Study of expression of endoglin (CD105) in oral squamous cell carcinoma

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Abstract

Context: Oral cancer is the 8th most common cancer in the world. An important feature of carcinogenesis is angiogenesis. Endoglin is a powerful marker of neovascularization in solid malignancies. This study was done to ascertain its role as an indicator of metastasis and prognosis.

Aim: This study aimed to evaluate and compare the expression of endoglin (CD105) in metastatic primary tumor, lymph node of the metastasized tumor, nonmetastatic primary tumor and in normal buccal mucosa immunohistochemically.

Settings and Design: The total sample size comprised 45 formalin-fixed paraffin-embedded tissue blocks, n = 10 metastasized primary tumor, n = 10 lymph nodes of metastasized primary tumor, n = 20 nonmetastasized oral squamous cell carcinoma and n = 5 normal buccal mucosa were studied.

Subjects and Methods: Immunohistochemistry for endoglin was performed and microvessel density (MVD) was determined by hot spot method. Microvessel density was compared between the groups.

Statistical Analysis: Statistical analysis was used using one-way ANOVA. P < 0.05 was statistically significant.

Results: Endoglin expression in metastatic cases (0.68 ± 0.10) was higher than nonmetastatic cases (0.45 ± 0.20) and the difference was statistically significant (P = 0.002).

Conclusion: This study shows that presence of endoglin determines the metastatic potential of the tumor and its prognosis, thus, could be considered as a potential target of therapy.

Keywords: Angiogenesis, endoglin, microvessel density, oral squamous cell carcinoma

INTRODUCTION

Cancer is the eight most common cause of morbidity and mortality in the world. Oral squamous cell carcinoma (OSCC) accounts for more than 90% of all oral malignancies. Carcinogenesis is a multistep process acquired by alteration such as mutations, amplification of proto-oncogenes and inactivation of tumor suppressor genes that results in development of unique tumor environment which, in turn, supports tumor growth. Angiogenesis and metastasis influence prognosis in carcinoma. Metastasis is initiated by invasion of cancerous cell into surrounding tissue due

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Endoglin, also known as CD105 (cluster of differentiation 105), is a 180 kDa homodimeric transmembrane glycoprotein composed of two disulfide-linked subunits and mapped to chromosome 9q34. It also acts as a co-receptor of transforming growth factor (TGF)-β 3 and activates TGF-β signaling via small mothers against decapentaplegic (SMAD) pathways. Endoglin plays a pivotal role in balance of activin-like kinases I (ALKI) and ALK5 signaling that phosphorylates SMAD 1/5/8 and regulates endothelial cell proliferation. It is considered to be a pleiotropic angiogenic factor expressed on activated endothelial cells during angiogenesis and not on quiescent or resting endothelial cells. Thus, it is considered specific for tumor angiogenesis. Loss of endoglin gene causes hereditary hemorrhagic telangiectasia type 1.

Microvessel density (MVD) is a measure of tumor angiogenesis. Various studies have shown that MVD correlates with tumor aggressiveness and poor prognosis. Measurement of MVD within isolated regions of highest vessel concentration, i.e. hot spots, was of a very high prognostic indicator. Various authors assessed MVD for CD105 antibody as a vascular marker that stained endothelial cells which are specifically undergoing angiogenesis in various carcinomas and do not react with normal blood vessels. This suggests that measurement of MVD using endoglin antibody is a specific and sensitive marker for neoangiogenesis. The present study has been taken up to assess tumor neo-vascularization and understand its prognostic signification immunohistochemically using monoclonal antibody to endoglin in OSCC with and without metastasis.

SUBJECTS AND METHODS

Patients and tissue samples
In this retrospective study, 45 formalin-fixed paraffin-embedded archival tissue blocks were obtained from the Department of Oral Pathology and Microbiology, Ragas Dental College and Hospital, Chennai. The samples were divided into four groups as under:

- **Group I:** (10 samples) – Primary site of metastasizing OSCC
- **Group II:** (10 samples) – Draining lymph node of group I
- **Group III:** (20 samples) – Primary site of nonmetastasizing OSCC
- **Group IV:** (5 samples) – Normal buccal mucosa.

Tonsil tissue specimen known to express endoglin-positive cells served as the control group. The approval for the study was obtained from the Institutional Review Board of Ragas Dental College and Hospital, Chennai.

Immunohistochemical determination
Five micron thick serial sections were cut from formalin-fixed paraffin-embedded blocks and transferred onto 3-aminoethyltriethoxysilane-coated slides. Antigen retrieval was done by transferring the slides to citrate buffer of pH 6.2 and autoclaved at 15 lbs pressure for 30 min. The sections were treated with 3% hydrogen peroxidase for 10 min to quench endogenous peroxidase activity. Prediluted primary antibody, namely mouse monoclonal CD105 antibody (4G11, biogenix), was added to the sections and incubated for 60 min in a hydrated chamber. The sections were then washed with tris buffer for 2–3 min and a super-enhancer reagent was added and incubated for 30 min. Then, the sections were washed with tris buffer for 2–3 min and incubated for 60 min with a secondary antigen that is polymer-Horseradish Peroxidase Immunohistochemistry, 3,3’ (HRP IHC) detection system. Chromogen Diaminobenzidine (DAB) was added and incubated for 20 min, then washed with tris buffer and counterstained with hematoxylin. Negative control sections were omitted of primary antibody. Tonsil tissue specimens known to express CD105 positive cells were used as a positive control. CD105 staining is localized to cell membranes of the endothelium of blood vessels.

![Figure 1: Hematoxylin and eosin-stained positive control tissue – tonsil. (×100) (a) and (×400) (b) respectively. Endoglin staining seen on the membranes of blood vessels in tonsil (×100) (c) and (×400) (d), respectively](image-url)
Criteria for evaluation of endoglin staining

Degree of positivity

The tissue section was divided into three regions namely intratumoral, peritumoral and resection margins. Evaluation of endoglin staining under light microscopy was done using hot spot method proposed by Weidner et al. In this method, the first step was to identify the area of highest neovessel density by scanning the whole tumoral section at lower power ($\times 100$) and in the second step, the individual microvessels were counted at a higher power ($\times 200$) in an adequate area in each of the above-mentioned regions of the tissue section. The immunostained vessels were counted twice in these hot spot areas in the three regions. Endoglin-negative vessels were also counted twice in the above-mentioned hot spot areas. The group II comprises of the lymph node of the primary tumor that has metastasized. As a result of metastasis the nodal architecture was lost hence, the node architecture was stratified into hilar region (representing the central region) and capsular region. In these regions, both positive and negative vessels were also counted in the above-mentioned hot spot areas.

The mean vascular density (MVD) was calculated by the formula $\frac{\text{No. of immunostained positive vessels}}{\text{Total no. of vessels}}$.

Total no. of vessels = no. of positive vessels + no. of negative vessels. The MVD was compared between the intratumoral, peritumoral and resection areas in each group and also between the groups.

Inclusion criteria

Any brown staining single cell or spot that stained by immunohistochemical marker was counted as a vessel.

Exclusion criteria

Vessels with muscular walls were excluded. Vessels lumen and red cells within this lumen were not used to define a microvessel. Areas of necrosis and hemorrhage also have to be excluded.

Statistical analysis

Statistical analysis was done using SPSS software version 11.0 (SPSS, IBM, Chicago, IL, USA). One-way ANOVA was done to compare the number of endoglin-positive vessels per unit area of the tissue section among the groups, namely primary OSCC that has metastasized (Group I) and primary OSCC that has not metastasized (Group III). Multiple comparisons were made between the groups. Wilcoxon signed-rank test was done to compare endoglin-positive vessels in the lymph node of the primary tumor (Group II). Multivariate linear regression analysis was utilized to predict mean values. $P < 0.05$ was considered to be statistically significant.

RESULTS

Demographic data and habit distribution among study groups

The study population included males predominantly in all the groups ($P = 0.615$). In Group I, 70% patients belonged to the age of 26–50 years and in Group III 75% of the patients were of 51 and above years of age. Mostly, males (85%) had habits such as smoking, chewing areca nut and alcohol consumption and females (37.5%) had the habit of chewing tobacco and areca nut, whereas in Group IV, none of the subjects had any habit ($P = 0.053$). The demographic details of Group II were similar to Group I as it was the lymph node of the primary tumor that has metastasized [Table 1].

The histopathological grading between Group I and Group III was done. In Group I, 20% cases were well...
differentiated and 40% each were moderately and poorly differentiated, whereas in Group III, 100% of cases were well-differentiated OSCC. This distribution in grading showed the aggressive behavior of the tumor in Group I and hence had a higher potential for metastasis [Table 2]. This difference was statistically significant ($P = 0.000$).

**Endoglin expression**

All the 45 samples were examined for the expression of endoglin. The cell membrane of the endothelial cells with brown staining was positive for endoglin. The malignant, inflammatory and mesenchymal cells were endoglin negative. The morphology of endoglin-positive blood vessels at the invasive front of the tumor (intra- and peritumoral) exhibited aberrant morphology with dilated, elongated blood vessels with gaps between the endothelial cells. No statistically significant correlation was found between endoglin staining with other variables such as age and histopathological grading. CD105 negative staining was seen in normal control-buccal mucosa.

**Evaluation of MVD**

Quantification of endoglin was done by determining MVD using hot spot method by Weidner et al.[20] MVD of endoglin was significantly higher in metastatic group (0.68 ± 0.10) in comparison to nonmetastatic group (0.45 ± 0.20), as depicted in Table 3 ($P = 0.002$). The MVD of endoglin-positive vessels was counted and compared by tissue localization in the study groups. Higher MVD was seen in the intratumoral region of Group I (0.73 ± 0.12) than in Group III (0.57 ± 0.18) and their difference was significant ($P = 0.022$). Similarly, significant difference ($P = 0.039$) was seen in the peritumoral region of Group I (0.67 ± 0.10) and Group III (0.55 ± 0.18). Higher MVD was seen in the resection margin of Group I (0.62 ± 0.10) than in Group III (0.45 ± 0.20) with a $P$ value of 0.002. Two cases in Group III did not have resection margin [Table 4]. The MVD in the lymph nodes of the metastatic tumor revealed significantly increased endoglin-positive vessels in the hilar region (0.57 ± 0.13) than in the capsular region (0.37 ± 0.07), as depicted in Table 5 ($P = 0.009$) [Figure 2].

When we studied endoglin expression and MVD by linear regression analysis, for gender and histopathological

![Figure 2](image-url)
Table 4: Comparison of endoglin-positive vessels by tissue localization between Group I and Group III

| Regions         | Study groups (mean±SD) | P     |
|-----------------|------------------------|-------|
|                 | Group I (n=10)         | Group III (n=20) |
| Intratumoral    | 0.73±0.12              | 0.57±0.18 | 0.022 |
| Peritumoral     | 0.67±0.10              | 0.55±0.18 | 0.039 |
| Resection margin| 0.62±0.10              | 0.45±0.20 | 0.002 |

SD: Standard deviation

Table 5: Comparison of endoglin-positive vessels by tissue localization in draining lymph node of metastasized primary tumor (Group II)

| Regions         | Mean±SD | P     |
|-----------------|---------|-------|
| Hilar region    | 0.57±0.13 | 0.009 |
| Capsular region | 0.37±0.07 |       |

SD: Standard deviation

grading differentiation in carcinomas, we found that compared to metastasized group, the nonmetastasized group had −0.175 lesser mean values of MVD. This negative correlation was statistically significant (P = 0.014).

**DISCUSSION**

Angiogenesis is an important requirement for the growth of malignant neoplasm to meet its nutritional demand. Angiogenic markers should ideally detect quality and quantity of the newborn vessels. Endoglin (CD105) is a suitable marker for angiogenesis in neoplasm as it is a TFG-β co-receptor expressed on activated endothelial cells only. Hence, it is specific and sensitive marker for neoangiogenesis, as confirmed by several recent studies. Sharma et al.[26] studied 80 cases in histologically confirmed OSCC and reported a male predominance, similar to our study in which 80% were male and 20% were female.

In this study, the mean ± standard deviation of endoglin-positive vessels in Group I (primary tumor that has metastasized) was 0.23 more compared to Group III (primary tumor that has not metastasized) and this difference was statistically significant. A similar study done by Marioni et al.[27] stated that the mean MVDs were 3.6 and 3.1 in metastatic and nonmetastatic groups, respectively. Martone et al.[28] and Eshghyar et al.[29] evaluated endoglin expression in patients suffering with OSCC and found that MVDs for endoglin were significantly higher in metastatic tumors than in nonmetastatic tumors.

Margaritescu et al.[25] studied the distribution and morphology of endoglin-positive vessels in the invasive front of the tissue section and found an increased MVD in the intratumoral region. They also observed irregular vascular architecture with tortuosity and elongation of vessels with incomplete or missing endothelial lining. Similarly, we examined and compared endoglin expression between intratumoral, peritumoral and resection margin regions of Group I and Group III and a significant difference was noted. The intratumoral vessels exhibited increased MVD compared to peritumoral and resection margin vessels. Owing to the fact that, there was not only quantitative difference in distribution of vessels and but also in the properties of endothelial cells resulting in aberrant morphology that disrupts the normal vascular architecture in tumor microenvironment and there is an increased opportunity for tumor cells to enter circulation. Further on observation, we found a decline in endoglin expression as we moved away from the invasive front. Similar findings were also observed by Kyzas et al.[30] and Nagatsu et al.[31] Lymphatic metastasis in OSCC results in tumor progression and is crucial for cancer staging, treatment and prognosis. In this context, we examined the draining lymph nodes of the metastasized neoplastic tissue for expression of endoglin. The normal architecture of the lymph nodes was completely destroyed by the invading tumor islands. Hence, MVD was calculated in the central and peripheral region presumably representing the hilar and capsular region. The MVD was significantly higher in the hilar region suggesting a high degree of neovascularization. This finding was coherent with study done by Miyahara et al.[32] where an increased expression of endoglin was identified in the lymph nodes predicting a more aggressive tumor behavior in OSCC. This finding reinforces the paradigm of quick vascular spread conferring a stage IV status to the malignancy.

Basnakar et al.[32] in their study also quantified MVD using endoglin. They also found increased MVD in tumor specimen than in dysplastic and normal mucosa. Similarly, Patil et al.[33] also observed MVD associated increased endoglin expression in malignant OSCC than in well-differentiated OSCC. They also proposed that calculation of MVD using endoglin is a more accurate parameter for determination of angiogenesis and its correlation in tumor progression. They compared VEGF and endoglin expression with the survival rate of the patient and found significant correlation. Increased expression of endoglin was associated with decreased survival rate indicating that endoglin is a prognostic marker in OSCC patients.
CONCLUSION

Endoglin (CD105) is an auxiliary TGF-β receptor transmembrane glycoprotein essentially expressed on activated endothelial cells participating in neoangiogenesis. Increased endoglin expression was seen in metastatic group than in nonmetastatic group of OSCC. Evaluation of MVD using endoglin can be an effective tool in quantification of neovascularization in carcinomas and also determines the metastatic potential of the tumor and its prognosis. Thus, endoglin could be considered as a potential target of therapy for OSCC.

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Conflicts of interest
There are no conflicts of interest.

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