Original Article

Endoglin (CD105) Positive Microvessel Density and Its Relationship with Lymph Node Metastasis in Squamous Cell Carcinoma of the Tongue

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Abstract

Background: Neovascularization is an important factor for predicting tumor behavior. Evidence suggests that endoglin (CD105) is a powerful marker of neovascularization and determination of microvessel density in several malignancies, and can be used as an agent to predict lymph node metastasis. However, it is controversial, particularly in head and neck squamous cell carcinoma.

We studied CD105-MVD in tongue squamous cell carcinoma and evaluated its correlation with lymph node metastasis in relation to sex, age, and histopathologic grade.

Methods: This study analyzed a total of 40 cases of tongue squamous cell carcinoma by dividing patients into two groups, a) with metastatic lymph nodes (N+) and b) without metastatic lymph nodes (N-). By CD105 immunostaining, microvessel density was determined in three different areas (intratumoral, invasive front and adjacent normal tissue) of all cases. Statistically, we evaluated the relation between microvessel density and lymph node involvement, in addition to other clinicopathologic factors by using the Kolmogorov-Smirnov test, *t*-test, and other analyses.

Results: CD105-MVD in the invasive front ($P \leq 0.001$) and intratumoral ($P \leq 0.006$) areas of the N+ group was significantly higher than in the N-group. In addition, there was a correlation between CD105-MVD and differentiation in the invasive front area ($P \leq 0.013$) No relation existed between CD105-MVD and other clinicopathologic features.

Conclusion: CD105-MVD, as a prognostic factor, may be helpful for determining the possibility of lymph node metastasis of primary SCC of the tongue.

Keywords: carcinoma, endoglin protein, neovascularization, pathologic, squamous cell

Introduction

Angiogenesis plays a central role in the growth and development of normal tissues and in progression of various pathologic processes.[1,2]

Neoangiogenesis supplies metabolic requirements for the growing tumor and provides a vascular pathway for hematogenous spread to distant sites.[3] It is a complex multistep process involving extracellular matrix remodeling, endothelial cell migration and proliferation, microvessel differentiation and anastomosis.[4] These processes are controlled by positive and negative angiogenic factors and their receptors.[2] Angiogenic factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor and platelet-derived growth factor are released by tumor cells and tumor associated inflammatory cells (particularly macrophages).[5]

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An important predictor of tumor behavior is intratumor microvessel density (MVD). The intensity of angiogenesis has been associated with poor prognosis in several cancers.[6,7–16]

Angiogenesis is determined by immunohistochemical assessment and usually performed by the method described by Weidner et al.[10] with several endothelial cell markers (pan-endothelial markers such as von Willebrand factor, CD31, and CD34). All the above factors have some problems related with specificity.[3] Antibodies against the above factors react with large and small vessels in addition to lymphatic, tumoral, and inflammatory cells, and may be confuse with tumor microvessels.[5]

CD105 is a 180-KDa homodimeric transmembrane glycoprotein, a receptor for two types of transforming growth factors (TGF-β1 and TGF-β3), and modulates TGF-β signaling by interacting with TGF-β receptors I and/or II.[17] It is a potent pleiotropic angiogenic factor expressed on activated endothelial cells during angiogenesis and considered to be a specific marker for the detection of tumor angiogenesis.[18]

CD105 is a powerful marker of neovascularization in solid malignancies and has been accepted as a more accurate factor than other panendothelial markers such as CD31 and CD34 in the evaluation of ongoing tumor angiogenesis.[3,5,6]

Recent studies have shown that the reactivity of CD105 in blood vessels of malignant tumors correlates with metastasis,[5,10,19–21] however, they are controversial studies.[1,2,6]

The purpose of this study was to evaluate microvessel density (MVD) in tongue squamous cell carcinoma (TSCC) using CD105 in N+ and N- groups of patients, and to investigate it's relation with sex, age, and histologic grade.

Materials and Methods

Samples

In this retrospective study, 40 formalin-fixed paraffin-embedded tissue blocks were selected according to inclusion/exclusion criteria from 62 patients with primary TSCC from the archives of the Cancer Institute of Tehran University of Medical Sciences between 1996 and 2006. All patients underwent hemiglossectomy and radical neck dissection.

Patients who had received radiotherapy and/or chemotherapy prior to surgery were excluded from the study. According to their records, all patients were free from distant metastasis (M0) but suspected of lymph node involvement and underwent radical neck dissection. Due to histopathological reports and slides the samples were divided into two groups N+ and N-. Tumor H&E sections were retrieved and graded according to Broder's criteria.[22] Clinical data were retrieved from patients' records. There were 22 men (mean age: 59.04; range: 19 – 80) and 18 women (mean age: 59.44; range: 31 – 84) with a mean age of 59.22±15.73 years (range: 19 – 84).

Immunohistochemistry

Sections (4 μm thick) were cut from the retrieved tumor blocks, dewaxed in xyline and rehydrated in graded alcohol. For blocking of endogenous peroxides, 0.3% hydrogen peroxide was used for 15 min. To unmask hidden epitopes, sections were digested with protease type protein kinase at 37°C for 10 min. Sections were incubated with monoclonal antibody against a 1:10 dilution of CD105 (clone SN6h, DAKO, Denmark) for one h at room temperature according to the manufacturer's instructions and subsequently developed using a streptavidin-biotin-peroxidase system. Visualization of the antibody complex was achieved with a diaminobenzidine (DAB, DAKO, Denmark) reaction, resulting in brown staining of activated endothelial cell membranes. Sections were counterstained by Meyer's hematoxylin. All sections were interpreted by a pathologist who was blinded to the clinicopathologic data.

Quantification of blood vessel staining

Sections were screened according to Weidner et al.[10] and van Hoef et al.[23] Briefly, at a magnification of 100×, the areas of highest endoglin staining were noted (hot spots). Four hot spot fields in each intratumoral, invasive front and normal adjacent tissue areas were chosen. Then, each of the hot-spot areas were assessed at 400× magnification. Any brown staining endothelial cell or endothelial cell cluster that was clearly separate from adjacent microvessels, tumor cells and other connective tissue elements was considered a single, countable microvessel. Areas of necrosis, hemorrhage and sclerosis were excluded and vessels with smooth muscle around their lumen were not counted.[24–26] The average of the vessel counts in four fields for each area was used as the final mean MVD value in each intratumoral, invasive front, and normal adjacent tissue.

Statistical analysis

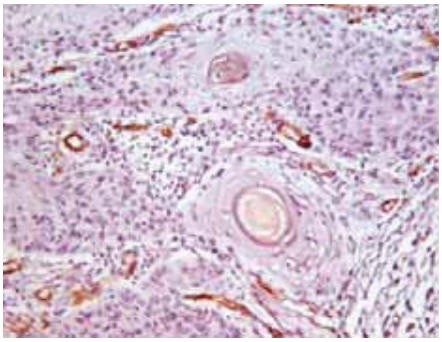
The software packages SPSS 11.5 was used for statistical analysis and graphical representation.

The independent samples *t*-test, Kolmogorov-Smirnov test, Pearson correlation, Chi-square, and if needed, Fisher exact tests were used to search for associations between CD105-MVD and the presence or absence of metastatic lymph nodes, tumor differentiation, gender, and age of patients. We also used Receiver Operating Characteristic curve (ROC), which may show a cut-off point for CD105-MVD.

Results

The specimens were from surgical resected tumors of 40 patients with TSCC. All tumors were primary, untreated lesions. Tumors were classified into two different groups based on the presence or absence of lymph node metastasis. CD105 stained microvessels were observed within the tumor, invasive front and the adjacent non-neoplastic tissue. Improperly stained slides or those with unreadable tissue in each of the previously mentioned areas were ignored. Therefore, our specimens were less than 40 in each area (Figure 1).

a



b

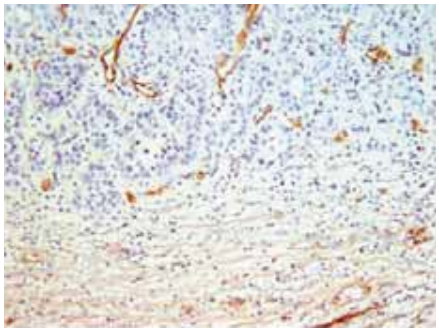


Figure 1. Immunostaining of primary squamous cell carcinoma of the tongue with CD105 antibody. (a) Intratumoral area (400x); (b) invasive front (400x).

These 40 cases were divided into two groups, N+ (n=20) and N- (n=20). Cases were divided into five differentiation groups (Table 1).

According to the Kolmogorov-Smirnov test, CD105-MVD had a normal distribution. Out of 22 men and 18 women, a total of 15 and 5 patients, respectively were N+, which was statistically significant ($P \leq 0.025$). No relation existed between tumor differentiation and lymph node metastasis ($P \leq 0.718$).

The mean age in the N+ and N- groups were 63.6±13.81 and 54.85±16.63 years. The mean CD105-MVD value in the intratumoral, invasive front and normal adjacent tissue was evaluated between the two groups, which was statistically different in the intra

Table 1. Frequency of tumor differentiation.

	Frequency	Percentage	Valid percent	Cumulative percent
Poor	9	22.5	22.5	22.5
Poor to intermediate	4	10.0	10.0	32.5
Intermediate	14	35.0	35.0	67.5
Intermediate to well	5	12.5	12.5	80.0
Well	8	20.0	20.0	100.0
Total	40	100.0	100.0	

Table 2. Mean CD105 MVD values in N+ and N- groups.

NODE	N	Mean	Standard deviation	*P*-value	95% CI lower	95% CI upper
CD105I*						
N+	19	16.6579	5.23888	0.006	-9.87906	-1.77738
N-	18	22.4861	6.83442			
CD105B**						
N+	19	10.7237	2.90342	0.000	-7.85723	-2.72481
N-	17	16.0147	4.57359			
CD105A***						
N+	19	7.6184	4.84651	0.610	-3.99790	2.38180
N-	17	8.4265	4.56701			

I*= intratumoral; B**=invasive front; A***=adjacent non-neoplastic tissue.

tumoral and invasive front area (Table 2).

There was a correlation between CD105-MVD and differentiation in the invasive front area ($P \leq 0.013$).

Pearson correlation analysis did not show any correlations between CD105-MVD and the three tissue fields. There was no relation between CD105-MVD and sex, age, or histopathological grade in both groups.

We have evaluated the value CD105-MVD diagnosis in the three areas with the ROC curve. In the intratumoral area, the surface under the curve was 0.744 (95% CI: 0.585 – 0.903) and 19.375 was determined to be an acceptable cut off point for this area (66.7% sensitivity, 73.7% specificity; Figure 2).

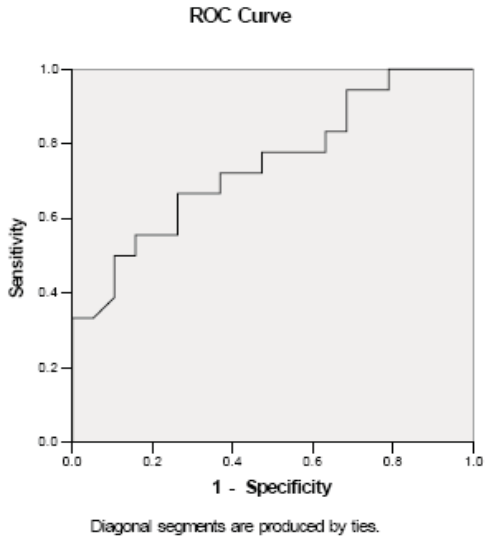


Figure 2. ROC curve 1 intratumoral area.

In the invasive front area, the surface under the curve was 0.814 (95% CI: 0.657 – 0.971) with 12.5 as an acceptable cut off point for this area (82.4% sensitivity, 78.9% specificity; Figure 3). If we used the mean of both above areas for diagnosis, the surface under the curve would be equal to 0.870 (95% CI: 0739 – 1.001) and 15.8125 was an acceptable cutoff point for these two areas (82.4% sensitivity, 84.2% specificity; Figure 4).

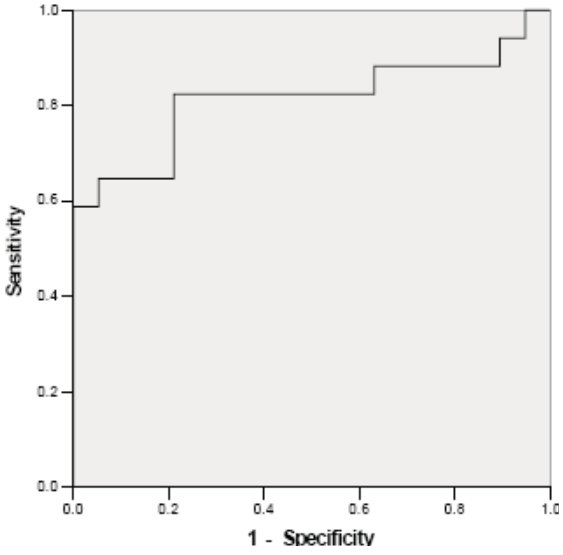


Figure 3. ROC curve 2 invasive front area.

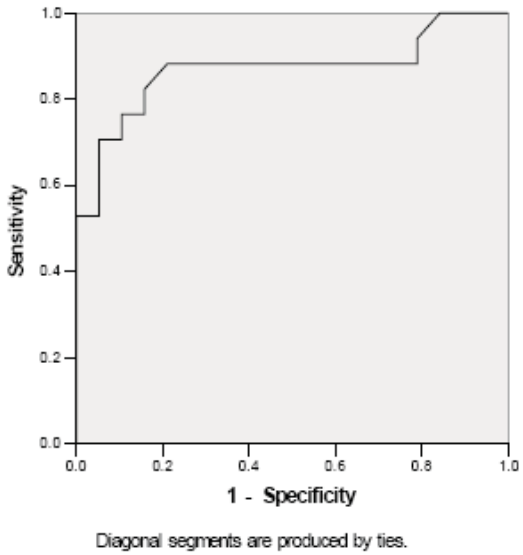


Figure 4. ROC curve 3 mean of intratumoral and invasive front areas.

Discussion

Tumor angiogenesis and its role in tumor progression and metastasis have been studied in a variety of neoplasms,[15,16,27–29] including head and neck SCC.[1,2,19]

The first quantitative evidence that angiogenesis in tumors can predict the probability of metastasis has been reported for melanoma,[8] of which other studies confirmed this for different tumors.[4,5] However, some reports were unable to find a relation between tumor prognosis and MVD.[30,31] Curiously another study has found an association with high MVD and better prognosis.[32] Thus, the controversy in this subject still exists.

There are several reasons for these discrepancies. The most probable are differences in the patient population studied, variability in the reactivity of endothelial cell antibodies, differences in tissue pretreatment procedures and MVD determination methods.[4,15,30,33,34]

Widely used pan-endothelial markers such as CD31, CD34, and von Willebrand factor react with endothelial cells in both normal and tumoral tissue but evaluation of these reactions has several limitations.

CD31 stains both large and small vessels equally, in addition to some carcinoma cells, however, the reliability of CD31 staining has been inconsistent between laboratories.[35]

CD34 is detected on the ongoing active angiogenesis endothelial cells but it also stains mesenchymal cells.[36,37]

The expression of CD34 and von Willebrand factor becomes less or absent in some microvessels of normal and many tumoral tissues.[5]

von Willebrand factor is not specific for blood vessels because it can stain lymphatics; in addition some tumor vessels cannot be stained with this factor.[5]

CD105 is specific for activated endothelial cells that participate in tumor angiogenesis.[38] It is a more specific and sensitive marker of neoangiogenesis, which has certain advantages over other pan-endothelial markers as have been confirmed by several recent studies.[39,40]

In our study, CD105-MVD was investigated in N+ and N- groups of TSCC by IHC. Microvessels of all the specimens were stained by CD105. CD105-MVD was significantly higher in the N+ group than the N- group, which may show the effect of MVD in lymph node metastasis.

Data from other carcinomas such as breast and cervical cancers suggest that CD105 expression is a valuable factor for identifying patients who are at the risk of metastasis.[39,40]

This study also suggests that CD105 can be a useful marker in predicting the risk of metastases in TSCCs. In the future, more samples with longer follow up should be assessed to confirm our thesis.

We found that CD105-MVD was higher in tumors than adjacent normal tissue, which was also confirmed by several previous investigations on different tumors.[1–3,5] This shows the effect of tumors and their stromal cells on microvessel formation (neoangiogenesis), which is more effective inside a tumor rather than at its periphery where differentiated vessels are located. Because of our study, we propose a cutoff point in patients with TSCC where patients' with MVD over 15.81 in both intratumoral and invasive front fields (based on CD105 staining) are candidates for additional therapies and more follow up.

The role of invasive front in oral SCC have been investigated by several investigators,[41–43] most of who believe that this part of the tumor has the main effect on its behavior and out come.[41–43] We found that MVD in the invasive front is significantly different between N+ and N- groups; the same as the intra tumoral area and in contrast with adjacent non-neoplastic tissue. This finding has also shown the importance of the invasive front area where the difference between the mean MVD of both groups was more than the intratumoral area.

To date only a few studies have investigated CD105 expression in the head and neck region.

Finally, CD105 seems to be a reliable predictor of lymph node metastasis in TSCC. We suggest reviewing more samples with long-time follow up in the future. Further studies are required to understand the mechanism of CD105 up-regulation and its potential role as a target of anti-angiogenic therapy.

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