Original Article

# GLUT-1 and Carbonic Anhydrase IX Immunoexpression in Oral Squamous Cell Carcinoma

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## Abstract

**Background:** Oral squamous cell carcinoma (OSCC) is the most prevalent malignancy and represents more than 91.5% of all oral malignancies. glucose transporter-1 (GLUT-1), a glucose transporter, increases the intake of glucose for use in aerobic glycolysis for quick cell division. Another protein that helps tumor cells survive is carbonic anhydrase IX (CAIX). **Objectives:** The aims of this study were to evaluate the expression of GLUT-1 and CAIX in OSCC patients and determine how these proteins' expression correlated with histopathological grading and clinical staging. **Materials and Methods:** 24 specimens of OSCC were carefully chosen during the period of 2014–2017. Histological grade and TNM (T :tumor, N: extent of spread to the lymph node, M: presence of metastasis) clinical staging were done. The immunohistochemistry procedure was completed by the ordinary procedure for paraffin-embedded tissue blocks. **Results:** The clinical stage of OSCC was observed to significantly correlate with the expression of GLUT-1 and CAIX ($P$ = 0.043 and $P$ = 0.007, respectively), whereas an insignificant correlation of these proteins with histopathological grade was observed. Stage IV patients have higher GLUT-1 expression. This expression was mainly observed in the membrane and cytoplasm of tumor cells. Regarding CAIX, the majority of tumor cells have combined nucleus and cytoplasmic staining. **Conclusion:** Positive expression of GLUT-1 and CAIX relates significantly to pTNM staging and insignificantly to histological grade of OSCC. As a result, the expression of GLUT-1 may be used as a biomarker for those who have OSCC.

**Keywords:** CAIX, GLUT-1, immunohistochemistry, squamous cell carcinoma

## Introduction

Cancer is a serious public health concern in terms of morbidity and death worldwide.[1] Oral squamous cell carcinoma (OSCC) is the most prevalent malignancy and represents more than 91.5% of all oral malignancies.[2] It is the most common type of metastatic malignancy to cervical lymph nodes.[3] It is a locally aggressive tumor that advances quickly and has a low oxygen concentration.[4] Cellular modifications brought on by hypoxia can allow tumor cells to adapt and survive in extended hypoxic conditions, which can lead to a more aggressive behavior that induces metastasis and invasion.[5] According to studies, it may be beneficial for these cells to get nutrients if proteins linked to cellular metabolism are expressed more frequently. The overexpression of glucose transporter-1 (GLUT-1) is associated with enhanced glucose absorption by malignant cells, which is used in aerobic glycolysis for quick cell division.[6] The majority of cells from healthy tissues do not readily display GLUT-1 with the exception of renal tubules, erythrocytes, testicular germinal cells, endothelial cells in blood-brain barrier vessels and the perineurium of peripheral nerves.[7] High expression of GLUT-1 in OSCC promotes an higher consumption of glucose via the membrane, providing the neoplastic cells with the required metabolic necessities for their quick multiplication, high energy needs, and aggressive behavior.[8-10] It is seen as a sign of a poor prognosis. Predictive and prognostic uses for the enhanced

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expression are possible.[8,11] Carbonic anhydrase IX (CAIX) is another protein activated by hypoxic condition and induces the reversible hydration of $CO_2$, supplying protons for extracellular acidosis and bicarbonate ions for intracellular neutralization. This enzyme helps tumor cells survive.[12] Studies have revealed a correlation between these proteins (GLUT-1 and CAIX) and the development of several cancer forms, particularly squamous cell carcinoma; therefore, the aim of this study is to evaluate the expression of GLUT-1 and CAIX in patients with OSCC and to assess how these proteins expression relates to clinical staging and histological grading and how it effect on the clinical behavior of tumor.

## Materials and Methods

Twenty four formalin-fixed and paraffin-embedded tissue blocks diagnosed as oral squamous cell carcinoma were obtained in the period 2014-2017 with age spectrum of 37-75 years (mean age was 67 years). From these 24 cases, four were removed after extensive local excision and extreme neck dissection and 20 were tissue specimens taken from alveolus, buccal mucosa, tongue, lateral border of tongue, floor of mouth, and gingiva. Clinical staging of these patients was performed in accordance with the American Joint Committee on Cancer staging method, and histological grading using Bryne's Invasive Tumor Front Grading system (1989 and 1992) was carried out on sections with H&E staining [Figure 1].

### Immunohistochemistry

From paraffin-embedded tissue blocks, 4-µm-thick slices were cut out for immunohistochemistry analysis. To prevent endogenous peroxidase activity, after paraffin wax removed, the sections were submerged in three percentage of hydrogen peroxide. After washing the tissue slices in phosphate-buffered saline (PBS), a few drops of protein block were added to the slides to prevent background staining that was not specific. Table 1 provides information on the GLUT-1 and CAIX antibodies' antigen retrieval method, catalog number and antibody dilution. The primary antibodies were incubated with the sections for an hour at room temperature in a wet chamber before they were

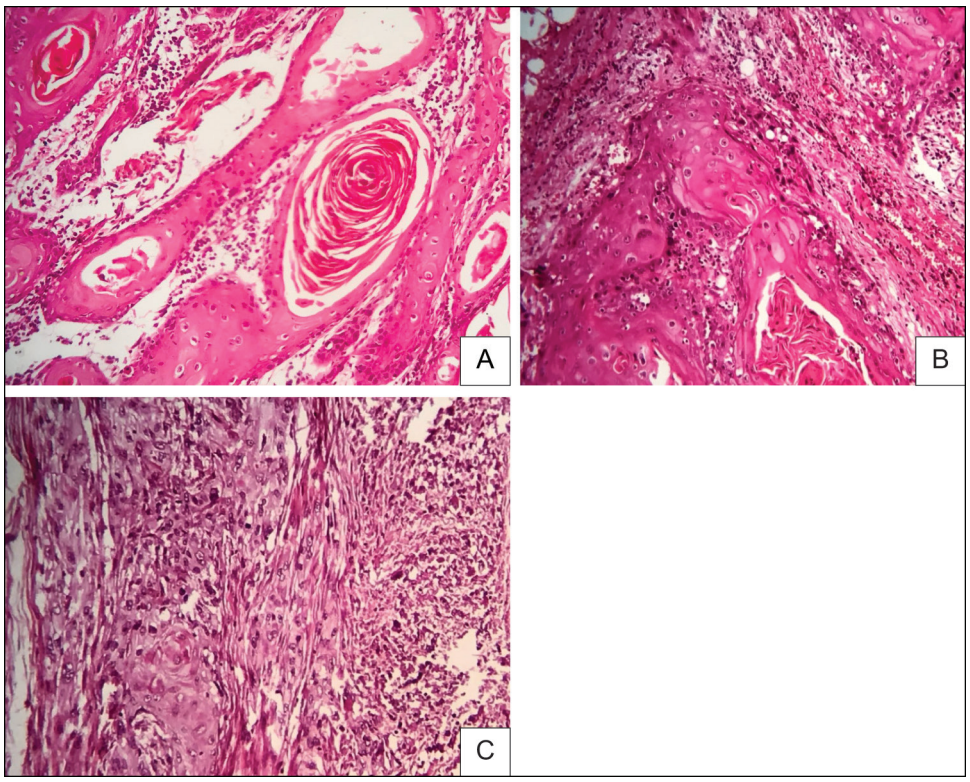


**Figure 1:** Photomicrographs of H&E staining of tissue specimens (A) well differentiated SCC. (B) Moderately differentiated SCC. (c) Poorly differentiated SCC (×200)

**Table 1: Catalog number, specificity, company, dilution, antigen retrieval, and incubation of the primary antibodies**

| Catalog number | Specificity | Company | Dilution | Antigen retrieval | Incubation |
|---|---|---|---|---|---|
| Ab15309 | GLUT-1 | Abcam | 1/200 | Not required | Overnight |
| Ab128883 | CAIX | Abcam | 5 µg/mL | Citrate, pH 6.0 Pascal, 90-95°C, 20 min | Overnight |

GLUT-1 = glucose transporter-1

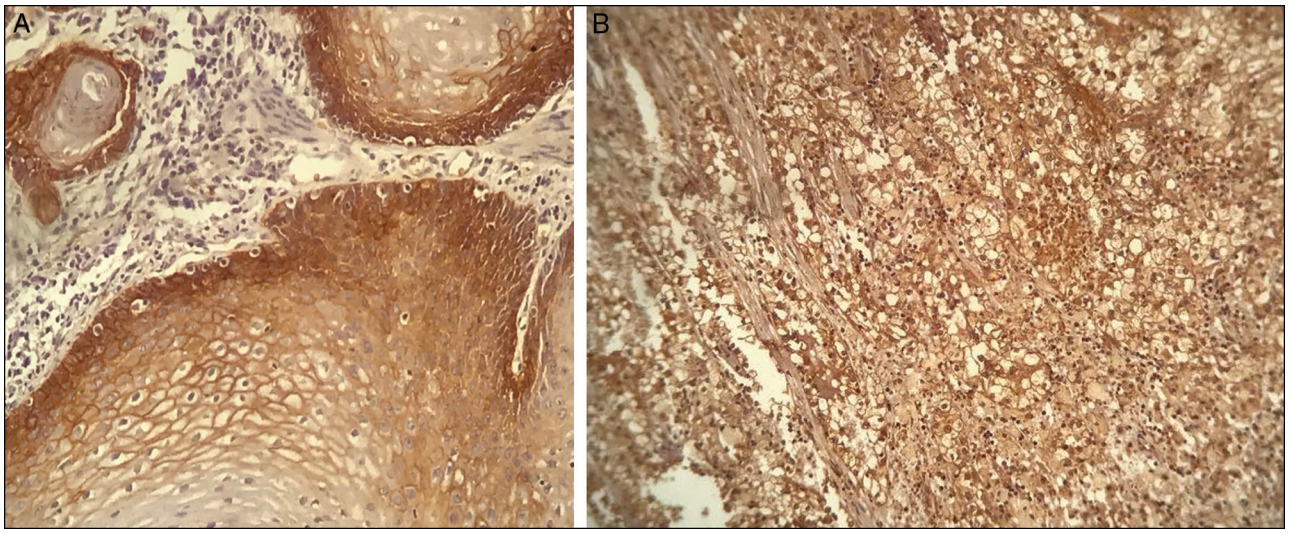


**Figure 2:** (A) Positive expression of glucose transporter-1 in esophageal carcinoma (positive control) [Immunohistochemistry (IHC) stain, ×100]. (b) Positive expression of CAIX in clear cell renal cell carcinoma (positive control) (IHC stain, ×100)

placed in a refrigerator overnight at 4°C. The tissue samples were subsequently given two PBS washes. Following the addition of the secondary antibody, the primary antibodies were bound in a humid chamber. The sections were then treated with the detection solution (streptavidin-horseradish peroxidase reagent), and the chromogen solution (liquid 3,3'-Diaminobenzidine + substrate, Abcam) was added, producing a brown reaction product. The sections were then counterstained with hematoxylin, and the covers were applied.

According to the manufacturer's instructions, positive control samples for GLUT-1 and CAIX were acquired from clear cell renal cell carcinoma (ccRCC) and squamous cell carcinoma of the esophagus, respectively [Figure 2]. As an internal positive control for the GLUT-1, erythrocytes in each region were used.

### Immunostaining assessment and statistical analysis

Using a light microscope, the tissue sections were examined. GLUT-1 immunostaining was estimated by determining whether the cell membrane, nucleus, and cytoplasm were stained or not. Haphazard fields were selected and three hundred cells were count up. Following that, a grade and proportion of positive cells were determined. The staining was assessed by two observers; in turn, the intensity of staining and the data' mean was calculated. On the basis of the percentage of tumor cells that displayed the protein, the intensity was grouped in total cases from 0 to 3, where 0 denoted negative stain (<10% positive tumor cells), 1 represented mild stain (<25% positive tumor cells), 2 represented moderate stain (25%–50% positive tumor cells), and 3 denoted intense stain (>50% positive tumor cells).[11]

Samples were declared positive for CAIX expression if tumor cells taken displayed brown staining on the cytoplasm, nucleus, membrane or all three compartments. A light microscope with a 400× magnification was used to examine five representative tumor sites. Specimens were scored according to the percentage of positive cells present: 0 (0% of positive cells), 1 (1%–10% of positive cells), 2 (11%–30% of positive cells), 3 (31%–50% of positive cells), and 4 (>50% of cancer cells stained).[13]

Following immunohistochemical examination, the findings were put on a database using the statistical analysis tool chi-square test and SPSS software version 24.0 (Statistical Package for the Social Sciences IBM Corporation, Chicago, USA), with the level of significance set at 95% ($a$ = 0.05).

### Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. The study protocol was approved by a local ethics committee according to the document number 832, on June 26, 2023.

## Results

Table 2 lists the fundamental features of OSCC patients. The majority of cases were male patients and aged above 50 (62.5%). Tongue (41.66%) was the most common and buccal mucosa and gingiva was the least frequent location. Majority of patients were in stage IV (83.33%) and most frequent histopathological grade was grade I (50%).

Immunohistochemistry findings of OSCC patients are summarized in Tables 3-5. The positive expression of GLUT-1 was detected in total cases. In the center of the tumor, the expression was significantly weaker

and mostly present around the tumor's periphery. The positive GLUT-1 expression was mainly detected in the membranous and cytoplasm of tumor cells. The internal positive control used was erythrocytes. Staining intensity was found to be one in three cases, two in 11 cases, and three in 10 cases of OSCC. This study demonstrated a significant *P* value of 0.038 for a positive relationship between OSCC and GLUT-1 expression [Figure 3].

Analysis of CAIX immunoexpression showed positive staining in all cases. There was a high prevalence of combined nucleus and cytoplasmic staining in neoplastic cells. The staining expression was score 1 in eight (33.3%) cases, score 2 in five (20.83%) cases, score 3 in eight (33.3%), and score 4 in three (12.5%) cases. This study demonstrated a significant *P* value of 0.000 for a positive correlation between CAIX expression and OSCC [Figure 3].

Among the four cases of stage III, staining of GLUT-1 was strong in three cases and moderate in 1 case. Among 20 cases of stage IV, seven cases exhibited strong staining (score 3), 10 cases were moderate and three cases showed weak stain. The expression patterns of GLUT-1 showed a strong association with the OSCC clinical stage ($P = 0.043$ by using Chi-square test) [Table 4]. In regard to CAIX staining, among 20 cases with stage IV, eight showed weak stain and five cases were moderate, whereas six cases showed intense staining. The expression patterns of CAIX showed significant association with the OSCC clinical stage ($P = 0.007$ by using Chi-square test) [Table 4].

Table 5 displays the expression of GLUT-1 and CAIX in relation to OSCC histological grade. In regarded to GLUT-1 expression, staining intensity was found to be 2 in five cases and 3 in seven cases among 12 cases with grade I staining. In eight patients with grade II, the staining intensity was 2 in five cases and 3 in three cases whereas in

**Table 2: Age, sex, and patient distribution for oral squamous cell carcinoma with different factors**

| Variables | Number (n) and (%) of patients |
|---|---|
| Age (years) | |
| <50 | 5 (20.8 %) |
| >50 | 19 (79.2%) |
| Sex | |
| Male | 15 (62.5%) |
| Female | 9 (37.5%) |
| Tumor site | |
| Buccal mucosa | 4 (16.66%) |
| Lateral border of tongue& tongue | 10 (41.66%) |
| Floor of mouth | 7 (29.16%) |
| Alveolus and gingival | 3 (12.5%) |
| Histopathological grade | |
| Grade I | 12 (50%) |
| Grade II | 8 (33.33%) |
| Grade III | 4 (16.66%) |
| TNM stage | |
| I | 0 (0) |
| II | 0 (0) |
| III | 4 (16.66%) |
| IV | 20 (83.33%) |

TNM = T: tumor, N: extent of spread to the lymph node, M: presence of metastasis

**Table 3: Distribution of cases of OSCC, accordance to the positive scores for glucose transporter-1 and carbonic anhydrase IX**

| | Glucose transporter-1 | | | | CAIX | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Score 0 | Score 1 | Score 2 | Score 3 | Score 0 | Score 1 | Score 2 | Score 3 | Score 4 |
| | n (%) | | | | n (%) | | | | |
| OSCC | 0 | 3 (12.5%) | 11 (45.83%) | 10 (41.66%) | 0 | 8 (33.3%) | 5 (20.83%) | 8 (33.3%) | 3 (12.5%) |
| *P* value | | | 0.038 | | | | 0.000 | | |

N: number of cases

$P < 0.05$ (S), $P < 0.001$ (HS)

**Table 4: Expression of glucose transporter-1 and CAIX in relation to various clinical phases in OSCC patients**

| | IHC scores | Stage I (%) | Stage II (%) | Stage III (%) | Stage VI (%) | P value |
|---|---|---|---|---|---|---|
| GLUT-1 | Score 0 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0.043 |
| | Score 1 | 0 (0) | 0 (0) | 0 (0) | 3 (12.5) | |
| | Score 2 | 0 (0) | 0 (0) | 1 (4.16) | 10 (41.66) | |
| | Score 3 | 0 (0) | 0 (0) | 3 (12.5) | 7 (29.16) | |
| CAIX | Score 0 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0.007 |
| | Score 1 | 0 (0) | 0 (0) | 0 (0) | 8 (33.3) | |
| | Score 2 | 0 (0) | 0 (0) | 0 (0) | 5 (20.83) | |
| | Score 3 | 0 (0) | 0 (0) | 2 (8.33) | 6 (25) | |
| | Score 4 | 0 (0) | 0 (0) | 2 (8.33) | 1 (4.16) | |

IHC = immunohistochemistry, GLUT-1 = glucose transporter-1

**Table 5: Expression of glucose transporter-1 and CAIX in relation to various histopathological grades OSCC patients**

| | IHC scores | Grade I (%) | Grade II (%) | Grade III (%) | P value |
|---|---|---|---|---|---|
| GLUT-1 | Score 0 | 0 (0) | 0 (0) | 0 (0) | 0.605 |
| | Score 1 | 0 (0) | 0 (0) | 3 (12.5) | |
| | Score 2 | 5 (20.83) | 5 (20.83) | 1 (4.16) | |
| | Score 3 | 7 (29.16) | 3 (12.5) | 0 (0) | |
| CAIX | Score 0 | 0 (0) | 0 (0) | 0 (0) | 0.529 |
| | Score 1 | 0 (0) | 4 (16.66) | 4 (16.66) | |
| | Score 2 | 5 (20.83) | 0 (0) | 0 (0) | |
| | Score 3 | 6 (25) | 2 (8.33) | 0 (0) | |
| | Score 4 | 1 (4.16) | 2 (8.33) | 0 (0) | |

IHC = immunohistochemistry, GLUT-1 = glucose transporter-1

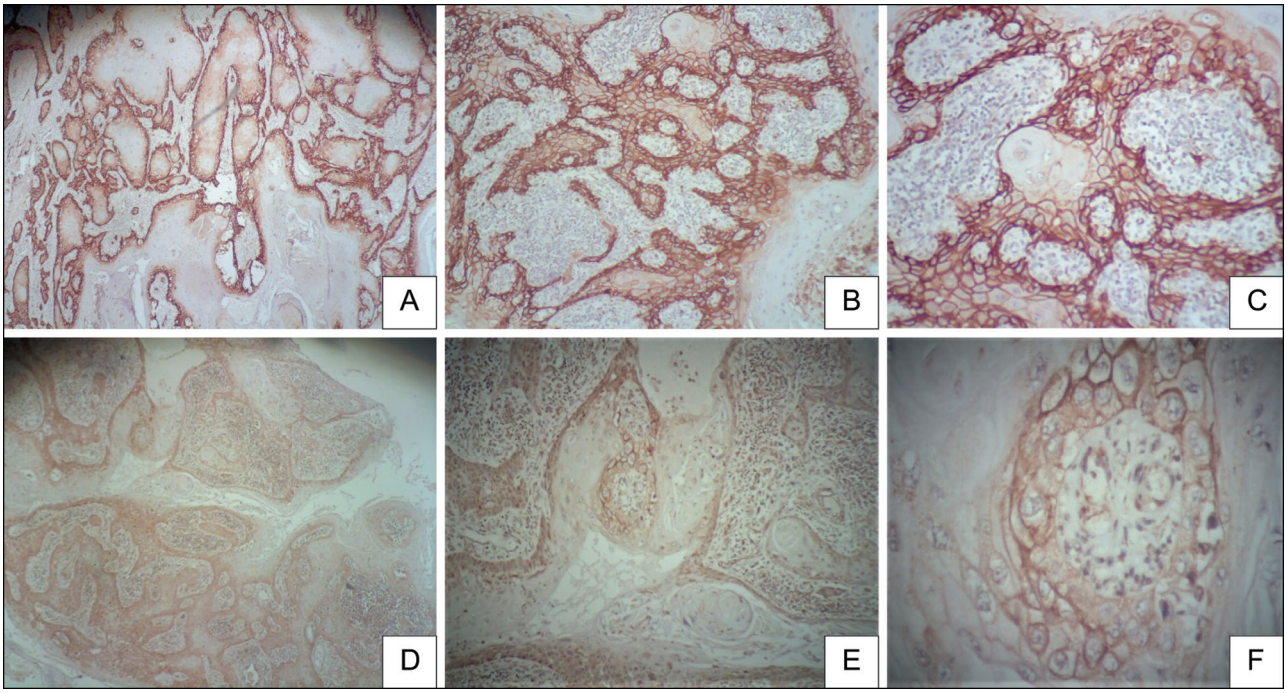


**Figure 3:** Positive expression of glucose transporter-1 in well differentiated OSCC (Grade I) [immunohistochemistry (IHC) stain]: (A) ×100, (B) ×200) (C) ×400. Positive expression of CAIX in well differentiated OSCC (Grade I) (IHC stain): (D) ×100, (E) ×200, (F) ×400

grade III cases ($n = 4$), staining intensity was 1 in three cases and 2 in one case. Comparing histopathological grades with GLUT-1 immunohistochemistry scores in OSCC patients led to statistically insignificant ($P = 0.605 > 0.05$) findings. Concerning with CAIX expression, out of 12 samples with grade I, staining intensity was detected to be 2 in five cases, 3 in six cases, and 4 in one case. Staining intensity was 1 in four cases, 3 in two cases, and 4 in two cases among eight cases with grade II. In all cases with grade III ($n = 4$), staining intensity was 1 with insignificant ($P = 0.529 > 0.05$) results were found when comparing histopathological grades with immunohistochemistry scores of CAIX in OSCC patients.

## Discussion

Tumor ischemia and hypoxia result from an insufficient flow of oxygenated blood brought on by the disordered vascular circulation that forms in tumors.[14] The transcription factor hypoxia-inducible factor (HIF-1) regulates the overexpression of a number of genes in tumors, including GLUT-1, CAIX, and vascular endothelial growth factor.[14,15] Pimonidazole, which has been described as a hypoxia marker, was shown to be linked with the expression of GLUT-1 and CAIX, according to research by Airley *et al.*[14] These results showed that one of the intrinsic indicators of hypoxia was GLUT-1.[16] It has been proposed that GLUT-1 is a marker of tumor development and aggressiveness.[17,18] It was stated to be related with a bad prognosis for OSCC patients. It is a key gene for the HIF, which regulates glucose input into cells under circumstances with increased metabolic demands, such as division of cells, malignant development, and nutritional deficiency. As a result, the relationship between malignancy and unregulated expression of

GLUT-1 is widely established.[19] When oral epithelial dysplasia is severe, the expression of GLUT-1 rises.[20] In severe dysplasia, it was extensive and intense, affecting the granular and corneal layers of the whole epithelium. The cells' low or missing glycogen content in sections of epithelium with dysplastic growths has been connected to this association with dysplasia grade.[20,21] In the present study, 79.2% of patients were over 50 years old and male patients made up the majority (62.5%). Similar results were seen by Malhotra *et al*.,[22] Shyam Sunder *et al*.,[23] Zini *et al*.,[24] Abbas *et al*.,[25] and Abdulhussain[26] and in contrast with Saeed and Abdullah.[27] The tongue (41.66%) was the most frequently affected tumor location in this research, which is in accordance with Azad *et al*.,[19] Alkawaz,[28] Taha and Younis[29] followed by floor of mouth (29.16%) and buccal mucosa (16.66%). In this study, out of 24 cases, 12 (50%) cases were in grade I, which is an agreement with Harshani *et al*.[11] and 20 (83.33%) cases were in pTNM stage IV. The expression of GLUT-1 was found in all cases (100%). Harshani *et al*.[11] reported similar findings, stating that the examination of GLUT-1 immunoexpression revealed positive staining in 100% of OSCC, with strong expression in 41.66 % of OSCC which is in agreement Ayala *et al*.[17] and in contrast with Tian *et al*.[30] Our findings are consistent with those of Reisser *et al*.,[31] who found that GLUT-1 staining was present in the periphery of tumor nests and was contributed in cellular differentiation, proposing that it may be able to estimate the level of histologic differentiation. In this study, the majority of the immunohistochemical staining was cytoplasmic and membrane with little nuclear staining, which is agreement with the report of Harshani *et al*.[11] and Usman *et al*.,[32] where all samples had membranous staining with just one case exhibiting nuclear staining. However, in this regard, our study differed from Ayala *et al*.,[17] who discovered that 49.7% of patients were nuclear stained and 50.3% of cases were membranous. Our study showed a positive association between TNM (T: tumor, N: extent of spread to the lymph node, M: Presence of metastasis) staging of OSCC and GLUT-1 immunoexpression with *P* value of 0.043, which is in agreement with Harshani *et al*.,[11] Angadi *et al*.,[33] Azad *et al*.,[19] Usman *et al*.[32] But it differs from the study conducted by Ohba *et al*.[34] as well as Demeda *et al*.[35] A *P* value of 0.605 in the Chi-square test indicated that there was no significant association between the histological grades of OSCC and GLUT-1 staining. Similar findings were made by the research done by Tian *et al*.,[30] Airley *et al*.,[36] Demeda *et al*.,[35] Angadi *et al*.,[33] and Usman *et al*.,[32] in which GLUT-1 staining was assessed in cases of OSCC and statistically no positive association was discovered between tumor differentiation or T grade classification and GLUT-1 staining. In contrast with Azad *et al*.[19] finding's that OSCC tumor differentiation and GLUT-1 appear to be correlated. Regarding the CAIX, it contributes to the microenvironment of malignancies by keeping the extracellular pH acidic and promoting the growth and spread of cancer cells and correlated with the tumor depth.[37,38] In this study, we noted CAIX expression in 100% of the cases and showed statistically positive correlation between CAIX expression and clinical stages. These results come in agreement with Lorenzo-Pouso *et al*.[37] who observed CAIX expression in 98% of cases and OSCC cases with stage IV, T4 or high N factor cancer had CAIX over expression (≥50%) and a propensity for a bad prognosis, whereas statistically insignificant correlation was observed between the CAIX and histopathological grades which disagree with previous study.

## Conclusion

In OSCC cases, GLUT-1 and CAIX expression were shown to be statistically significantly correlated, and their expression increased with more advanced clinical stages. The enhanced expression of these proteins indicated an increase in glycolytic activity and aggressive behavior of tumor cells of OSCC with an increase in the stage of tumor. However, when GLUT-1 and CAIX expression was compared with tumor grade, no significant outcomes could be seen. However, in order to obtain more definitive results, research with bigger sample sizes must be carried out.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

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