Original Article

Immunohistochemical Evaluation of Angiogenesis and Cell Proliferation in Tongue Squamous Cell Carcinoma

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Abstract

Objectives: Squamous cell carcinoma (SCC) is the most common malignant neoplasm of the oral cavity and a public health threat. Tumor progression is believed to be influenced by angiogenesis as well as tumor cell proliferation; however, the correlation of these two factors in tongue SCC still remains unclear. This study aimed to assess the correlation of these two factors in tongue SCC.

Materials and Methods: Twenty-four paraffin block sections of tongue SCC were stained with monoclonal antibodies against CD105 and Ki-67. In order to assess the expressions of CD105 and Ki-67 to evaluate CD105 microvessel density (MVD), positively stained microvessels were counted in a predominantly vascular area (hot spot) in each specimen at ×400 magnification. The proliferation index was expressed as a percentage of Ki-67 positive cells. Data were analyzed by t-test and Pearson's correlation coefficient (P<0.05).

Results: The CD105 MVD was related to histological grading as well as Ki67 labeling index (LI; P= 0.045 and P=0.047, respectively). Both CD105 MVD and KI67 LI were unrelated to sex (P=0.41 and P=0.78, respectively) and age (P=0.20 and P=0.36, respectively) of the patients. No correlation was found between CD105 MVD and Ki67 LI (P=0.86).

Conclusion: The mean CD105 MVD was significantly lower in poorly differentiated tumors. This finding suggests that CD105 MVD may serve as a valuable prognostic factor in tongue SCC. Absence of correlation between MVD and tumor cell proliferation indicates that these processes may be guided by unrelated mechanisms.

Keywords: Carcinoma, Squamous Cell; Tongue; Angiogenesis Inducing Agents; ENG protein, human; Cell Proliferation; Ki-67 Antigen

INTRODUCTION

Oral cancer has been identified as a significant public health threat [1]. Squamous Cell Carcinoma is the most common type of oral cancer that more frequently involves the posterior, lateral and ventral surfaces of the tongue [2]. Angiogenesis, the formation of new capillary vessels from pre-existing ones, is essential for the progression of most solid tumors [3].

Tumor progression has also been shown to be influenced by the extent of cell proliferation [4].

It is, therefore, possible that alteration of angiogenesis may influence or be influenced by the extent of tumor cell proliferation [5]. The process of angiogenesis is the result of an imbalance between positive and negative angiogenic factors produced from both the tumor and normal cells [6].
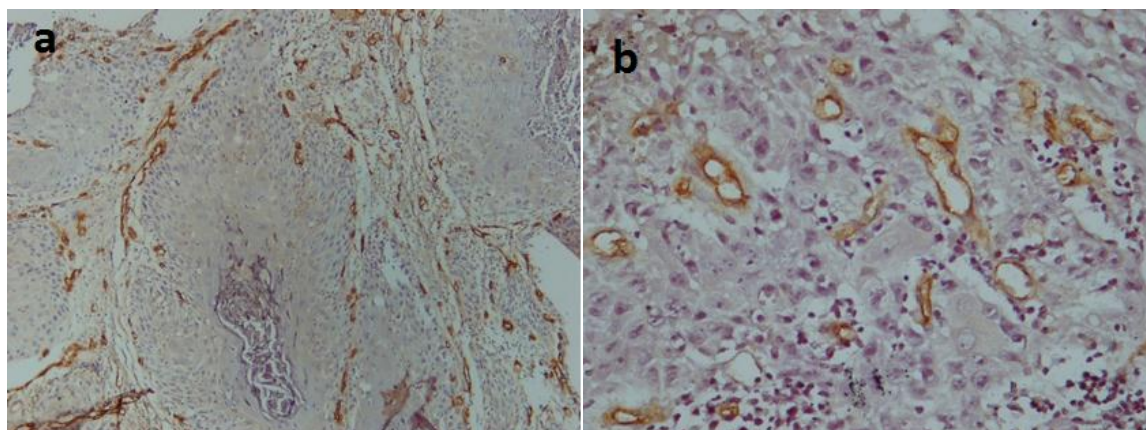
Fig. 1. CD 105 expression in well differentiated squamous cell carcinoma (a) (×100), and poorly differentiated squamous cell carcinoma (b) (×400).

Microvessel density has been reported to be an independent prognostic indicator of outcome in a variety of human malignancies [7]. Vascular density in tumors has been assessed by staining with pan-endothelial antibodies against antigens such as von Willebrand factor (Factor VIII), CD34 and CD31 [8].

Endothelial cells are highly heterogeneous, and it has therefore been suggested that pan-endothelial markers are not ideal to estimate the presence of pathologic or activated vessels in a tumor [9]. CD105 (endoglin) is a 180 -kDa homo-dimeric membrane glycoprotein and receptor complex of transforming growth factor-β1 [10].

It is expressed by the activated endothelial cells in angiogenesis and is considered a specific marker in ongoing angiogenesis of tumors [11]. Tumor cell cycle analysis has indicated that tumors with a higher proliferation rate show more aggressive clinical behaviors compared to tumors with a low proliferation rate [12].

Immunostaining studies using antibodies that recognize the Ki-67 nuclear antigen, a marker of cell proliferation, have provided a reliable method to characterize malignant tumors [12, 13]. Ki-67 is expressed in all phases of cell cycle (G1, S, G2 and M), but is undetectable in G0 [14,15].To date, there are rare studies about the relationship between MVD and tumor cell proliferation by immunohistochemical analysis in tongue cancer. Our study was aimed to evaluate the expressions of CD105 and Ki-67 to correlate CD105 MVD with Ki-67 labeling index and to analyze the relationship between proliferative and angiogenic activity of cancer cells and clinicopathological features.

MATERIALS AND METHODS

Twenty-four cases of tongue SCC were selected from the archives of the Department of Oral Pathology, Tehran University of Medical Sciences, between 1993 and 2007 for this retrospective study.

Table 1. Correlation of Ki-67 and CS105 expression with histological grade

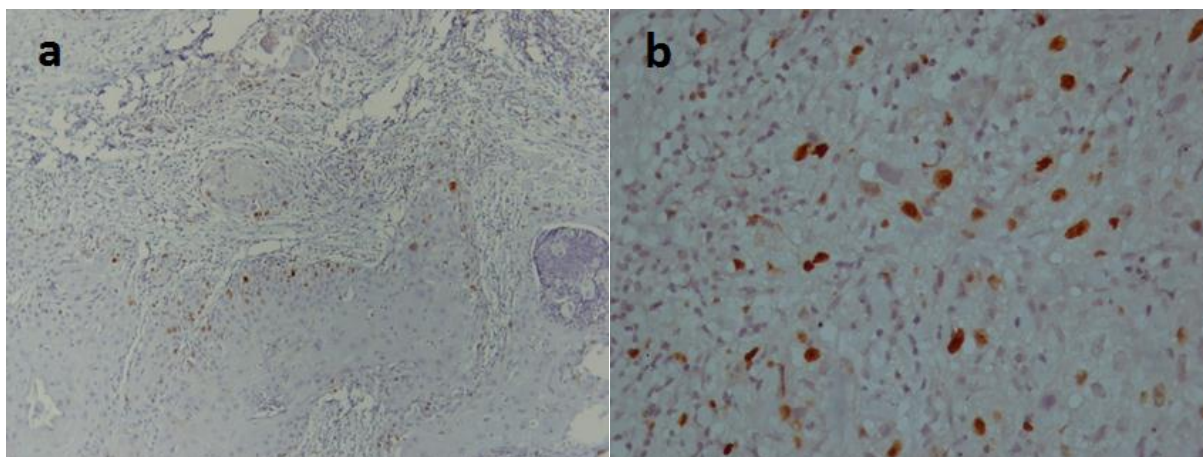|	Grade	No.	Mean	Minimum	Maximum	Standard deviation	P-value
CD 105 MVD	Grade I	12	46.7	7.75	88.75	21.76	0.045
	Grade II, III	12	27.02	0	64.5	23.47	
Ki-67 LI	Grade I	12	15.45	0.7	32.6	9.99	0.047
	Grade II, III	12	24.62	8	50.7	11.38	

P-value<0.05 considered significant.

Fig. 2. Ki-67 LI in well differentiated squamous cell carcinoma (a) (×100), and poorly differentiated squamous cell carcinoma (b) (×400).

There were 10 men and 14 women, with a mean age of 60.25 years (range, 36-83 years). All histology slides were reviewed by two authors to confirm the diagnosis.

Tumors were graded as well differentiated (grade 1), moderately differentiated (grade 2) and poorly differentiated (grade 3).

Immunohistochemical Staining:
Staining procedure for endoglin and Ki-67 was performed by Envision technique (horseradish peroxides-based two-step immunostaining method) on 4-µm-thick sections.

The tissue sections were deparaffinized in xylene and rehydrated in descending ethanol series. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide methanol solution.

After antigen retrieval by protease k enzyme, the sections were incubated with monoclonal mouse anti-CD105 (clone SN6h 1:10 diluted, Dako, Glostrup, Denmark) at room temperature. For immunostaining of Ki-67, epitopes were unmasked by microwave irradiation in 10 mM citrate buffer, pH=6, and then MIB-1 (ready to use, Dako, Glostrup, Denmark) was used for one hour. The reaction products were visualized with 3, 3´-diaminobenzidine (DAB) as the chromogen counterstained with Mayer's hematoxylin and mounted. For the negative controls, non-immune mouse serum was used instead of the primary antibodies. As positive controls, pyogenic granuloma and breast carcinoma sections were respectively used for CD105 and Ki-67 immunostaining.

Quantification of CD105-Immunostained Microvessels
The MVD was evaluated under light microscopy according to the procedure described by Weidner et al [8].

After scanning the immunostained section at low magnification (×40), four areas of carcinoma with the greatest number of immunostained microvessels (hot spots) were selected by two observers.

Then, they independently evaluated the slides for microvessels at ×400 magnification. Any brown staining endothelial cell or endothelial cell cluster that was clearly separate from the adjacent microvessels, tumor cells and other connective tissue elements was considered as a single, countable microvessel. The average value of the vessel count in four fields for each case was recorded as the CD105 MVD.
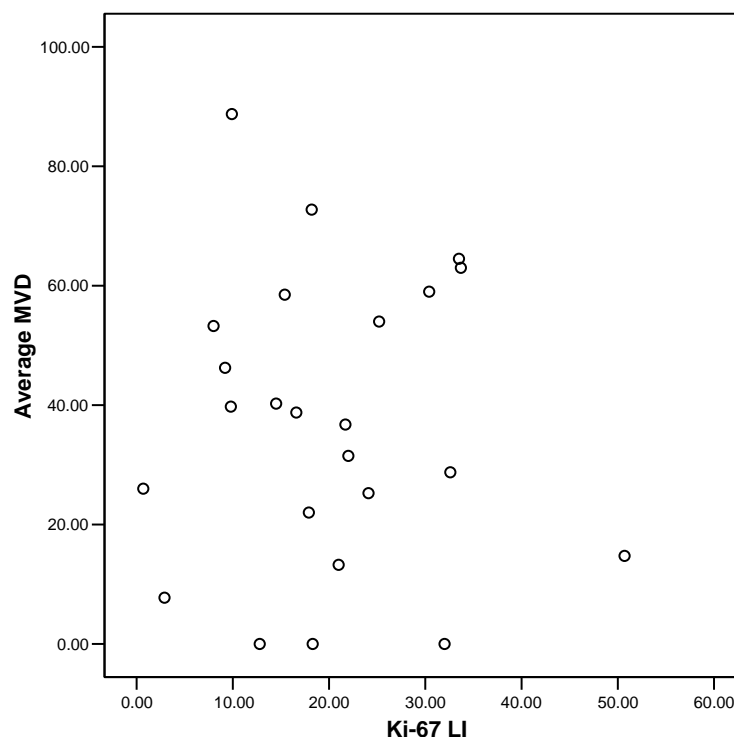
Fig. 3. Correlation between CD105 microvessel density and Ki-67 proliferation index in tumor cells.

Quantification of Ki-67 LI:
The Ki-67 LI was calculated in the tumor areas showing the highest density of stained cells, as determined by an initial scan with low magnification. The number of positively stained nuclei was counted in 1000 tumor cells at high magnification (×400) and was expressed as a percentage of the total positive cells.

Statistical Analysis:
The data were analyzed by the SPSS version 15.0. The correlations between the expressions of CD105 or Ki-67 and clinicopathologic parameters (gender and grade) were evaluated by t-test.
The Pearson's correlation was used to assess the relationship between CD105 MVD and Ki-67 LI and also between the expressions of CD105 or Ki-67 and age. A P-value less than 0.05 was considered significant.

RESULTS
A total of 24 histopathologically graded samples including 12 cases of well-differentiated SCC, eight cases of moderately differentiated SCC and four cases of poorly differentiated SCC were analyzed. The reason that there were not enough cases of high grade SCC is that this grade of tumor is relatively rare; thus, we decided to classify the samples equally into low grade and intermediate & high grade categories for subsequent evaluation. Low-grade carcinomas had a mean± standard deviation (SD) MVD of 46.70 ± 21.76, while for intermediate and high-grade tumors this value was 27.02 ± 23.47. High expression of CD105 correlated with higher degree of differentiation (P= 0.045) (Table 1, Fig. 1).
The mean CD105 MVD value was 36.86 ± 4.96. No statistical correlation was found between the expression of CD105 and age or gender. The Ki -67 LI ranged from 0.7 to 50.7

with a median of 20.04 ± 2.34. Considering pathological grading, the mean Ki-67 Li was 15.45 ± 9.99 in low-grade carcinomas and 24.64 ± 11.38 in intermediate and high-grade tumors. The Ki-67 LI was significantly higher in intermediate and high-grade group than in low-grade group (P= 0.047) (Table 1, Fig. 2). However, the LI was not significantly correlated to gender or age of patient. Besides, no significant correlation was observed between angiogenesis and cell proliferation (P = 0.86, r = − 0.03, Fig. 3).

DISCUSSION

Squamous cell carcinoma as the most common oral cancer has been identified as a public health threat [1]. It has been well established that angiogenesis and proliferative activity play key roles in tumor growth; however, the correlation of these two factors in tongue SCC still remains unclear. Microscopic evaluation of the tongue SCC as the most common site of intra-oral involvement suggests that most of the cases are histologically well differentiated [16]. The present study was performed on 24 cases of tongue SCC including 12 (50%) cases of well differentiated SCC followed by moderately differentiated and poorly differentiated (33.3% and 16.7%, respectively) cases. Angiogenesis is a complex and multi-stage mechanism that sustains tumorigenic potential in neoplasms [5,17].

According to the relationship of angiogenesis with tumor progression and metastasis, angiogenesis assessment in neoplasms might help predict tumor prognosis; however, there are contradictory findings about the clinical significance of the SCC angiogenesis in the head and neck region [7]. These contradictions might be related to the racial differences in the sample populations and differences in quantification methods for the stained endothelium and antibody used as angiogenesis marker [18]. Pan-endothelial markers such as CD34, CD31 and von Willebrand factor are expressed in both activated endothelial cells

and normal blood vessel endothelium and can cause a falsely elevated MVD value [18].

In the current study, we used anti-CD105 antibody because it reacts specifically with activated endothelial cells in angiogenic tissues with negligible or no reaction with the normal tissue [3]. Using MVD for quantitative angiogenesis assessment and evaluation of CD105 positive microvessels, no statistically significant correlation was found between the expression of CD105 and patient's sex and age. Same findings were reported in other studies [6, 18,19]. We used a descriptive system recommended by Neville et al, [2] for histopathological grading of SCC and found a negative correlation between CD105 MVD and grading. CD105 MVD was significantly higher in low-grade tumors. Controversial findings about the relationship of grading and MVD are reported in different studies [19, 20, 21]. Correlation of CD 105 MVD with lymph node metastasis and prognosis has been reported in tongue and esophageal carcinomas, respectively [22,23]. Cell proliferation is regarded as one of the most important biological mechanisms in oncogenesis. Prognostic significance of tumor cell proliferation rate has been shown in different kinds of malignancies [24,25]. Tumor cell cycle analysis has indicated that tumors with a higher proliferation rate show more aggressive clinical behaviors compared to tumors with a low proliferation rate [12]. Ki-67 is a non-histonic protein expressed by proliferative cells, which is present in all phases of the cell cycle except for G0 [26]. In the current study, Ki-67 antigen expression was used to assess cell proliferation rate. Ki-67 expression was significantly related to histological grading and was significantly lower in the low-grade group. Tumuluri et al, [26] and Huang et al. [15] reported increased cellular proliferation along with decreased tumor cell differentiation in oral and esophageal SCCs, respectively. Another study on laryngeal SCC reported no relationship between ki-67 LI and histological

grading [27]. Because of controversial findings, an investigation using different methods of histopathological grading to evaluate their correlation with Ki-67 LI is suggested. In our study, there was no significant relationship between ki-67 LI and CD105 MVD. In accordance with this finding, Tadbir et al. described no correlation between these two markers in salivary gland tumors [28]; however, Ravi et al, [5] and Macluskey et al. [29] suggested that tumor progression in the oral cavity depends on co-occurrence of angiogenesis and cell proliferation. In summary, considering the indirect role of CD 105 MVD in prognosis via the grading system, and if these findings are confirmed by other studies using follow up or other established prognostic factors, CD 105 MVD can be a good prognostic indicator to identify patients with poor prognosis and guide for appropriate therapy. Further investigations using multifactorial grading systems and excisional biopsies are required to identify the relation of clinical stage, tumor recurrence and patients' survival rate with CD105 MVD and Ki-67 LI.

CONCLUSION

The mean CD105 MVD value was significantly lower in poorly differentiated tumors. This finding suggests that CD105 MVD may represent a favorable prognostic factor in tongue SCC. Ki-67 LI was significantly higher in intermediate and high-grade group than in low-grade group. The lack of correlation between MVD and tumor cell proliferation indicates that these processes may be regulated by separate mechanisms.

ACKNOWLEDGMENT

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