RESEARCH ARTICLE

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HLA-G Expression is Associated with an Unfavorable Prognosis of Oral Squamous Cell Carcinoma

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Abstract

Background: HLA-G, a major histocompatibility complex of non-classical class Ib, plays a key role in the development of the primary tumors to metastatic stages. The aim of this study was to investigate HLA-G expression in oral squamous cell carcinomas and its relationship with clinicopathological factors. **Methods:** After immunohistochemical staining for HLA-G with 63 formalin fixed and paraffin embedded blocks (33 oral squamous cell carcinoma and 30 normal oral mucosa samples), staining intensity, percentage of stained cells and final immunoreactivity score were evaluated, along with other variables. **Results:** Staining intensity, percentage of stained cells and final immunoreactivity scores in oral squamous cell carcinomas were higher than those in normal oral mucosa (all P=0.001). The staining intensity in the parenchyma of squamous cell carcinoma cells was significantly associated with the clinical tumor stage (P=0.022) and the group with lymphatic metastasis exhibited a higher staining percentage (P=0.026). Staining intensity and immunoreactivity score (IRS) exhibited a significant but inverse correlation with survival rate (P=0.004 and P=0.018, respectively) and a significant direct relationship with clinical stage (P=0.001 and P=0.001). **Conclusion:** The results supported a role of HLA-G in development of oral squamous cell carcinomas and metastasis to lymph nodes. It might be useful in molecular-targeted therapy.

Keywords: HLA-G- immunohistochemical staining- metastasis, prognosis- squamous cell carcinoma

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Introduction

Squamous cell carcinoma is the most common type of oral cancer with poor prognosis (Neville et al., 2016; Seyedmajidi et al., 2017 ; Seyedmajidi et al., 2018); oral and oropharyngeal squamous cell carcinoma represent about 3% of cancers in men and 2% of cancers in women (Regezi et al., 2017). Since most of the early-stage oral squamous cell carcinomas usually do not cause visible changes in the oral cavity, its early detection is very important for survival (Neville et al., 2016). An increased incidence of primary oral squamous cell carcinoma (OSCC) has been reported in young patients in Iran (Seyedmajidi et al., 2014). The patients suffer from several complications after treatment of oral cancers (Motallebnejad et al., 2014).

Nowadays there is much interest in novel molecular markers for predicting patient prognosis and estimating overall survival rate in different cancers (Amini Shakib et al., 2015).

HLA-G is a major histocompatibility complex (MHC) from type I non-classical class, which plays a key role in the progression of primary tumor into metastatic stage in cancer cells and inhibits the function of immune cells (Cai et al., 2012). In this regard, few studies have been performed in other body areas. Andrea Souza et al evaluated the increased expression of HLA-G in oral squamous cell carcinoma. They concluded that increased expression of HLA-G is associated with its potential for metastasis in oral squamous cell carcinoma (Gonçalves et al., 2014).

Increased expression of HLA-G in gastric carcinoma with poor prognosis was shown in a study by Yie et al., (2007). In addition, a study by Strand et al revealed the role of HLA-G as a marker to detect the treatment efficacy in cervical carcinoma (Davidson et al., 2005). It has been reported that expression of HLA-G is associated with metastasis to lymph nodes, grade of the tumor, clinical stage and recurrence risk of oral squamous cell carcinoma (Gonçalves et al., 2014). In another study, increased expression of HLA-G was reported in association with decreased survival rate of patients in hepatocellular carcinoma (Cai et al., 2009).

TNM is commonly used to predict clinicopathological status in cancers. However, the predictive value of the staging system is not sufficient. There are some molecular

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markers that can be effective in improving the efficacy of clinical staging system (Davidson et al., 2005). Therefore HLA-G marker might be used to enhance the efficacy of this system.

There are limited studies on the possibility of the use of HLA-G as a prognostic marker. Since few studies have been performed on HLA-G expression in oral squamous cell carcinoma and no comprehensive study has been performed on the relationship between HLA-G expression and clinicopathological factors and most studies have been performed on other areas of the body, the aim of this study was to investigate the expression of HLA-G in oral squamous cell carcinoma and its relationship with some of the clinicopathological factors such as age and gender of patients, clinical stage of tumor, grade of tumor, regional lymph node metastasis, distant metastasis and survival rate of patients.

Materials and Methods

After ethical approval from the ethics in research committee of Babol university of medical sciences (No. MUBABOL.REC.1394.292), this cross-sectional study was performed on 33 formalin-fixed and paraffin-embedded samples of oral squamous cell carcinoma that were collected from the archives of the oral pathology department of Babol dental school. Thirty formalin-fixed and paraffin-embedded samples of normal oral mucosa with minimal inflammation from clinical and histopathological aspects that were obtained from patients undergoing crown lengthening surgery were also included in the study.

Medical records of patients diagnosed with oral squamous cell carcinoma were collected from the archives of Shahid Rajaee hospital of Babolsar (north of Iran) and after recording personal data, the paraffin blocks were collected from Babol dental school and several hospitals of Mazandaran province.

The necessary information, including age and gender of patients, location of tumor, clinical stage, grade of tumor, metastasis to cervical lymph nodes, distant metastasis and overall survival rate of patients, was obtained from the patients' records, histopathological report or by contacting the patients. Survival rate, death and cause of death were determined through contacting the patients or their family members.

Two sections were prepared from each paraffin block. The first section (4 microns in thickness) was stained with hematoxylin and eosin to confirm the initial diagnosis, and appropriate blocks containing sufficient amounts of the samples were selected. The second section was prepared for immunohistochemical evaluation.

The sections were deparaffinized and placed in a microwave oven in citrate buffer (pH=6.1) for 15 minutes for antigen retrieval. Then, they were washed in Tris Buffered Saline (TBS) (pH=7.2) and treated with 3% hydrogen peroxide for 10 min to block the endogenous peroxidase activity. Subsequently, the sections were incubated with diluted primary antibodies for 1 hour, followed by TBS wash, using diluted (1:50) Anti-HLA-G antibody [4H84] (ART NO: ab52455, Abcam CO, USA). Secondary antibodies (Goat Anti-Rabbit IgG H and L (HRP) ART NO: ab97051-1 mg Abcam CO, USA) were applied and incubated for 30 minutes with substrated diaminobenzidine (DAB) as chromogen (liquid DAB + substrate chromogen system; Dako, Denmark). The sections were eventually washed and counterstained with Meyer's hematoxylin. Finally the slides were mounted with DPX mountant.

Human trophoblasts were used as positive controls and negative controls were obtained by the omission of primary antibodies. All the slides were evaluated separately by two pathologists who were blinded to the clinical features of samples under an optical microscope (Olympus BX41, Tokyo, Japan) at ×400. Immunohistochemical expression of HLA-G was assessed by random selection of five histological fields and the percentage of cells with cytoplasmic and membranous staining was considered.

For immunohistochemical assessment, the percentage of stained cells in tumor (in the parenchyma and stroma of the tumor, in epithelial cells and lamina propria in normal oral mucosa) were scored as follows:

Score 0: Tumors in which 0% of cells were stained.

Score 1: Tumors that were stained <25%.

Score 2: Tumors that were stained ≥25%

Staining intensity of cytoplasm and membrane of tumoral cells by HLA-G was graded as follows: no staining (score 0), poor (score 1), moderate (score 2) and severe (score 3).

The final score (Immunoreactivity score or IRS) was obtained by multiplying scores of staining intensity and percentage of stained cells: 0 as no expression, ≤2 as low expression and >2 as high expression (Cai et al., 2012; Gonçalves et al., 2014).

Data were analyzed with SPSS 20, using chi-squared test, t-test, Spearman's rank correlation coefficient, nonparametric Mann-Whitney test and Kaplan-Meier survival analysis. Chi-squared test was used to assess the relationship between gender, age, clinical stage of tumor, tumor location and the expression of HLA-G; Spearman's correlation coefficient was used to evaluate survival rate of the patients. Mann-Whitney was used for qualitative variables. Statistical significance was set at $P<0.05$.

Results

Sixty-three samples were evaluated, including 33 paraffin blocks of oral squamous cell carcinoma from 12 male patients (36.4%) and 21 female patients (63.6%), with a mean age of 70.91±17.05 years, and 30 samples of normal oral mucosa with minimal inflammation from clinical and histopathological aspects that were obtained from crown lengthening procedures (performed on 30 patients with an average age of 27.33±8.77 years).

Oral squamous cell carcinoma specimens examined in this study consisted of 25 patients (75.8%) with grade I, 5 patients (15.2%) with grade II and 3 patients with grade III. Location of the tumor in 15 samples was the tongue, 10 cases were from the buccal mucosa, 6 cases were from the gingiva and retromolar pad and 2 cases were from the floor of the mouth.

In this study, HLA-G staining was evaluated in both the

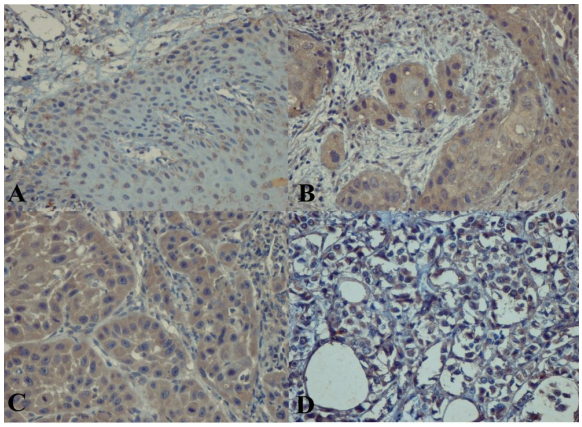

Figure 1. HLA-G Immunohistochemical Staining in (A) normal oral mucosa, (B) well-differentiated oral squamous cell carcinoma, (C) moderately differentiated oral squamous cell carcinoma and (D) poorly differentiated oral squamous cell carcinoma (at a magnification of ×400).

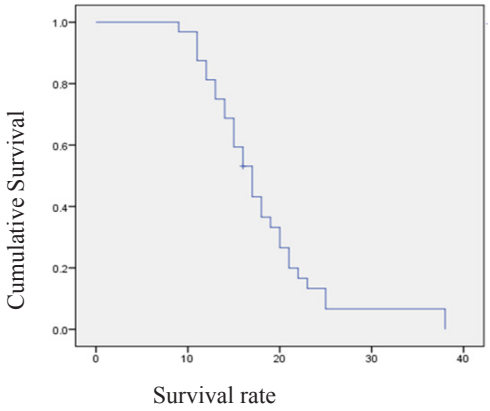

Figure 2. Evaluation of Patient Survival Rate in Terms of HLA-G Expression by Kaplan-Meier Method

parenchyma and stroma of oral squamous cell carcinoma (Figure 1, A to D).

The frequency distribution of the percentage of stained

Table 1. Frequency Distribution of the Percentage of Stained Cells and Staining Intensity of Cells and IRS in the Parenchyma and Stroma of Oral Squamous Cell Carcinoma and Normal Oral Mucosa

Group	Expression	Scoring	Frequency (number)	Frequency (percent)
Oral squamous cell carcinoma	The percentage of stained cells in the tumor parenchyma	Without staining	0	0%
		Staining Less than 25 percent of tumoral cells	1	3%
		Equal to or more than 25 percent of tumoral cells	32	97%
	Staining intensity of cells in the tumor parenchyma	Without staining	0	0%
		Weak staining	7	21.20%
		Moderate staining	19	57.60%
		Severe staining	7	21.20%
	Immunoreactivity score (IRS) in tumor parenchyma	Lack of expression	0	0%
		Low expression	6	18.20%
		High expression	27	81.80%
	The percentage of stained cells in the stroma	Without staining	1	3%
		Staining in less than 25 percent of tumoral cells	11	33.30%
		Staining at equal to or more than 25 percent of tumoral cells	21	63.60%
	Staining intensity of cells in the stroma	Without staining	1	3%
		Weak staining	19	57.60%
		Moderate staining	13	39.40%
		Severe staining	0	0%
	Immunoreactivity score (IRS) in the stroma	Lack of expression	1	3%
		Low expression	21	63.60%
		High expression	11	33.30%
Normal oral mucosa	The percentage of stained cells	Without staining	29	96.70%
		Staining in Less than 25 percent of tonality	1	3.30%
		Staining at equal to or more than 25 percent of tonality	0	0%
	Staining intensity of cells	Without staining	29	96.7%%
		Weak staining	1	3.30%
		Moderate staining	0	0%
		Severe staining	0	0%
	Immunoreactivity score(IRS)	Lack of expression	29	96.70%
		Low expression	1	3.30%
		High expression	0	0%

Table 2. Comparison of HLA-G Expression in Normal Oral Mucosa and Parenchyma and Stroma of Oral Squamous Cell Carcinoma

Group	Mean Rank of Oral squamous cell carcinoma (parenchyma)	Mean Rank of Normal oral mucosa	P value	Mean Rank of Oral squamous cell carcinoma (stroma)	Mean Rank of Normal oral mucosa	P value
Variable						
percentage of stained cells	46.98	15.52	0.001>	43.36	16.2	0.001>
intensity of staining	46.89	15.62	0.001>	46.24	16.33	0.001>
IRS	34.5	26.5	0.001>	46.21	16.37	0.001>

Table 3. Comparison of the Mean Expression of HLA-G in the Parenchyma and Stroma of Oral Squamous Cell Carcinoma

Group	Mean Rank of Oral squamous cell carcinoma (parenchyma)	Mean Rank of Oral squamous cell carcinoma (stroma)	P value
Variable			
percentage of stained cells	39.02	27.98	0.001>
intensity of staining	41.48	25.52	0.001>
IRS	41.59	25.41	0.001>

cells and staining intensity in normal oral mucosa, the parenchyma and stroma of oral squamous cell carcinoma are presented in Table 1.

Comparison of HLA-G expression in normal oral mucosa and parenchyma and stroma of oral squamous cell carcinoma in terms of percentage and staining intensity of cells and IRS is illustrated in Table 2. This comparison was performed with Mann-Whitney test. Mean scores of two groups were compared.

The results showed that the percentage of stained cells, staining intensity and IRS in the parenchyma of tumor cells were significantly higher than those in normal oral epithelial cells (P<0.001). Also the results showed that the percentage and staining intensity of cells and IRS in the stroma of squamous cell carcinoma were significantly higher than those in normal oral mucosa (P<0.001).

Comparison of HLA-G expression of parenchyma and stroma of oral squamous cell carcinoma in terms of the percentage and staining intensity of cells and IRS is summarized in Table 3. This comparison was performed with Mann-Whitney test. The mean scores of the two groups were compared.

The results showed that the percentage of stained cells, staining intensity and IRS were significantly higher in the parenchyma compared to the stroma (P<0.001).

Moreover, comparison of the percentages of stained cells and staining intensity and IRS was performed in the presence or absence of lymphatic and distant metastasis in the same way.

Table 4 shows that there was no significant difference in the percentage of cell staining between the two groups (P=0.175), although the group with lymphatic metastasis showed higher expression. The group with lymphatic metastasis exhibited a significantly higher staining intensity (P=0.026). In term of the IRS, the group with lymphatic metastasis showed higher expression with no significant difference (P=0.080).

Table 5 shows no significant difference in the percentage of stained cells between the two groups (P=0.976). The group with distant metastasis showed a significantly higher staining intensity (P=0.022). In terms of the IRS, the group with distant metastasis exhibited higher expression, but this difference was not significant (P=0.181).

The relationship between survival duration, age of patients, HLA-G expression and metastasis was assessed using Spearman's correlation coefficient. The results are presented in Table 6.

Chi-squared test revealed a significant relationship between IRS in the parenchyma and clinical stage of tumors (P=0.001). On the other hand, this test showed no significant relationship between the IRS in the stroma and clinical stage of tumors (P=0.529).

There was no significant relationship between IRS

Table 4. Comparison of Two Groups with and without Lymph Node Metastasis in Terms of the Percentage of Stained Cells, Staining Intensity and IRS

Group	With lymph node metastasis (Mean Rank)	Without lymph node metastasis (Mean Rank)	P value
Variable			
percentage of stained cells	34.44	30	0.175
intensity of staining	36.65	27.16	0.026
IRS	35.61	28.5	0.08

Table 5. Comparison of the Two Groups with and without Distant Metastasis in Terms of the Percentage of Stained Cells and Staining Intensity and IRS

Group	With distant metastasis (Mean Rank)	Without distant metastasis (Mean Rank)	P value
Variable			
percentage of stained cells	32.44	32.54	0.976
intensity of staining	38.56	28.62	0.022
IRS	35.86	30.35	0.181

in the parenchyma and tumor grades (P=0.693). Similar results were obtained in the stroma (P=0.891).

The results showed that smoking and gender of patient had no significant impact on survival (P>0.05). The time interval between the initial diagnosis of oral squamous cell carcinoma and death of the patient due to this disease or until the last follow-up is defined as the 'overall survival'.

The Kaplan-Meier analysis was used to determine the impact of HLA-G expression on patient survival based on the total follow-up period that is independent of other known risk factors. According to the results, the decline in survival rate was not statistically significant (P=0.845) (Figure 2).

Discussion

The present study showed that expression of HLA-G in oral squamous cell carcinoma was higher compared to normal oral mucosa, which confirmed the role of HLA-G in carcinogenesis. In other words, the expression of HLA-G in terms of the staining intensity, percentage of stained cells and immunoreactivity score in the parenchyma and stroma of squamous cell carcinoma exhibited a very significant difference from its expression in normal oral mucosa. Singer et al found that evaluation of HLA-G expression might be used as an adjunctive molecular index in the cytological assessment to differentiate malignancies of abdominal cavity from benign lesions (Singer et al., 2003).

Development of oral squamous cell carcinoma is related to genetic changes due to a lack of control mechanism for cell growth and differentiation (Ibrahim et al., 2004). Development of tumors is attributed to a series of genetic events with abnormal activity of oncogenes and metastasis-related genes and inactivation of tumor suppressor genes (Kumar et al., 2015). Molecular markers can play an indispensable role in predicting tumor aggressiveness. By identifying these biological markers, the capability of clinical staging system can be increased and prediction of prognosis and progression of oral squamous cell carcinoma is improved (Cai et al., 2012; Singer et al., 2003; Kumar et al., 2015). HLA-G biological marker is one of them. HLA-G is a protein responsible for presentation of antigens to T cells and belongs to the major histocompatibility complex class I. Its overall structure consists of a 45 kDa glycosylated heavy chain attached with a non-covalent bond to a beta-2 microglobulin (a free polypeptide found in serum) (Delves et al., 2017). HLA-G is a tolerogenic molecule that its expression leads to a mechanism that causes tumor optimal survival. This is due to the interaction of HLA-G which occurs with IL-2 and IL-4 on the surface of T cells, NK cells, dendritic cells and neutrophils leading to the issuance of negative signals in order to reduce the activity of the immune system against tumor cells (Yoon et al., 2007).

In fact, HLA-G as an anti-tumor antigen is found in a nonpathogenic position at maternal-fetal interface in intravillus cytotrophoblasts, chorionic endothelium of placenta, thymus epithelial cells and bone marrow hematopoietic cells. It is also present in other organs with

Table 6. Assessment of the Relationship between the Survival Duration, Age of Patients, Smoking, HLA-G Expression and Metastasis Using Spearman's Correlation Coefficient

Variable	The percentage of stained cells in parenchyma		The intensity of staining in parenchyma		IRS in parenchyma		The percentage of stained cells in parenchyma		The intensity of staining in parenchyma		IRS in parenchyma	
	Correlation coefficient	P-Value	Correlation coefficient	P-Value	Correlation coefficient	P-Value	Correlation coefficient	P-Value	Correlation coefficient	P-Value	Correlation coefficient	P-Value
Age of patients	0.001	0.5	-0.051	0.388	-0.099	0.291	-0.008	0.484	0.145	0.214	0.2	0.136
Overall survival	-0.078	0.336	-0.497	0.002	-0.374	0.018	0.22	0.113	0.087	0.318	0.163	0.186
Tumor stage	0.238	0.095	0.712	0.001	0.636	0.001	-0.106	0.282	-0.059	0.373	-0.055	0.382
The degree of tumor differentiation	-0.273	0.045	0.15	0.202	0.099	0.292	-0.188	0.151	0.109	0.276	0.047	0.399
Lymph node metastasis	0.204	0.132	0.571	0.001	0.545	0.001	0.198	0.139	-0.004	0.491	0.008	0.482
Distant metastasis	0.149	0.209	0.577	0.001	0.397	0.012	-0.095	0.302	0.012	0.474	-0.025	0.445
Smoking	0.086	0.319	0.001	0.5	0.026	0.445	-0.188	0.151	0.109	0.276	0.047	0.399

major levels of immune cells like the cornea, the matrix of the nails and pancreas (Cai et al., 2012).

In a remarkable way, the expression of HLA-G in a variety of malignancies, including gastric carcinoma (Gonçalves et al., 2014; Yie et al., 2007), ovarian carcinoma and endometrial carcinoma (Davidson et al., 2005), has been diagnosed with worse clinicopathological signs and symptoms. Recent studies have shown that HLA-G can cause suppression of the immune system and help tumor cells hide from anti-tumor monitoring of the immune system (Ibrahim et al., 2004; Barrier et al., 2006).

In this study, the expression of HLA-G exhibited a direct relationship with the progression of clinical stage of tumor and an inverse relationship with tumor prognosis; in other words, increased expression of HLA-G, increased the stage and decreased survival rate of patients. Our results are consistent with those reported by Goncalves et al., (2014) They reported that increased expression of HLA-G resulted in an increase in the risk of concealment of tumor cells from monitoring of the immune system, leading to unfavorable prognosis of squamous cell carcinoma.

Another consequence of increased HLA-G staining intensity in the parenchyma of oral squamous cell carcinoma was a significant decrease in patient survival rate, consistent with the results of a study by Näsman et al., (2013) They showed that in oropharyngeal squamous cell carcinoma, patients with no expression of HLA-G/CLASS I had a significantly higher survival rate.

In this study, HLA-G expression in the parenchyma of squamous cell carcinoma exhibited a direct relationship with distant metastasis and an inverse relationship with the survival rate of the patients, consistent with the results reported by Gonçalves et al., (2014); Ibrahim et al., (2004) and Yoon et al. (2007), while lymphatic metastasis showed no significant relationship with HLA-G expression.

HLA-G expression that is found clearly in tumor parenchyma exhibited diffuse distribution in neoplastic islands of oral squamous cell carcinoma but it was significantly lower in the stroma. HLA-G expression in the parenchyma and stroma exhibited no significant relationship with the grade of tumor differentiation.

In this study, the survival rate had no significant relationship with age and gender of patients, while the survival rate had a significant but inverse relationship with the expression of HLA-G and distant metastasis. It appears the survival rate in non-pathological conditions is dependent on age and gender but in the case of oral squamous cell carcinoma it is dependent on the disease.

In the present study, HLA-G expression in the parenchyma of squamous cell carcinoma exhibited a direct relationship with the clinical stage of disease, while it was inversely related to tumor differentiation. However, there was no significant relationship between marker expression in the stroma and clinical stage of the tumor.

In conclusion, based on the results of the current study, high expression of HLA-G in oral squamous cell carcinoma samples compared to normal oral mucosa indicates its role in carcinogenesis. In addition, the inverse relationship between high expression of HLA-G and the survival rate of patients suggests this marker as an independent prognostic marker to determine the prognosis and survival rate of patients diagnosed with squamous cell carcinoma. Thus, considering the results and with further studies in this regard, HLA-G might be used for molecular targeted therapy.

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