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Immunohistochemical Expression of Glypican-3 in Oral Squamous Cell Carcinoma, Oral Dysplastic Epithelium, and Non-Cancerous Oral Epithelium

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ABSTRACT

Background and Objective: Oral Squamous cell carcinoma (OSCC) is the most common oral cancer, and its early detection by molecular histopathological techniques can improve treatment outcomes. Glypican-3 (GPC3) plays a dual role (oncofetal and tumor suppressive) in various cancers. The aim of this study is to compare the expression of GPC3 between OSCC, dysplastic epithelium and non-cancerous oral epithelium.

Methods: In this cross-sectional study, the immunohistochemical expression of GPC3 was assessed in 87 paraffin-embedded tissue blocks of OSCC, as well as dysplastic and tumor-free margins of lesions obtained from 29 patients. Clinical and demographic data including age and gender of patients, lesion location, tumor size, lymph node involvement, and clinical stage, were extracted from patient records. The score of GPC3 staining was determined based on the percentage of stained epithelial cells and the intensity of staining. The final GPC3 expression score was calculated by multiplying the staining intensity score by the score obtained from the percentage of stained epithelial cells. Scores of 4 or less were considered as low expression of GCP3 and scores between 5 and 12 were considered as high expression of GPC3.

Findings: The expression of GPC3 was higher in both OSCC and dysplastic epithelium compared to normal oral mucosa ($p<0.001$). However, no statistically significant relationship was found between GPC3 expression and clinicopathologic parameters. High expression of GPC3 was observed in 45% of OSCC samples and 41% of dysplastic epithelium samples but only low expression of GPC3 was observed in non-cancerous oral epithelium samples.

Conclusion: The results of the study showed that GPC3 expression is higher in OSCC and dysplastic epithelium compared to non-cancerous oral epithelium. Therefore, GPC3 can be considered as an oncofetal gene contributing to initiation of carcinogenesis and its development, as well as the progression of OSCC.

Keywords: *Squamous Cell Carcinoma, Dysplastic Epithelium, Non-Cancerous Oral Epithelium, Glypican-3.*

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Introduction

Dysplastic epithelium refers to morphologically altered tissues that are more likely to transform into cancerous lesions than normal epithelium. It has been noted that oral squamous cell carcinoma (OSCC) can originate from the dysplastic epithelium (1, 2). This cancer is one of the most common tumors of epithelial origin rising in the head and neck region (3). Early diagnosis is vital to effective treatment of this cancer (4). OSCC can develop in the tongue, floor of the mouth, buccal mucosa, gingiva, palate, and other parts of the mouth. Due to the invasive nature of OSCC, it can spread to areas beneath tissues and other regions, including cervical lymph nodes, liver, and lungs (2). The pathogenesis of OSCC involves the accumulation of genetic alterations occurring over years and causing dysplastic changes in cellular morphology (5). Survival in OSCC patients diagnosed in the last stages is significantly shortened, many of whom show a poor response to treatment and a high recurrence rate (6). Appropriate treatment depends on the clinical stage and includes wide excisional surgery, radiotherapy, or a combination of both. Different chemotherapeutic agents may also be used as complement therapy (4). In some special cases, molecular targeted therapy may also be applicable (3, 7-9).

Glypican-3 is a surface protein of the glypicans family belonging to the heparin sulfate group that binds to the cell membrane**.** This protein regulates several signaling pathways by facilitating or suppressing the attachment of these growth factors to their corresponding receptors. The expression of this protein increases during embryogenesis and organogenesis but subsides during adulthood (10). This molecule is known to regulate cellular proliferation (1) and morphogenesis (11).

Early diagnosis, regular screening, molecular-pathological laboratory techniques, and suitable therapeutic modalities play an important role in the outcome of OSCC (4). Glypican-3 plays a dual role (oncofetal and tumor suppressive) in various cancers. The overexpression of GPC3 has been shown in lung squamous cell carcinoma (12). Also, in OSCC, the expression of this marker has been reported to be higher in the tumoral tissue than in tumor-free tissue (5).

In hepatocellular carcinoma, GPC3 may be a promising marker and can be used as a diagnostic marker (13). Furthermore, GPC-3's re-expression in HCC and its involvement in key tumorigenic processes underscore its value as a biomarker for early diagnosis and a target for therapeutic intervention (14). In renal cell carcinoma, GPC3 was reported to be downregulated compared to normal tissues, suggesting a tumor-suppressive role for GPC3 (15, 16). These findings suggest that the function of GPC3 may differ based on the tissue of origin in which it is expressed (16).

There are ambiguities regarding the role of GPC3 in OSCC carcinogenesis. In order to help diagnose and even treat OSCC, we intended to investigate the expression of GPC3 in OSCC tumoral tissues in comparison with dysplastic epithelium and non-cancerous oral mucosa.

Methods

After approval by the Ethics Committee at Babol University of Medical Sciences with the code IR.MUBABOL.HRI.REC.1401.009, this cross-sectional study was conducted on 87 paraffin-embedded blocks of OSCCs (29 cases), dysplastic margins (29 cases), and their tumor-free margins (29 cases) belonging to 29 patients. The samples were obtained from the archives of pathology department of Shahid Beheshti Hospital, Babol, Northern Iran. These samples belonged to newly diagnosed patients with primary OSCC who received no treatment yet. Clinical and demographic data including age and gender of patients, lesion location, tumor size, lymph node involvement, and clinical stage, were extracted from patient records.

The sample size was calculated using the following formula and based on a previous study investigating GPC3 expression in OSCC (10). Considering α=0.05, P=73.3% (GPC3 expression in OSCC), and d=0.25, the sample size was estimated as 23 and due to the availability of sufficient samples, 29 samples were included. First, 4-µm thick slices were prepared from paraffin-embedded blocks and stained with hematoxylin-eosin (H&E) to confirm the diagnosis and check for inclusion criteria. The samples were then approved by an oral and maxillofacial pathologist. Histological selection criteria were lesion confirmation and grade determination in the case of OSCC and grade of dysplasia, as defined by Neville et al. (1).

For immunohistochemical staining, 4-µm tissue slices were prepared from paraffin-embedded blocks and inserted into xylene for deparaffinization. Then tissue slices were dehydrated by being immersed in alcohol solutions with different dilutions. Then, slices were washed with distilled water, and antigen retrieval was conducted using Dako Cytomation solution (pH=9) for 20 minutes. Intrinsic peroxidase activity was suppressed by 3% H_2O_2. Next, the tissue sections were incubated with anti-GPC3 antibodies (Mouse anti-Glypican-3 IgG1, Ready-to-Use, Zytomed Systems, Berlin, Germany) for 30 minutes.

After that, they were exposed to secondary antibody (HRP-Polymer System, Cell marque, Sigma-Aldrich, California, USA) and DAB (Diaminobenzidine, liquid DAB + Substroke Chromogen System; Dako, Denmark) for staining reaction and Mayer's hematoxylin (for background staining) for 15 minutes. Next, the tissues were placed in a TBS (Tris Buffer Saline) phosphate buffer. Finally, the slides were covered with a coverslip and examined by a pathologist under an optical microscope (Labomed, Labo America Inc., USA).

Membrane and cytoplasmic staining for GPC3 was considered positive. To evaluate GPC3 staining, the percentage of positive epithelial cells and the intensity of the staining of these cells were considered. The staining intensity was estimated semi-quantitatively according to scores of 0: no staining, 1: positive staining with light yellow color, 2: positive staining with yellow color, 3: strong positive staining with brown color. The percentage of stained epithelial cells was determined by counting GPC3-positive cells in five microscopic fields (400x magnification). The scores were defined as follows: 0: When 10% or less of the epithelial cells were stained, 1: When between 11-25% of the epithelial cells were stained, 2: When 26-50% of the epithelial cells were stained, 3: When 51-75% of the epithelial cells were stained, 4: When more than 75% of the epithelial cells were stained.

The final GPC3 score was calculated as the product of the score for the intensity of epithelial cell staining and the score for the mean percentage of epithelial cells stained. Scores of 4 or less were considered as low GCP3 expression, and scores between 5 and 12 were considered as high GPC3 expression (15). The positive control consisted of gastric epithelium, and the negative control was obtained by omitting the primary antibody.

Data were analyzed by the statistical software SPSS version 22. The expression levels of GPC3 in OSCC, dysplastic epithelium, and non-cancerous epithelium were presented using descriptive statistics and compared between tissue samples using the chi-square test, one-way ANOVA, and independent t-test. The statistical significance level was designated as $p<0.05$.

Results

A total of 87 samples, including 29 paraffin-embedded tissue blocks of OSCC, 29 blocks of dysplastic margins, and 29 blocks of normal oral mucosa margins, from 13 male patients (44.8%) and 16 female patients (55.2%) with a mean age of 68.38±14.74 years (age range of 25 to 88 years), were included in the study (Table 1). Of all OSCC samples examined in this study, 4 were Stage I, 13 were Stage II, 4 were Stage III, and 8 were Stage IV. The mean tumor size was 3.34±1.30 cm, and 6 (20.7%) of them had lymph node

involvement. In blocks of dysplastic margins, 19 (65.5%) showed severe dysplasia, 10 (34.5%) revealed moderate dysplasia, while there was no case of mild dysplasia. Regarding the percentage of stained epithelial cells, more than 50% of epithelial cells showed positive staining for GPC3 in 58% and 62% of OSCC and dysplastic epithelium samples, respectively. In non-cancerous oral epithelium, 93.1% of the samples showed <10% staining. (Table 2). Accordingly, there was a statistically significant difference between OSCC, dysplastic, and non-cancerous epithelium in terms of the percentage of stained epithelial cells ($p<0.001$). Also, there was a statistically significant difference between the groups regarding the intensity of staining; epithelial cells in OSCC showed stronger staining for GPC3 compared to dysplastic and non-cancerous epithelium. Also, the intensity of staining was higher in dysplastic epithelial cells compared to non-cancerous counterparts ($p<0.001$).

Table 1. Frequency distribution of squamous cell carcinoma by oral site of involvement

Location of tumor	Number(%)
Floor of the mouth	7(24.1)
Gingiva and alveolar ridge	3(10.3)
Buccal mucosa	11(37.9)
Labial mucosa	2(6.9)
Tongue	3(10.3)
Palate	3(10.3)
Total	29(100)

Table 2. Percentage of stained epithelial cells and staining intensity of epithelial cells in oral squamous cell carcinoma and dysplastic and non-cancerous oral epithelium

Samples	Expression	Grading	Number(%)
Oral Squamous Cell Carcinoma	Percentage of stained epithelial cells	≤10%	1(3.5)
		11-25%	4(13.79)
		26-50%	7(24.13)
		51-75%	9(31.03)
		>75%	8(27.58)
	Staining intensity of epithelial cells	No staining	0(0)
		Light yellow	9(31.03)
		Yellow	11(37.93)
		Brown	9(31.03)
Dysplastic epithelium	Percentage of stained epithelial cells	≤10%	2(6.89)
		11-25%	6(20.68)
		26-50%	3(10.34)
		51-75%	13(44.82)
		>75%	5(17.24)
	Staining intensity of epithelial cells	No staining	1(3.46)
		Light yellow	11(37.93)
		Yellow	11(37.93)
		Brown	6(20.68)
Non-cancerous epithelium	Percentage of stained epithelial cells	≤10%	27(93.10)
		11-25%	2(6.9)
		26-50%	0(0)

		51-75%	0(0)
		>75%	0(0)
		No staining	15(51.74)
	Staining intensity of epithelial cells	Light yellow	13(44.82)
		Yellow	1(3.44)
		Brown	0(0)

Finally, according to the information obtained from the intensity of staining of epithelial cells and the percentage of stained epithelial cells, the comparison of the final score between the three groups of OSCC, dysplasia, and normal mucosa indicated a statistically significant difference (p<0.001) (Table 3 and Figure 1).

Table 3. Comparison of the final score of GPC3 expression between the three groups

Group	Final score	p-value*
Oral squamous cell carcinoma	5.17±3.06	<0.001
Dysplastic oral epithelium	4.52±3.04	<0.001
Non-cancerous oral epithelium	0.18±0.03	<0.001

***Chi-square**

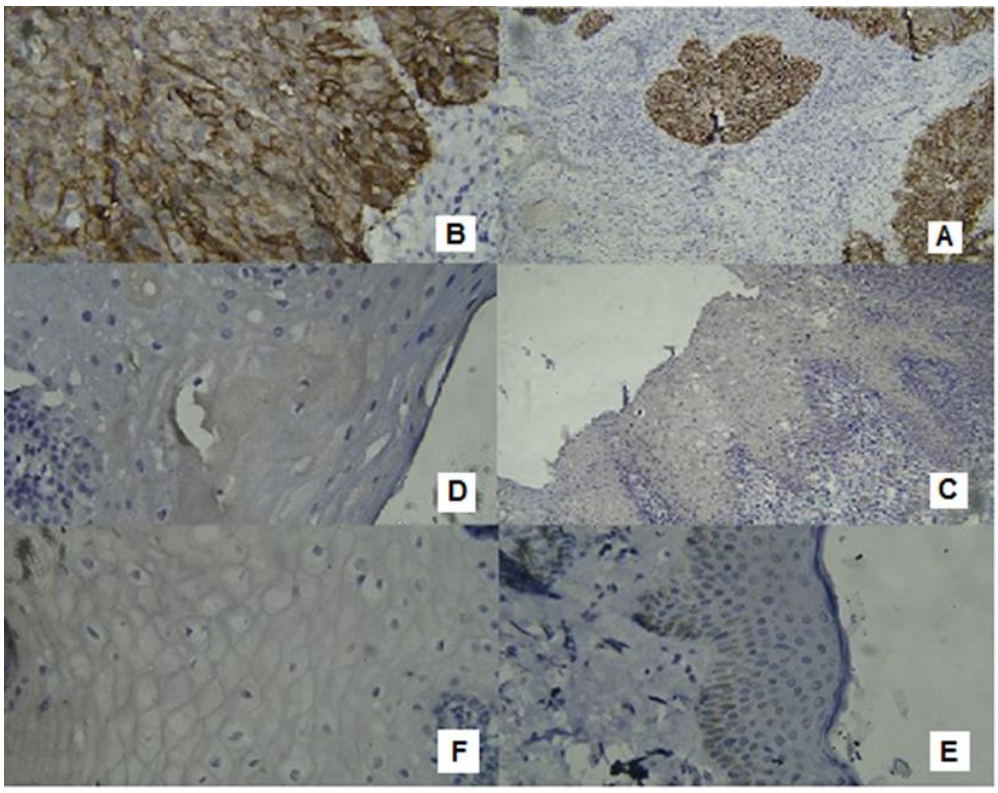

Figure 1. Immunohistochemical staining with GPC3 marker. A: Squamous cell carcinoma at 100x magnification, B: Squamous cell carcinoma at 400x magnification, C: Dysplastic oral epithelium at 100x magnification, D: Dysplastic oral epithelium at 400x magnification, E: Normal oral mucosa at 100x magnification, F: Non-cancerous oral mucosa at 400x magnification

The comparison of GPC3 expression between OSCC, dysplastic epithelium, and non-cancerous epithelium samples revealed a significant difference ($p<0.001$) (Table 4). In addition, a significant relationship was observed between the OSCC versus normal oral mucosa and oral dysplasia versus normal oral mucosa groups ($p<0.001$ and $p<0.001$, respectively). However, the expression of the desired marker in OSCC and dysplastic epithelium samples was close to each other and no statistically significant difference was obtained in their comparison.

Regarding GPC3 expression in OSCC samples, there was no statistically significant relationship between GPC3 expression and clinicopathological factors (Table 5). In dysplastic epithelium samples, GPC3 expression was comparable between those with moderate and severe dysplasia, showing a statistically significant difference close to significance level ($p=0.051$).

Table 4. The expression level of GPC3 in study groups

	Low expression (final score≤4) Number(%)	High expression (final score 5-12) Number(%)	p-value*
Oral squamous cell carcinoma	16(18.4)	13(14.9)	<0.001
Dysplastic oral epithelium	17(19.5)	12(13.8)	<0.001
Non-cancerous oral epithelium	29(33.3)	0(0)	<0.001

*Chi-square

Table 5. Comparison of GPC3 expression in oral squamous cell carcinoma samples based on clinicopathological factors

Variable	Low expression	High expression	p-value
Tumor size (Mean±SD)	3.44±1.11	3.22±1.53	0.665**
Lesion site			
Mouth floor	3	4	0.595*
Gingiva and alveolar ridge	2	1	
Labial mucosa	0	2	
Buccal mucosa	7	4	
Tongue	2	1	
Palate	2	1	
Lymph node involvement			
Involved	3	10	0.775**
Not involved	13	3	
Clinical stage			
I	1	3	0.450*
II	9	4	
III	2	2	
IV	4	4	
Differentiation degree			
Poor	4	1	0.220*
Moderate	4	7	
Good	8	5	

*Chi-square, **Independent t-test

Discussion

In the present study, the expression level of GPC3 was assessed in OSCC, dysplastic epithelium, and non-cancerous oral epithelium. It was observed that the expression of this marker was higher in OSCC samples and dysplastic oral epithelium compared to non-cancerous oral epithelium. Moreover, the intensity of staining and the percentage of positive epithelial cells were significantly higher in OSCC compared to dysplastic and non-cancerous oral epithelium. According to these findings, it seems that GPC3 is involved both in the creation of dysplastic changes in the oral mucosa and in the development and progression of OSCC. In similar studies, researchers have affirmed that GPC3 expression has been higher in tumoral samples compared to normal tissues, reflecting the role of this protein in carcinogenesis (1, 10, 12, 17, 18).

Wu et al. stated that glypicans were aberrantly expressed in OSCC tissues, indicating the role for these molecules in carcinogenesis (17). Sales et al. also showed that GPC3 protein was expressed in OSCC tumor cells but not in tumor-free tissues. Glypicans such as GPC3 play an important role in the biological processes of tumor cells not only by regulating the hedgehog (HH) pathway but also by acting as both oncoprotein and tumor suppressor (1). Butler et al. also found that GPC3 was specifically secreted by neuroendocrine cells and could act as an oncofetal protein in prostate cancer (19).

Another finding was that GPC3 expression in patients with OSCC had no significant correlation with clinicopathological parameters (lesion location, tumor size, lymph node involvement, degree of differentiation, and clinical stage of patients). Andisheh-Tadbir et al. also detected GPC3 expression in more than 70% of OSCC samples; however, the reaction was negative in non-neoplastic tissues. These researchers also observed no association between GPC3 expression and clinicopathological factors such as grading and lymph node metastasis (10), which was in line with the results of our study.

Aviel-Ronen et al. investigated GPC3 expression in lung adenocarcinoma and normal lung tissues and identified no positivity for GPC3 in normal tissues; however, most lung carcinoma samples were positive for this protein. Similar to our findings, these researchers witnessed no correlation between GPC3 expression and clinicopathological characteristics such as age, sex, disease stage, and outcome (12). Wang et al. investigated the biological role of GPC3 and its carcinogenic mechanisms in liver cancer and found that hepatic tumors expressed high levels of GPC3, which was accompanied by cancer cells' poor differentiation and high proliferation (18).

In contrast with our results and those of the above-mentioned studies, Jot et al. declared a significantly lower GPC3 expression in OSCC than in normal oral mucosa, suggesting a tumor suppressive role for this marker, and justifying its downregulation in OSCC (20). Also, Gonzales et al. suggested that GPC3 could inhibit cellular proliferation and promote a tumor suppressor role. These researchers showed that apoptosis induction in mesothelioma and breast cancer cells required the binding of OCI-5/GPC3 to its receptor on the cell membrane (21). Valsechi et al. also stated that GPC3 expression was decreased in clear cell renal cell carcinoma compared to normal renal tissues, suggesting that a reduction in cellular proliferation in cell lines with the overexpression of GPC3 could indicate the tumor-suppressive role of this protein (15).

Regarding the possible role of GPC3 in the malignant transformation of salivary gland tumors, Andisheh-Tadbir et al. stated that GPC3 expression in malignant salivary tumors (adenoid cystic carcinoma and mucoepidermoid carcinoma) was significantly higher than in pleomorphic adenoma, and in pleomorphic adenoma it was higher than normal salivary glands, and they did not observe a relationship between GPC3 expression and clinicopathological parameters (22), which is consistent with the results of the present study in terms of the higher incidence of GPC3 in malignant neoplasms compared to noncancerous oral mucosa.

A number of studies have investigated the possible role of GPC3 in cancer diagnosis and treatment. For instance, Filmus et al. identified GPC3 expression in hepatocellular carcinoma (HCC) tissues but not in benign, cirrhotic, or normal hepatic samples. Accordingly, they proposed two GPC3-targeting treatment approaches for HCC, including anti-GPC3 monoclonal antibodies, which significantly prevented the growth of GPC3-positive HCC xenografts in mice. The second approach was based on a GPC3-peptide vaccine consisting of two GPC3-derived peptides that were able to stimulate cytotoxic T lymphocytes without causing autoimmunity, preventing HCC development (11).

In another study, Zheng et al. concluded that anti-GPC3 antibodies could not completely eradicate hepatic tumor cells; however, GPC3-based immunotherapies (e.g., CAR-T cell therapy) were more effective in this regard (23). Also, Scheper et al. found that a Furin inhibitor could be effective in the treatment of hepatoblastoma via GPC3-mediated suppression of cellular proliferation (24).

Sawada et al. evaluated the safety and efficacy of a GPC3 peptide vaccine in patients with advanced HCC and stated that this vaccine was well-tolerated by patients and measurable anti-tumor immune responses and anti-tumor effects (25). Noting that GPC3 was reactive in around half of HCC patients, but it was not detected in the sera of patients with liver cirrhosis, Nakatsura et al. suggested that this protein could play a role as an oncofetal tumor marker and a target for immunotherapy (26). Considering the results of these studies regarding various human cancers, it can be noted that GPC3 promotes a dual role, acting as an oncoprotein in some cancers and a tumor suppressor in others. In the present study, our results support an oncogenic role for GPC3 in OSCC.

Considering the higher levels of GPC3 in OSCC and dysplastic oral epithelium samples compared to non-cancerous samples, it can be concluded that GPC3 acts as an oncogene in OSCC, contributing to both the initiation, primary development and progression of OSCC. Further studies can help determine if this protein can be used as a tumor marker or as an immunotherapy target in OSCC.

Conflict of Interest: The authors declare that they have no conflict of interest.

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