Original Article

Correlation of P53 and Granzyme B expression in Oral Squamous Cell Carcinoma with Clinicopathologic Findings

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Abstract

Background: The present study aimed to evaluate the correlation of P53 and granzyme B (GB) expression, and also the relationship between P53 expression and GB cell density with lymph node metastasis, histologic grade, and inflammation intensity in oral squamous cell carcinoma (OSCC).

Methods: Immunohistochemical technique with P53 and GB antibodies were performed on stored paraffin blocks from 48 patients with OSCC (with lymph node metastasis n = 24; without lymph node metastasis n = 24). The density of GB expression was quantified both in invasive front (peritumoral) and within cancer nests (intratumoral).

Results: P53 positivity was seen in 13 (54.16 %) cases of the nonmetastatic group and 14 cases (58.3 %) in the metastatic group. A significant correlation was seen between P53 immunoexpression and histologic grade (P = 0.047), but there was no significant correlation between P53 expression with lymph node metastasis and inflammation intensity. The density of GB^+ cells in the peritumoral zone correlates with a higher intratumoral GB expression (P = 0.001) and was significantly higher in the nonmetastatic group (P = 0.029). No significant correlation between GB and P53 immunoexpression, lymph node metastasis, or inflammation intensity was seen.

Conclusion: The present study showed that the presence of a higher density of GB^+ cells infiltrating the peritumoral area may have an important role against tumoral cells, prevent lymph node metastasis, and better prognosis in OSCC patients.

Keywords: Granzyme B, lymph node metastasis, oral SCC, P53

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Introduction

Oral squamous cell carcinoma (OSCC) constitutes the sixth most common cancer worldwide and accounts for approximately 92000 new cases and 42000 deaths in Europe annually.[1,2] The development of malignant tumors is controlled by a multifactorial biologic system that depends on genetic abnormality and interaction between tumor cells and host immune response.[3]

Antigen specific $CD8^+$ T cells play a crucial role in host defense against malignancies. In the T cell- mediated cytotoxicity process two major pathways are involved which the most important one is a secretory lysosomes, termed lytic granules. The cytotoxic granules contain perforin and a family of highly specific serine proteases known as granzymes which in human, granzyme B (GB) has received the most attention.[4]

GB secreted mainly by cytotoxic T-lymphocytes (CTLs) and natural killer (NK) cells, which kill abnormally proliferating cells due to its ability to induce apoptosis.[5]

On the other hand, many apoptosis- related genes are regulated by the tumor protein 53 (TP53), such as those encoding death receptors and proapoptotic Bcl-2.[6]

Moreover, P53 accumulates in cytoplasm, where it directly activates the proapoptotic protein Bax to promote mitochondrial outer membrane permeabilization.[7] However, despite this knowledge, the functional relationship between GB and P53 remains unknown.

The aim of this study was first to evaluate the correlation of P53 and GB expression in OSCC; second, we investigated the relationship between P53 expression and GB^+ cell density with lymph node metastasis, histologic grade, and inflammation intensity.

Patients and Methods

Patients

This study was based on samples retrospectively collected from 48 patients with primary OSCC who were diagnosed at the Department of Oral and Maxillofacial Pathology, Beheshti Dental Faculty, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Patients without complete clinicopathologic data and insufficient paraffin-embedded tumor material were excluded from the study. The patients were divided into two groups: 1) 24 cases of OSCC without lymph node metastasis (nonmetastatic group), and 2) 24 cases of OSCC with lymph node metastasis (metastatic group). Clinicopathologic information on each case including age, gender, location of tumor, histologic grade, nodal status, and inflammation intensity was obtained from the patients' file and reviewing slides.

Immunohistochemistry

Four μm thick tissue sections were made from 10 % formalin-fixed and paraffin-embedded OSCC tissue, deparaffinizied in xylene, rehydrated in graded ethanol series, and then treated with 3 % hydrogen peroxide for 40 minutes (this step was done only for

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Table 1. Characteristics of the two groups of oral squamous cell carcinoma patients

Variable	Metastatic group	Nonmetastatic group	P-value
Sex			**0.021**
Male	8 (16.6 %)	16 (33.3 %)	
Female	16 (33.3 %)	8 (16.6 %)	
Age (year)			**0.723**
Mean	61.42	63.04	
Range	31–85	24–78	
Site of the tumor			**0.078**
Tongue	12 (25 %)	5 (10.4 %)	
Floor of the mouth	2 (4.1 %)	0 (0 %)	
Other areas	10 (20.8 %)	19 (39.5 %)	
Histologic grade			**0.245**
Well differentiated	11 (22.9 %)	6 (12.5 %)	
Moderately differentiated	5 (10.4 %)	8 (16.6 %)	
Poorly differentiated	8 (16.6 %)	10 (20.8 %)	
Inflammation intensity			**0.876**
I	4 (8.3 %)	5 (10.4 %)	
II	10 (20.8 %)	9 (18.75 %)	
III	10 (20.8 %)	10 (20.8 %)	

P-value < 0.05 considered significant.

Table2. Relation between P53 and GB expression with clinicopathologic findings using multiple regression analysis (beta coefficient and P- value)

Variable	P53		GB intratumoral area		GB peritumoral area	
	P	**Beta**	**P**	**Beta**	**P**	**Beta**
Histologic grade	0.047	-0.31	0.404	-0.132	0.844	0.029
Inflammation intensity	0.28	-0.16	0.462	0.114	0.576	0.081
Lymph node metastasis	0.966	-0.007	0.446	-0.124	0.029	-0.344
Gender	0.778	0.044	0.557	0.096	0.631	0.074
Age	0.362	0.132	0.822	0.035	0.431	-0.114

P-value < 0.05 considered significant.

P53 antibody). The sections were heated in 10 mM citrare buffer (pH: 6) two times for seven and five minutes in a microwave oven at 450 and 800 Watts, respectively. After cooling into room temperature, the slides were incubated with the following primary antibodies: monoclonal mouse human GB (clone 11F1; Novocastra, UK diluted at 1: 60) and monoclonal mouse human antibody P53 (clone DO-7, Novocastra, UK, ready to use) for one hour. After washing in TBS, P53 sections were treated with Novolink polymer detection system (Novocastra), but for GB sections envision + system (DAKO) was used as secondary antibody. Finally, the sections were visualized on stable DAB solution and counterstained with Meyer's hematoxylin.

Evaluation of immunohistochemistry

The expression of GB was assessed in lymphocytes as cytoplasmic granular pattern. The densities were quantified by expressing the number of positive cells/mm^2 in the stroma near the invasive front of OSCC (peritumoral) and within cancer nests (intratumoral) separately. All counts were performed in 10 alternate microscopic high power fields (× 400) using an integration graticule, and then a mean number for each case was identified for statistical analysis.[8] The P53 immunoreactivity in tumor cells was assessed by counting an average of 1000 tumor cell per case and 200 tumor cells per field, and then scored as follows: - (0 % – 25 %), + (26 % – 50 %), ++ (51 % – 75 %), and + + + (76 % – 100 %).[9]

Histologic grade

Grade of differentiation was categorized according to WHO guidelines (2005) into well, moderately, and poorly differentiated.[10]

Inflammation intensity

The intensity of inflammatory reaction around tumor nests was categorized in three groups by counting eight fields as follows: I) discrete: presence of less than 500 inflammatory cells; II) moderate: presence of 500 – 1000 inflammatory cells, and III) intense: presence of more than 1000 inflammatory cells.[11]

Statistical analysis

Mann-Whitney test was performed to compare the age, histologic grade, inflammation intensity, P53 and GB expression in the metastatic and nonmetastaic groups. Relation of lymph node metastasis with gender and tumor site was assessed by chi-square test. To evaluate the correlation of P53 expression (dependent variable) with clinicopathologic variables (independent variables), multiple ordinal regression test was used. Multiple linear regression (enter method) test was applied to determine the correlation of GB $^+$ cell density (dependent variable) with the same independent clinicopathologic variables. Correlation between P53 and GB expression was analyzed by Spearman test. All statistical analyses were performed using SPSS11.5 software. The significant level was set at P less than 0.05.

Results

The general characteristics of 48 patients included in this study have been shown in Table 1. The mean age of metastatic and nonmetastatic groups was 61.42 and 63.04, respectively. Most of the patients in the metastatic group were females (66.6 %); in contrast men (66.6 %) constituted the majority of the nonmetastatic group. Data analysis considering clinicopathologic parameters (Table 1), only showed a significant correlation between gender and lymph node metastasis.

The expression of P53

P53 immunostaining in total samples was seen in more than half of the cases (56.2 %) (Figure1). Table 2 shows data analysis of P53 immunoreactivity correlation with clinicopathologic findings. There was a significant correlation between P53 expression and histologic grade (P = 0.047). However, there were no significant

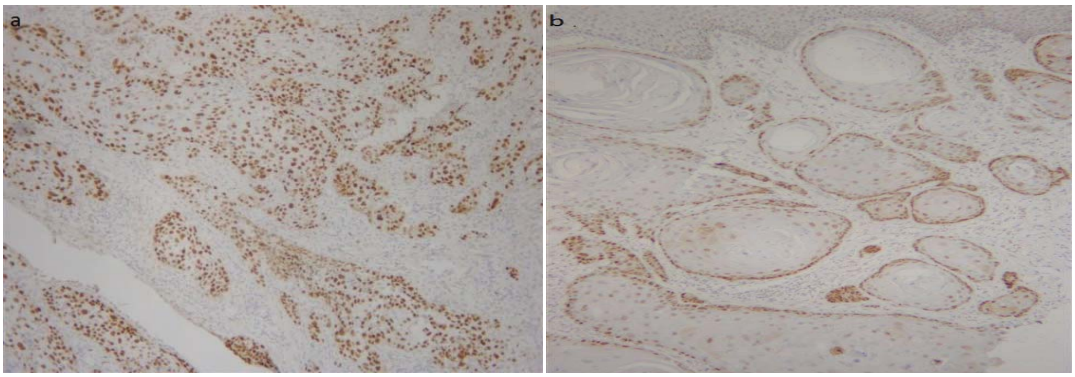

Figure 1. Immunohistochemical expression of P53 in OSCC. **a)** Nuclear P53 staining in tumoral islands (×100); **b)** Nuclear expression of P53 mainly in peripheral cells of tumor nests (×100)

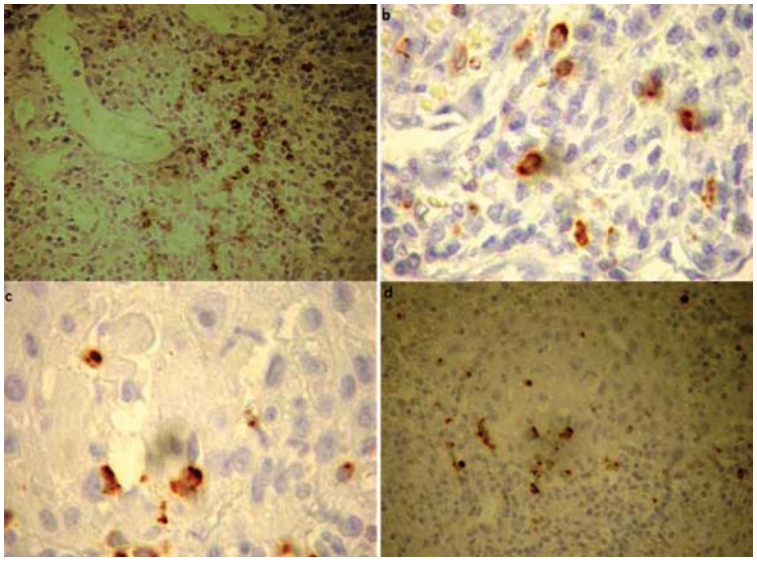

Figure 2. a, b) Granzyme B positive cells in peritumoral area in lymphocytes as granular cytoplasmic staining (× 40 and 400); **c, d)** Few granzyme B positive cells in intratumoral area (× 400 and 40).

relations between P53 expression with lymph node metastasis, age, gender, and inflammation intensity.

The expression of GB

The expression of GB in lymphocytes was assessed as sparsely cytoplasmic granular pattern in two separate zones (peritumoral and intratumoral) (Figure 2). Correlation of GB^+ cell density in peri- and intratumoral areas with clinicopathologic variables are also summarized in Table 2.

Figure 3 and Figure 4 show median values and ranges of GB expression in the metastatic and nonmetastatic groups in peri- and intratumoral areas. Data showed that the density of GB^+ cells in the peritumoral zone was significantly higher in the nonmetastatic OSCC group in comparison with the metastatic OSCC ($P = 0.029$). In contrast, GB^+ cells in the intratumoral zone showed no relation with lymph node metastasis .

In line with these data, the OSCC group with higher number of peritumoral GB^+ cells showed a higher number of GB^+ cells in the intratumoral area ($P = 0.001$). We couldn't find any correlation neither between intratumoral GB^+ cell density and metastatic lymph node nor inflammation intensity, histologic grade, age, and gender. Additionally, there was no significant correlation between P53 expression and GB^+ cell density in both areas ($P = 0.05$).

Discussion

Approaches to treatment of cancer based on the immune system have focused on cytologic effector cells such as CTLs.[12]

In principle, the final goal of chemotherapy as well as hormonal and radiotherapy is induction of apoptosis in tumoral cells. In addition, there are significant numbers of new therapeutic concepts which directly target pro- or antiapoptotic signaling pathways in tumoral cells.[13,14]

Apoptosis pathway mediated via cell intrinsic proapoptotic pathways are largely controlled by P53 and Bcl-2 proteins. It is well known that TP53 has a central role in protecting cells from a variety of stress stimuli such as DNA damage, oncogene activation, or irradation. It is activated as a transcription factor that allows its stabilization and accumulation in the nucleus to regulate target gene expression.[2,15] In addition, to control apoptotic activity, it accumulates in the cytoplasm to activate Bax and releasing cytochrome c. Mutated TP53 often lacks these functions and represents the most frequently identified alteration in human cancer and tumorigenesis.[16] P53 is also a key determinant in antitumor CTL-mediated response that regulates induction of FAS receptor expression, Bid translocation, and CD95–induced activation to target mitochondrial pathway in tumor cells. It has also been established that CTL-tumor target cell interaction resulted in P53

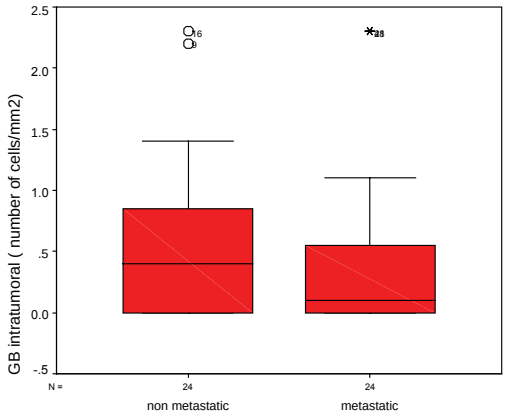

Figure 3. Intratumoral GB expression in metastatic and nonmetastatic groups.

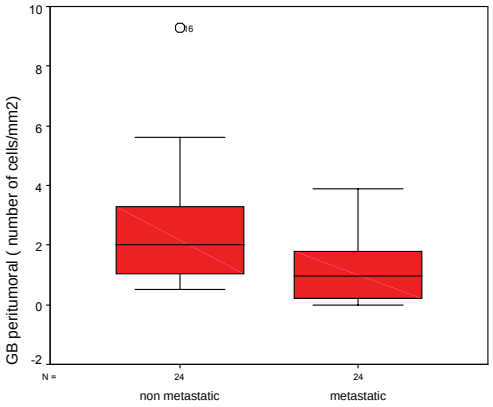

Figure 4. Peritumoral GB expression in metastatic and nonmetastatic groups.

accumulation and activation by GB.[5,17]

Of the five human granzymes, GB is the most potent one in inducing apoptosis in two ways, first it cleaves BH3 domain protein Bid resulting to cytochrome c and activation of procaspase9/3. Besides, GB has substrates such as intracellular domain of notch 1 that contributes to caspase independent –cell death. It is a type of transmembrane receptor which is controlled by ligand binding or cancer-prone oncogenic mutation.[18–21]

Studies have shown that high apoptotic activity of OSCC neoplastic cells is associated with lower lymph node metastasis and increase survival rate. The presence of regional and distant metastasis in head and neck cancer is related to decrease cytotoxiticity of peripheral blood mononuclear cells due to down regulation of GB, perforin, and FAS ligand.[22,23]

According to the existing data, we now have studied the expression relation of P53 and GB with lymph node metastasis and other clinicopathologic findings in OSCC patients.

Our findings revealed a lower number of GB^+ cells in the peritumoral microenvironment of OSCC with lymph node metastasis comparing with the nonmetastatic group. These findings are consistent with observations of Costa, et al.[12], Kondo, et al.[24], Batinac, et al.[8], van Houdt, et al.,[25] and Mulder, et al.[26] In contrast, no clinicopathologic associations with peri- and intratumoral GB^+ cells in OSCC were investigated by Cruz, et al.[27] The current study showed a statistically significant correlation between P53 expression and histologic grade which was similar to other studies[28,29] but P53 expression did not show any correlation with lymph node involvement, inflammation intensity, age, and gender. These results are similar to Bolslakov, et al. and other researchers' studies,[2,16,30] but different from Motta, et al.[31] This result can be attributed to several factors. First, we considered cutoff point for P53 expression over 25 % stained cells based on our reference[9] whereas in Motta, et al.'s study this level has been identified over 10 % stained nuclei. Second, detection of P53 by immunohistochemistry staining does not always display TP53 mutation; even different TP53 mutation hotspot can affect P53 expression in immunohistochemistry staining.[32] Third, many authors have suggested that P53 mutation and expression is responsible for initiation of OSCC and not progression of that.[28,33]

The lack of a significant correlation between P53 and GB expression in peri- and intratumoral areas was seen in current study. Many different in vitro studies with various results to investigate the function and relation of P53 and CTL- mediated cell death have been done.

Meslin, et al. in an in vitro study on melanoma cell line found that P53 activation is mediated by GB and P53 plays efficient role in regulation of CTL-mediated apoptosis of tumoral cells.[5] In contrast, Theiry, et al. concluded that restoration of wild type (wt) P53 can induce a significant potentiation of CTL- mediated cytotoxicity, but it seems that it only can run tumor cell death by activating FAS- mediated pathway not with GB cytotoxicity pathway.[15] Investigation of protease inhibitors and cytokines of apoptosis induced by wt P53 on M1 myeloid leukemic cells demonstrated that GB targets tumoral cells. GB can cleave proteins to an aspartyl residue just like interleukin converting enzyme (ICE) like proteases and activates these apoptosis inducing proteases which are not blocked by inhibitors which seem to be involved in wt P53 –induced apoptosis.[32,34]

Hua, et al.'s research proved that P53 cannot be cleaved by GB to P40, P35, and P13 which have strong proapoptotic activity. They also found that P53 is a physiologic substrate of granzyme K (GK) but not GB and triggering of P53 by GK comparing GB shows more killing capability. Besides, P53- deficient cells were more resistant to CTL-mediated cytolysis than P53 restored cells.[35]

Induction of reactive oxygen species (ROS) as an important factor for activating granzymes –mediated proapoptotic and cell death was brought up by Aguillo, et al. Intracellular accumulation of ROS starts P53 activation and GB-induced cell death accompanied by a caspase-dependent pathway with extramitochondrial origin probably via activation of NADPH oxidase as mentioned by the authors.[36]

To sum up, different results of the above- mentioned studies can imply a combination of multiple biologic processes and factors which affect CTL-induced tumor target cell killing mediated by granzymes which are independent and interact in their functions. However, P53 has a strong role in intrinsic apoptosis pathway, yet extrinsic apoptotic pathway via granzymes specially GB may act via caspase –independent function such as cleavage of notch 1 even in the absence of perforin.[21] Moreover, wt P53 restoration in P53 mutant tumoral cells enhance tumor susceptibility to granzyme-induced cytolysis, whereas deficient P53 may disable GB-mediated cell death. Taken together, our results regarding the lack of correlation of P53 and GB can be justified.

In conclusion, our results suggest that the presence of a higher density of GB^+ cells infiltrating the peritumoral area may have an important role against tumoral cells independent of P53 expression, prevent lymph node metastasis, and may be considered as a prognostic factor in OSCC patients.

According to our current data based on the relationship among

lymph node status and tumor infiltration, studies evaluating other related apoptotic factors with CTL activity and also immune correlations between vaccine-induced tumor specific T-cell responses and clinical outcome are recommended. Besides, investigation of expression correlation of restored P53 in tumoral cells with GB^+ cell density in in vivo may be helpful to design a new treatment choice of OSCC.

Acknowledgment

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Abbreviations

OSCC: *Oral Squamous Cell Carcinoma;* ***GB:*** *Granzyme B;* ***CTLs:*** *Cytotoxic T- Lymphocytes;* ***NK:*** *Natural Killer cells.*

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