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Tumor Angiogenesis in Oral Squamous Cell Carcinomas: The Significance of Endothelial Markers and Hotspot Selection

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Background: The intensity of angiogenesis affects the prognosis for many malignant tumors, but there is no consensus about the association between the prognosis of oral squamous cell carcinoma (OSCC) and the intensity of angiogenesis. This discrepancy might be caused by different endothelial markers used and by hotspot selection. The aim of this study was to investigate the sensitivity and specificity of different endothelial markers for evaluating microvessel density (MVD) in OSCCs. We also examined the effect of hotspot area selection in association with clinicopathological features of OSCCs. **Methods:** We collected 84 OSCC specimens for immunohistochemical staining for three common endothelial makers: van Willebrand factor (vWF), CD31 and CD34. Peritumoral and intratumoral vascular hotspot areas were selected separately for MVD counting. **Results:** There was no significant association between peritumoral MVD and clinicopathological parameters. However, the intratumoral MVDs determined using CD31 and CD34 were significantly associated with tumor size ($P = 0.003$ and $P < 0.0001$, respectively), with histological differentiation ($P = 0.0025$ and $P = 0.018$, respectively) and with tumor stage ($P = 0.001$ and $P < 0.0001$, respectively). In addition, the intratumoral MVD counted using CD34 immunostaining was significantly associated with lymph node metastasis of OSCC ($P = 0.005$). **Conclusions:** Tumor angiogenesis and the density of newly formed vessels are of potential prognostic relevance in the assessment of malignant neoplasia. The endothelial marker CD34 was better in the assessment of tumor vascularization of OSCCs. Furthermore, hotspot selection, especially intratumoral MVD, is important in examining OSCC progression.

Key words: angiogenesis, endothelial markers, hotspot selection, oral squamous cell carcinoma

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common malignancy in the oral cavity. The tumor has a severe impact on the patient's quality of life as it causes aggressive destruction and postoperative deformity. Despite considerable improvements in diagnosis, treatment and understanding of the molecular mechanisms of this malignancy, the high morbidity rate and the five-year survival rate of less than 50% have not improved in the past two decades[1,2]. This poor outcome is related primarily to the characteristic features of this oral cancer, which has a high degree of local invasion into surrounding tissue and a high incidence of metastasis to cervical lymph nodes. Thus, it is necessary to understand the molecular mechanism of oral cancer progression and find a prognostic indicator for more aggressive therapy in selected cases.

It is well known that tumor growth depends on angiogenesis and that the ingrowth of new capillaries increases the opportunity for tumor cells to enter the circulation, then metastasize to a distant site[3,4]. Many retrospective studies have shown that the intensity of angiogenesis in many human malignant tumors is proportional to the tumor stage, recurrence rate, capacity for metastasis and death[5-8]. Most of these studies have focused on the product of angiogenesis, microvessel density (MVD). MVD is measured histologically by staining the blood vessels in a given tissue and determining its vascularity[9]. In most of these studies, MVD was found to have independent prognostic significance compared with traditional prognostic markers.

However, in oral cancers the relationship of tumor angiogenesis with cancer progression is not consistent. For example, Albo et al. found that tumor angiogenesis was directly related to clinical outcome including early and

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extensive recurrence or metastasis[10]. William et al. reported that tumor angiogenesis showed a strong correlation with regional recurrence[11]. In addition, López-Graniel et al. observed significant correlations between MVD and recurrence of the tumor, lymph node metastases and tumor size[12]. However, some studies found that tumor angiogenesis in oral cancer was not associated with these parameters of progression[13-15]. For example, Moriyama et al. did not find any correlation between MVD and tumor size, degree of differentiation and lymph node metastasis[13]. Leedy showed that tumor angiogenesis did not determine lymph node metastasis[14]. Tahan and Stein compared tumorous tissue MVD with nontumorous tissue MVD. They concluded that tumor vascularity could not predict the risks of metastasis[15]. Taken together, the discrepancy between these studies may be due to methodological variations including the use of different endothelial markers for detecting tumor vascularity, and selection of the vascular hotspot area for counting tumor vascularity. Therefore, we tested three commonly used markers, von Willebrand factor (vWF), CD31 and CD34, to investigate their specificity and sensitivity in the staining of microvessels in OSCCs. We also aimed to identify the most significant area as the hotspot to evaluate any correlations between MVD and clinicopathological parameters in OSCCs.

METHODS

Patients and Specimens

Formalin-fixed, paraffin wax-embedded tissue blocks were obtained from 84 patients (75 men and 9 women, mean age 54±12 years, range 39-79 years) with OSCC. Diagnosis of OSCC was based on histological examination of hematoxylin and eosin-stained tissue sections. All patients were diagnosed and/or treated at the Tri-Service General Hospital (Taipei, Taiwan) from January 1980 to December 2006. Specimens were obtained from either incisional biopsies or total surgical excision of the tumors. None of the patients had received any form of tumor-specific therapy before the initial biopsies. The histological differentiation and clinical staging of OSCC were determined according to our previous reports[16].

Immunohistochemistry

All specimens were fixed in 10% neutral formalin, embedded in paraffin wax and cut in 5μm serial sections. Immunohistochemistry was performed using the DAKO EnVision stain system (Dako, Copenhagen, Denmark). Briefly, sections were dewaxed, rehydrated and then heated in a plastic slide holder (Dako) containing 10 mM citrate buffer (pH 6.0) in a microwave oven for 30 min to retrieve antigenicity. The endogenous peroxidase activity was blocked by immersing the sections in 3% H_2O_2 in methanol for 10 min. After washing in 10 mM Tris-buffered saline, pH 7.4, sections were incubated with 10% normal goat serum to block nonspecific binding. Sections were then incubated sequentially with primary monoclonal mouse anti-human vWF, CD31 and CD34 antibodies (250 μg/mL, used at 1/200 dilution, Dako) for 60 min, followed by incubation with the horseradish peroxidase-labeled polymer (Dako) for 30 min. The chromogen 0.02% diaminobenzidine hydrochloride (Dako) containing 0.03% H_2O_2 was used to visualize the peroxidase activity. The preparations were lightly counterstained with hematoxylin, mounted with Permount and examined using light microscopy.

MVD

Determination To determine the MVD, the stained cancer tissue sections were initially screened at low power (100×) to identify the areas of highest vascularization (hotspots). Peritumoral and intratumoral MVDs were counted separately. Five high power (400×) fields were then chosen randomly and the number of microvessels in each (0.09 mm^2) was counted for each case with the use of an ocular grid[17]. The MVD for each sample was the mean of the five values obtained and recorded as the MVD for each patient. For cutoff point analysis, the median values were used to categorize the tumor into “high” and “low” MVD groups. Immunostaining results were evaluated by one investigator (YSS) without prior knowledge of the tumor’s histopathology or the patient’s clinical status.

Statistical Analysis

The proportions of tumors with high or low MVD counts were analyzed using the χ^2 test to compare these with clinicopathological features. Statistical significance was set at $P < 0.05$.

RESULTS

vWF, CD31 and CD34 Immunohistochemistry Positive immunostaining for vWF, CD31 and CD34 was observed in endothelial cell-lined microvessels for all tumors that were stained. More positive hematopoietic cells and background staining were seen in CD34 immunostained specimens and some inflammatory cells were costained by the anti-CD31 antibody. In general, the anti-CD31 and -CD34 antibodies detected more peritumoral MVD than did anti-vWF. The distribution of tumoral vascularity was highly

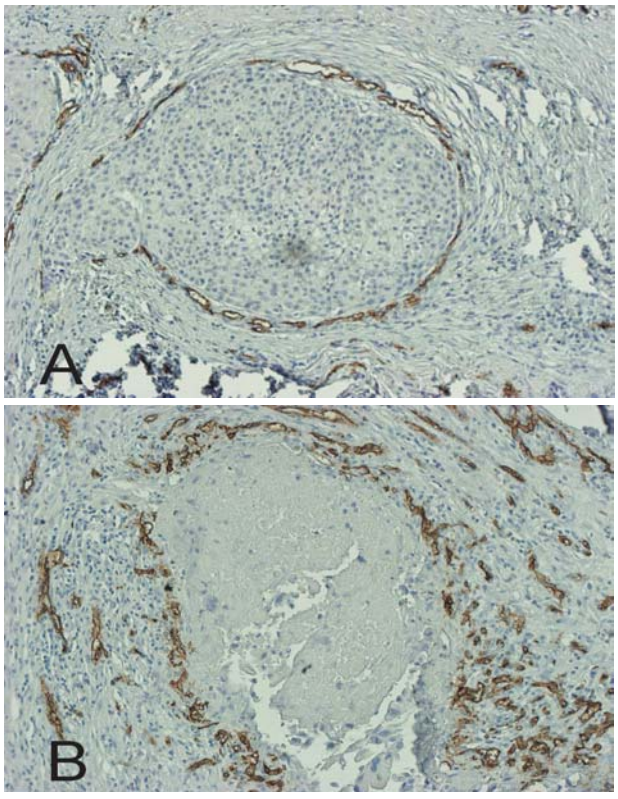

Fig. 1 (A). Peritumoral MVD was observed predominantly in front of a tumor nest. (B). Hotspot of intratumoral MVD was found in area of tissue necrosis.

Table 1. Correlation between peritumoral MVD and clinicopathological features of OSCC

Characteristic	N = 84	Peritumoral MVD					
		vWF		CD31		CD34	
		Low	High	Low	High	Low	High
Gender							
Male	75	40	35	42	33	29	46
Female	9	2	7	5	4	5	4
Age							
≦ 54	48	20	28	33	15	28	20
＞ 54	36	11	25	25	11	19	17
Tumor size							
≦ 4 cm	46	25	21	23	23	20	26
＞ 4 cm	38	17	21	24	14	14	24
LN status							
No	44	19	25	24	20	20	24
Yes	40	23	17	23	17	14	26
Differentiation							
Well	36	23	13	22	14	15	21
Moderate	30	13	17	17	13	15	15
Poor	18	6	12	8	10	4	14
Stage							
Low	43	19	24	20	23	19	24
High	41	23	18	27	14	15	26

LN, lymph node

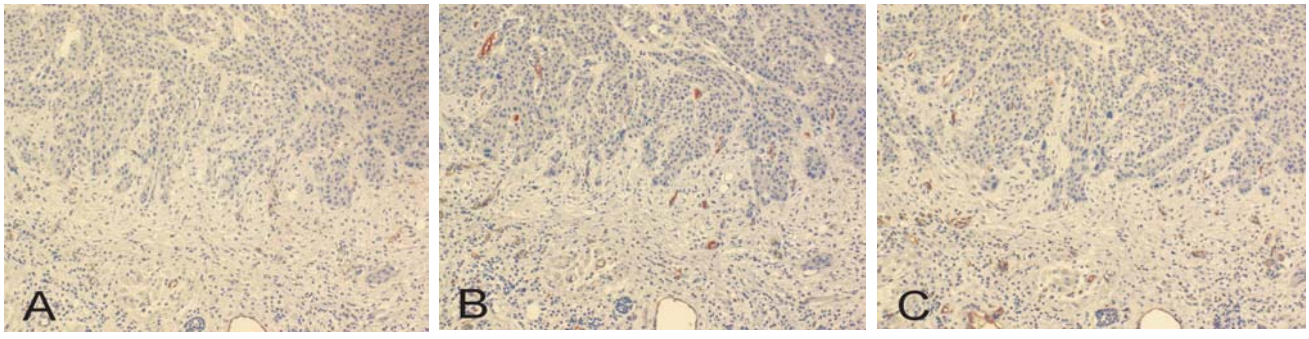

Fig. 2 The serial sections of the same peritumoral location stained with vWF(A), CD31(B) and CD34(C).

heterogeneous in cancerous tissues found among different tumors and among different areas in the same tumor. Notably, a high peritumoral MVD tended to be observed in the stroma around a tumor nest and high intratumoral MVD was frequently noted surrounding areas of tissue necrosis (Fig. 1).

Association of Peritumoral MVD with Clinicopathological Features The peritumoral MVD count per high power field (0.09 mm^2) in all 84 tumors detected by antibodies to vWF, CD31 and CD34 ranged from 8.0 to 29.0, 14.0 to 31.0 and 12.0 to 35.0, respectively, with means of 17.8, 21.4 and 24.2, respectively. Representative sections of vWF, CD31 and CD34 immunostaining results are shown in Fig. 2. We used the median values of 17.5, 21.0 and 24.0, respectively, as the cutoffs to separate tumors into those with high and low peritumoral MVD counts. The association of peritumoral MVD with each patient's clinicopathological features is shown in Table 1, but no statistically significant association was found.

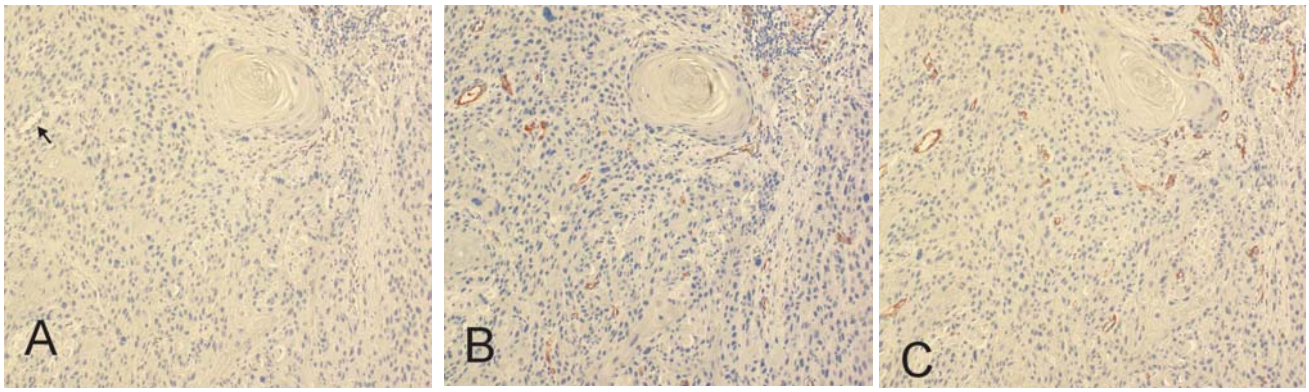

Fig. 3 The serial sections of the same intratumoral location stained with vWF(A), CD31(B) and CD34(C).

Association of Intratumoral MVD with Clinicopathological Features

Similar to peritumoral MVDs, immunostaining for CD34 and CD31 revealed greater intratumoral MVD than did vWF. Most of the intratumoral vessels that were not immunostained for vWF, but were positive for CD31 and CD34, were small vessels (Fig. 3). The intratumoral MVD counts per high power field immunostained for vWF, CD31 and CD34 ranged from 8.0 to 22.0, 11.0 to 29.0 and 13.0 to 35.0, respectively, with means of 14.2, 19.0 and 22.5, respectively. Median values of 14.0, 19.0 and 22.0, respectively, were used as the cutoffs to separate tumors into those with high and low peritumoral MVD counts.

The intratumoral mean MVDs revealed by vWF, CD31 and CD34 immunostaining according to clinicopathological parameters are shown in Table 2. MVD determined using vWF immunostaining showed no significant association with tumor stage, tumor size or histological differentiation. When intratumoral MVDs were determined using CD31 and CD34 immunostaining, the MVD was significantly associated with tumor size ($P = 0.003$ and $P < 0.0001$, respectively), histological differentiation ($P = 0.0025$ and $P = 0.018$, respectively) and tumor stage. ($P = 0.001$ and $P < 0.0001$, respectively). In addition, intratumoral MVD counted using CD34 immunostaining was associated with the rate of lymph node metastasis of OSCCs ($P = 0.005$).

Table 2. Correlation between intratumoral MVD and clinicopathological features of OSCC

Characteristic	N = 84	Intratumoral MVD					
		vWF		CD31		CD34	
		Low	High	Low	High	Low	High
Gender							
Male	75	28	47	51	24	41	34
Female	9	3	6	7	2	6	3
Age							
≦ 54	48	23	18	23	18	23	18
＞ 54	36	23	18	23	18	23	18
Tumor size							
≦ 4 cm	46	17	29	[a]38	8	[b]35	11
＞ 4 cm	38	14	24	20	18	12	26
LN status							
No	44	17	27	34	10	c31	13
Yes	40	14	26	24	16	16	2
Differentiation							
Well	36	17	19	[d]29	7	[e]26	10
Moderate	30	8	22	21	9	15	15
Poor	18	6	12	8	10	6	12
Stage							
Low	43	16	27	[f]37	6	[g]35	8
High	41	15	26	21	20	12	29

LN, lymph node. [a], $P=0.003$; [b],$P < 0.0001$; [c],$P=0.005$; [d],$P=0.025$; [e],$P=0.018$; [f],$P=0.001$; [g],$P < 0.0001$

DISCUSSION

It has been suggested that angiogenesis is necessary for continued tumor growth[5-8]. However, for OSCCs no consistent results between tumor angiogenesis and disease progression, prognosis or metastasis have been established[10-15]. We evaluated peritumoral and intratumoral MVD to relate angiogenesis to tumor progression and found that intratumoral MVD values counted using CD31 and CD34 immunostaining were significantly associated with tumor size, histological differentiation and clinical stage of the OSCC. Previously, a significant increase in peritumoral vascularity during the transition from normal tissue through the dysplastic state to early cancer was reported[16]. Nonetheless, in our study peritumoral vascularity did not increase with tumor growth or further disease progression. In contrast to peritumoral vascularity, intratumoral vascu-

larity increased with tumor progression from early and/or small tumors to advanced and/or large tumors, which indicated that increasing the intratumoral vascularity is more important than peritumoral vascularity during the progression of OSCCs.

Our study also confirmed other reports that vascular hotspots of OSCC are encountered predominantly at the peripheral tumor margin[5,9] and that a high intratumoral MVD[9,17] is frequently found near the region of necrosis in such tumors. Tissue necrosis is a consequence of tumor hypoxia[18] and tumor vessels are structurally and functionally different from their normal counterparts[19,20]. The distinctive characteristics of normal arterioles, capillaries and venules do not apply to most tumor vessels, which are often highly disorganized, tortuous and dilated, with uneven diameter, excessive branching and shunts and a defective wall structure[19,20]. This might be caused by an imbalance of angiogenic regulators, such as vascular endothelial growth factor and angiopoietins. Consequently, tumor blood flow is chaotic and variable[21] and leads to hypoxic and acidic regions[22,23]. Molecular response to hypoxia is mediated by the stabilization of hypoxia-inducible factor (HIF)[18,24,25]. Overexpression of HIF was found to be associated with tumor aggression and poor prognosis in several tumors. Notably, diffuse expression of HIF in the malignant tissue was found in the invasive fronts of tumor masses as well as in areas immediately adjacent to central necrotic tumors[18,25]. Therefore, it is possible that hypoxia or HIF overexpression is involved in the induction of angiogenesis in OSCCs.

The vascularity of tumor tissue can be analyzed by immunostaining of endothelial cells. Two categories of human endothelial cell-specific antibodies are currently available: the pan-endothelial cell markers and antibodies that bind selectively to activated or proliferating endothelium[26-38]. The pan-endothelial markers including Factor VIII-related antigen (FVIII-RA), CD31 and CD34, are characterized by equal intensity of staining for small and large vessels and reactivity in both frozen and paraffin wax-embedded samples. Detection in the latter type of specimens is of clinical importance in that it facilitates the use of archival specimens. For detection of OSCC vascularity, we used vWF, CD31 and CD34 immunostaining to evaluate tumoral MVD. Our data showed that CD34 and CD31 immunostaining techniques were more sensitive for evaluating the tumor blood vessels of OSCCs, and MVDs determined using these markers were significantly associated with tumor growth and disease progression. Previously, a comprehensive study of endothelial markers pointed out that immunostaining for vWF is problematic because this factor is not expressed by all endothelial cells, which limits its reliability for identifying the endothelial cells of microvessels[39]. In agreement with another report[14], we also found that some tumor vessels did not stain for vWF when a distinct lumen (black arrow in Fig. 3A) was recognized clearly. These results were also supported by a report that tumor vascularity as measured by vWF immunostaining did not correlate with neck nodal metastasis in early tongue cancers[14]. In addition to oral cancers, a recent report for breast cancers using antibodies to CD31, CD34 and FVIII-RA also demonstrated that immunostaining for CD31 and CD34 was better than factor VIII-RA in finding associations with survival[40].

In terms of immunological markers of tumor blood vessels, vWF is a multimeric glycoprotein synthesized exclusively in endothelial cells and megakaryocytes and stored in Weibel-Palade bodies in the cytoplasm of endothelial cells and in platelet α-granules[41]. Histologically, vWF antibody shows variable staining of large vessels and capillaries. Several studies have proven that vWF, although highly specific for vasculature, is absent from part of the capillary endothelium in tumorous tissues[36]. CD31, a 130-kDa transmembrane glycoprotein, is a platelet endothelial cell adhesion molecule type 1 of the immunoglobulin superfamily expressed on the surface of circulating platelets, monocytes, neutrophils and selected T-cell subsets and is a constituent of the endothelial intercellular junctions. CD31 is found in large amounts on endothelial cells and is less abundant on platelets and most leukocytes. Generally, CD31 immunostaining specifically recognizes small and large vessels with equal intensity. The disadvantages associated with staining for the CD31 antigen include costaining of inflammatory cells, but these can be distinguished from endothelial cells by morphology and frequent antigen loss caused by fixatives containing acetic acid. Antigen retrieval could effectively abolish this problem but a careful selection of the most suitable tissue fixation procedures should still be performed prospectively[17]. CD34, regarded as a common diagnostic endothelial marker, is a 115-kDa transmembrane glycoprotein present on lymphohematopoietic stem and progenitor cells, leukemic cells, endothelial cells and embryonic fibroblasts. Immunohistochemically, CD34 is primarily expressed on small or newly formed vessels and endothelial cells of endothelial tumors, while endothelial cells of small and large vessels in normal and tumor tissue have been reported to be stained with equal intensity[42-47]. As an endothelial marker, the disadvantage of CD34 immunostaining specificity is the presence of CD34 antigen in perivascular stroma cells as well as other stromal elements[17]. Therefore, careful and experienced evaluation is important in evalu-

ating CD34 immunostaining results.

In conclusion, tumor angiogenesis and the density of newly formed vessels are of potential prognostic relevance in the assessment of malignant neoplasia. To study the significance of tumor angiogenesis, one must pay attention to the selection of suitable counting areas as well as to the blood vessel markers used. Compared with other monoclonal antibodies, that for the endothelial marker CD34 is better in the assessment of tumor vascularization of OSCCs. In addition, hotspot selection in the intratumoral area is necessary to examine any involvement in OSCC progression.

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