represent negative; 1–5 points represent weak positivity and 6–12 points represent strong positivity**.** The results of GLUT-1 expression were tabulated and statistically analyzed.

Statistical analysis

The data were obtained from overall GLUT-1 expression in normal oral mucosa and oral leukoplakia using ANOVA test, Mann–Whitney test and *t*-test to see the level of significance using SPSS (Statistical Package for the Social Sciences) 17.0 software, IBM, Chicago, Illinois, USA. Differences with $P < 0.05$ were considered as statistically significant.

RESULTS

All the 10 normal individuals demonstrated weak membrane positivity in focal areas of the basal layer for GLUT-1. The percentage of GLUT-1-positive cells ranged from 2% to 9%. The data on overall GLUT-1 expression in normal oral mucosa revealed weak positivity in all 10 cases (100%) [Table 3].

The oral leukoplakia cases showed immunopositivity in all 23 cases (100%), of which 10 cases (39.14%) demonstrated focal positivity and 13 cases (60.86%) of diffuse positivity with expression confined to basal layer in 4 cases (17.4%) and up to suprabasal layer in 19 cases (82.6%). GLUT-1 was expressed in cell membrane of the epithelial cells in 14 cases (60.86%), 8 cases (34.78%) showed membrane and cytoplasmic positivity and 1 case (4.35%) showed both membrane and cytoplasmic positivity. Of 23 cases of oral leukoplakia, 3 cases (13.04%) demonstrated mild positivity, 11 cases (47.82%) demonstrated moderate positivity and 9 cases (39.14%) demonstrated intense positivity. The percentage of GLUT-1 positive cells in oral leukoplakia cases ranged from 7% to 88%. The overall GLUT-1 expression score of oral leukoplakia revealed strong positivity in 13 cases (56.52%) and weak positivity in 10 cases (43.48%) [Table 3].

All the 10 cases of mild epithelial dysplasia demonstrated expression of GLUT-1 up to suprabasal layer which expressed focal positivity in 4 cases (40%) and diffuse positivity in 6 cases (60%). The epithelium demonstrated 7 cases (70%) of membrane positivity and 3 cases (30%) of membrane and cytoplasmic positivity. Of 10 cases of mild epithelial dysplasia, 4 cases (40%) revealed moderate positivity, 6 cases (60%) revealed intense positivity and none of the cases revealed low intensity of staining positivity. The percentage of expression of GLUT-1-positive cells ranged from 25% to 88%. The overall GLUT-1

expression score of mild epithelial dysplasia demonstrated strong positivity in 7 cases (70%) and weak positivity in 3 cases (30%) [Table 4].

Moderate epithelial dysplasia demonstrated expression of GLUT-1 up to suprabasal layer in 9 cases (90%) and in basal layer in 1 case (10%). Focal expression for GLUT-1 was observed in 4 cases (40%), and 6 cases (60%) demonstrated diffuse positivity. GLUT-1 expression was observed in 5 cases of epithelial cell membrane and 5 cases of cytoplasm respectively. The intensity of staining was observed as low intensity in 1 case, moderate intensity in 7 cases (70%) and intense in 2 cases (20%). The percentage of GLUT-1 positive cells ranged from 15% to 63%. The overall GLUT-1 expression score of moderate epithelial dysplasia demonstrated equal number of 5 cases (50%) each of strong positivity and weak positivity [Table 4].

All the 3 cases (100%) of severe epithelial dysplasia demonstrated expression of GLUT-1 confined to basal layer. Of 3 cases of severe epithelial dysplasia, 2 cases (66.67%) demonstrated focal positivity and 1 case (33.33%) demonstrated diffuse positivity. The expression of GLUT-1 was observed in cell membrane in 2 cases (66.67%), and 1 case (33.33%) showed both membrane and cytoplasmic positivity. The intensity of staining was observed as low intensity in 2 cases, intense in 1 case and none of the cases showed moderate intensity expression. The percentage of expression of GLUT-1-positive cells ranged from 7% to 32%. The overall GLUT-1 expression score of severe epithelial dysplasia demonstrated strong positivity in 1 case (33.33%) and weak positivity in 2 cases (66.67%) [Table 4].

Table 1: Total sample size

Category	Sample size	Total sample size
Normal mucosa[control group]	10	33
Oral leukoplakia	23	

Table 2: Histopathological grades of oral leukoplakia

Histopathological grades of oral leukoplakia	Sample size	Total sample size
Mild epithelial dysplasia	10	23
Moderate epithelial dysplasia	10	
Severe epithelial dysplasia	3	

Table 3: Glucose transporter protein-1 expression in normal oral mucosa and oral leukoplakia

	Normal mucosa		Oral leukoplakia	
	No	%	No	%
Focal positivity	10	100%	10	39.14%
Diffuse positivity	0	0%	13	60.86%
Cell membrane positivity	10	100%	14	60.87%
Cytoplasmic positivity	0	0%	8	34.78%
Both cell membrane and cytoplasmic positivity	0	0%	1	4.35%
Mild intensity	10	100%	3	13.04%
Moderate intensity	0	0%	11	47.82%
Intense positivity	0	0%	9	39.14%
Strong positivity	0	0%	13	56.52%
Weak positivity	10	100%	10	43.48%
Negative	0	0%	0	0%

Statistics results

The overall score of GLUT-1 expression observed in normal mucosa and oral leukoplakia were also compared statistically using ANOVA test and was significant at $P = 0.002$.

The Mann-Whitney test was utilized to analyze the results of GLUT-1 expression in normal individuals, and the study group, i.e., oral leukoplakia which revealed a statistical significance of $P = 0.000$.

The data of overall score of GLUT-1 expression observed in normal mucosa and oral leukoplakia were compared statistically using t-test and was significant at $P = 0.000$.

DISCUSSION

OSCC represents the 6th most common cancer worldwide, and it is the most common malignancy of oral cavity accounting approximately 90% of all oral malignancies.

Table 4: Glucose transporter protein-1 expression in different grades of oral leukoplakia

	Mild epithelial dysplasia		Moderate epithelial dysplasia		Severe epithelial dysplasia	
	No	%	No	%	No	%
Focal positivity	4	40%	4	40%	2	66.67%
Diffuse positivity	6	60%	6	60%	1	33.33%
Cell membrane positivity	7	70%	5	50%	2	66.67%
Cytoplasmic positivity	3	30%	5	50%	0	0%
Both cell membrane and cytoplasmic positivity	0	0%	0	0%	1	33.33%
Mild intensity	0	0%	1	10.00%	2	66.67%
Moderate intensity	4	40%	7	70.00%	0	0%
Intense positivity	6	60%	2	20%	1	33.33%
Strong positivity	7	70%	5	50%	1	33.33%
Weak positivity	3	30%	5	50%	2	66.67%
Negative	0	0%	0	0%	0	0%

A considerable number of PMD such as oral leukoplakia, erythroplakia and oral submucous fibrosis transform into OSCC.[3] In spite of advancement in the molecular techniques, the mechanism of malignant transformation in PMD is not understood completely. Malignant transformation rate of leukoplakia differs based on geographic distribution and the race in which the study is conducted, and it ranges from 0.13% to 17.5%.[6] Although new biomarkers are emerging to assess the risk of malignant changes in the PMD, epithelial dysplasia is a paramount indicator for analyzing it. It is an established fact that much ahead of the expression of cytological abnormalities and tissue architectural changes, a number of molecular and biochemical alterations may occur during the tumor development. Identifying high-risk PMD and early intervention at this stage could play a vital role in reducing the mortality, morbidity and expense of oral cancer.[6] The carcinogenesis is a multistep event which depends on disruption of general cell-imposed barriers such as antiproliferative response, programmed cell death and senescence.[13] The cellular energy metabolism is the major process that is affected during the transition from normal tissue to malignant cells; especially, glucose metabolism is frequently disturbed in tumor cells. The increase in glucose metabolism is considered as the 7th hallmark of cancer.[7] Glucose is a hydrophilic component and it cannot pass through the bilipid layer by simple diffusion. Therefore, for the glucose transport, special carrier proteins distributed in the cell membrane such as the glucose transporter are required to transmit the glucose from the extracellular compartment into cytosol. The entry of glucose into cells occurs by a saturable and stereospecific nonenergy-dependent process of facilitated diffusion through specific transmembrane glucose transporter proteins.[8] When there is an increase in glucose metabolism, it leads to increases in glucose transporter activity.[27] There are 14 GLUT members, and the first member of the GLUT family is GLUT-1. Normal cell proliferation in tissues is controlled by the availability of growth-regulating factors and by the cell-to-cell interaction. The present study is undertaken to evaluate the expression of GLUT-1 in normal epithelium and oral leukoplakia. The GLUT-1 expression in the normal oral mucosa was observed as focal staining in basal layer. Its expression was mainly seen in the cell membrane of the basal cells distributed in the rete pegs region [Figure 3]. These findings are concurrent with the results of Voldstedlund and Dabelsteen.[28] However, they reported a parabasal expression of GLUT-1 in different stratified squamous epithelium.[28] The parabasal expression of GLUT-1 is not observed in any of the normal individuals involved in the current study. The percentage of GLUT-1-positive basal cells of the normal oral mucosa ranged from 2% to 9%. Such a finding was not reported in any literature for a comparison. The overall GLUT-1 score

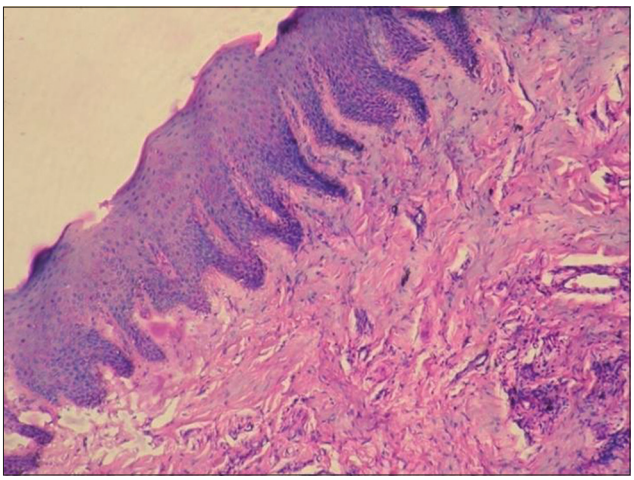

Figure 1: Normal oral mucosa showing epithelium and connective tissue (H&E, ×10)

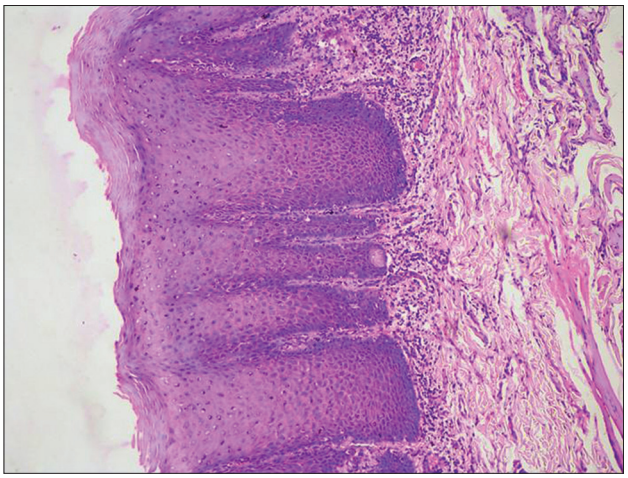

Figure 2: Oral leukoplakia showing dysplastic epithelium and underlying connective tissue (H and E, ×10)

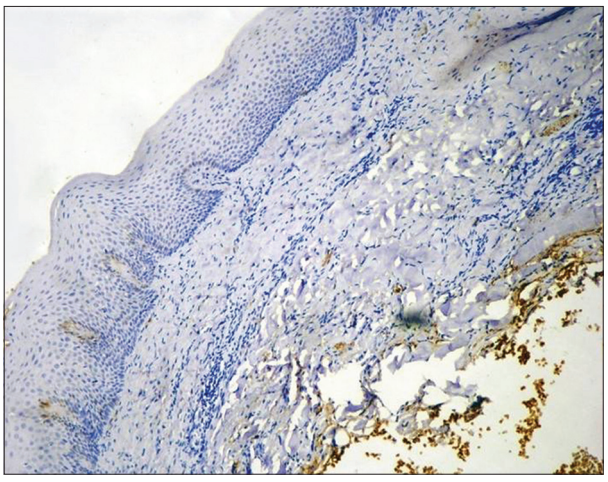

Figure 3: Normal oral mucosa showing GLUT-1 expression in focal areas of basal layer of the epithelium (×10)

of weak positivity in the normal epithelium is similar to the findings observed by Christoph Reisser.[10] The oral leukoplakia cases included in the current study showed GLUT-1 positivity in all the cases. In oral leukoplakia, the distribution of GLUT-1 was observed in basal cells and suprabasal layers [Figure 4]. The expression was noticed predominantly in suprabasal layers, which accounts for 82.6% of all the oral leukoplakia cases. The intensity of GLUT-1 staining was mostly moderate and intense in oral leukoplakia. The highest percentage of GLUT-1-positive cells in this category which accounts for 88% was a prominent finding than the percentage observed in the normal individuals. The statistical evaluation of GLUT-1 expression between normal individuals and oral leukoplakia was found to be significant ($P = 0.000$). Christoph Reisser reported a strong expression and distribution of GLUT-1in both basal and suprabasal areas in moderately dysplastic epithelium whereas the highly dysplastic epithelium expressed GLUT-1 in all the layers. However, the mild dysplastic epithelium expressed GLUT-1 in the basal cells exclusively. In the present study, the suprabasal cells' expression of GLUT-1 was observed in the epithelium of all grades of dysplasia. The suprabasal expression of GLUT-1 in moderate dysplasia is in accordance with the findings of Christoph Reisser. GLUT-1 overexpression in the basal layers of severe dysplasia indicated that there is an increased glucose uptake to meet the metabolic activity of basal cells, which is specifically involved in the cell proliferation. The GLUT-1 score showed both strong positivity and weak positivity in oral leukoplakia. Christoph Reisser stated that the expression of GLUT-1 is based on the extent of dysplasia; it has an inverse correlation with glycogen content in nondysplastic area of epithelium. They also suggested that GLUT-1 expression may be a reliable marker for diagnosis of premalignant alterations in squamous dysplasia of head and neck region. The severe dysplasia included in the study showed lower percentage of GLUT-1 positive cells, which did not correlate with the findings of christoph Reisser et al who observed GLUT-1 expression in all the layers of epithelium. However, the reason for such low expression cannot be justifiably discussed due to limited sample size. The normal oral mucosa never expressed GLUT-1 in cytoplasm of the epithelial cells, whereas few cases of leukoplakia showed expression in cytosol of epithelial cells.

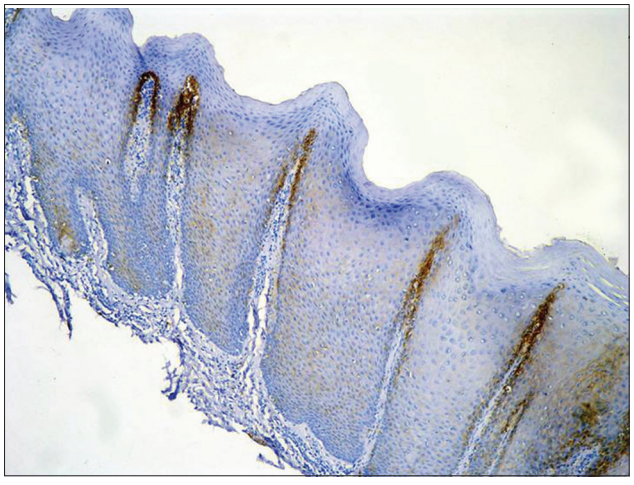

Figure 4: Oral leukoplakia demonstrating GLUT-1 expression in focal areas of the basal layer of the dysplastic epithelium (×10)

CONCLUSION

The pronounced expression of GLUT-1 in leukoplakia may be used as a reliable marker to identify the high-risk group for malignant transformation. To reduce the mortality and morbidity of OSCCs, GLUT-1 may be a target for anticancer strategy in conjunction with the existing treatment modalities. Due to inadequate sample size of severe dysplasia, the study results could not be able to give a conclusive statement on the significance of GLUT-1 in different grades of oral leukoplakia. In future, undertaking studies with a larger sample in GLUT-1 and related molecules may provide an insight on the role of GLUT-1 in malignant transformation of PMD.

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Conflicts of interest

There are no conflicts of interest.

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