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A Clinical and Preliminary Study for Anti-angiogenesis Therapy: Endostatin, VEGF and Microvessel Density in the OSCC in Different Stages and Differentiations

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Authors' contributions

This work was carried out in collaboration among all authors. Author DZ designed the study, performed the statistical analysis. Author HH wrote the protocol and wrote the first draft of the manuscript. Authors JW and XD managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was to analyze the association among ES, VEGF, Microvessel Density (MVD), clinicopathologic characteristics, angiogenesis and prognosis of OSCC.
Methods: Eight normal samples of oral epithelia and 52 Oral Squamous Cell Carcinoma (OSCC) samples were analyzed by immunohistochemical evaluation to study the expression and significance of Endostatin (ES) and Vascular Endothelial Growth Factor (VEGF) during the development of OSCC.
Results: Statistically significant differences were found as $p<0.05$ between VEGF expressions and clinicopathologic stages of OSCC and as $p<0.01$ between VEGF expressions and lymph node

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metastases of OSCC. And Statistically significant discrepancy was also found as $p<0.05$ between MVD and differentiation degrees and lymphnode metastases of OSCC, as well as $p<0.01$ between VEGF expressions and MVD. Additionally MVD increased gradually in accordance with the progression of the Cancer. While there was no obvious correlation between ES and VEGF, ES and MVD, as well as between ES and the development of OSCC.

Conclusion: By MVD et al evaluation, VEGF is one of the major angiogenesis factors for angiogenesis and lymphonode metastasis of oral carcinomas, as an important indicator for the development and malignancy of OSCC, while ES is of significance for anti-angiogenesis in tumor therapy.

Keywords: Endostatin; vascular endothelial growth factor; microvessel density; OSCC; immunohistochemistry.

ABBREVIATIONS

MVD : Microvessel Density
OSCC : Oral Squamous Cell Carcinoma
ES : Endostatin
VEGF : Vascular Endothelial Growth Factor

1. INTRODUCTION

Cytokines trigger the movement of cancer cells by connecting to receptors through signal transductions, thus participate in the occurrence, development and transfer of the tumor cell. Among which, VEGF is a very important growth factor which has important effects on tumor angiogenesis and vascular permeability [1-3], with receptors as VEGFR-1 and VEGFR-2. Angiogenesis [4,5] is mainly mediated by VEGF through the heterodimer formed from VEGFR-1 and VEGFR-2. In addition, VEGF-C(with receptors as VEGFR-2 and VEGFR-3) which mediates pericancer lymphatic vessel [6-8], enhances the invasion of lymphatic vessel, through strong chemical absorption of lymphatic endothelial cells, thus plays a cooperative role, together with the formation of capillary vessel, promotes the survival and growth of minor tumor metastasis.

Endostatin [9,10] is considered to be the strongest angiogenesis inhibiting factor, it inhibits angiogenesis and thus inhibits growth and metastasis of 65 kinds of different tumors. It is an endogenous inhibitor of angiogenesis, which is secreted by vascular endothelial cells of the tumor to lead to stagnate of G1 phase of the endothelial cell, and inhibits the proliferation of the endothelial cell and can also reduce Bcl-2, Bcl-xl antiapoptotic proteins. Mechanisms of angiogenesis inhibition include either binding to the heparin-like sulfate proteoglycan to inhibit angiogenesis, or inducing the apoptosisi of endothelial cell. Endostatin competes with bFGF through the structure of heparin binding site, and blocks signal conduction of caryomitotic growth factor and the growth of endothelial cell and then inhibit angiogenesis. Endostatin is a 20 ku (karmen unit) proteolytic fragment of Carboxyl terminus of collagen XVIII, produced from the hydrolysis of collagen XVIII by a variety of proteases (including matrix metalloproteinases, cysteine protease and serine protease). The secretion of proteolytic enzymes increases with the development of tumor malignance, degrading the collagen XVIII in vascular basement membrane and leading to the enhancement of Endostatin production.

Angiogenesis [11] is vital to the growth and metastasis of substantial tumor. The basic process of angiogenesis includes the activation of vascular endothelial cell, the degradation of extracellular matrix, the proliferation and migration of endothelial cells, lumen formation and the formation of the extra-vascular membrane. Blocking any step of this link can inhibit tumor angiogenesis. Hanahan [12] thought that angiogenesis is regulated by both angiogenesis promoting and inhibiting factors. Studies by Folkman [13] found that when the size of tumor grew to 2 mm^3, it needed new blood vessels to obtain nutrition and oxygen to sustain the rapid growth of tumor, otherwise tumor tissue would remain dormant or degraded. Evidently, inhibiting tumor angiogenesis to cut off the blood support of the tumor can prevent the rapid growth and metastasis of the tumor. This is the anti-angiogenic therapy. Correspondingly, antibody CD34 is often used to mark the tumor vascular endothelial cells, to quantify the extent of tumor angiogenesis. In this research we detected the expression of VEGF, Endosatin and the microvascular density (MVD) in the tumor tissues of 52 cases of OSCC patients and eight cases of normal oral mucosal tissue, to analyze the relationships between the expression of

VEGF, Endostatin and MVD and the clinical pathological features of OSCC and to provide a theoretical basis for clinical diagnosis and antiangiogenic tumor treatment.

2. MATERIALS AND METHODS

2.1 Clinical Materials

All the subjects have provided written informed consent for planned studies which had been approved by Institutional Review Board.

Among 8 cases of normal control group, including 4 male and 4 female, 4 cases aged above 60 and 4 cases aged below 60, averagely 55-year-old. They were hospital workers with no chronic systemic disease, salivary gland disease, untreated dental caries nor periodontal disease, whose oral mucosal membrane were normal, non-smoking, not in the female menstrual period.

The OSCC group, 52 patients pathologically diagnosed with oral squamous cell carcinoma were registered in the Second Affiliated Hospital of Medical College of Zhejiang University between 2000 and 2007 who accepted no preoperative radiotherapy, chemotherapy or other treatment. These 52 cases included 33 male and 19 female, medially aged 64, averaged 60.77±13.35. Specimens were collected and pathologically confirmed after surgical resection, in which 25 cases were well differentiated OSCC, 20 cases were moderately differentiated OSCC, 6 cases were poorly differentiated, 1 case was unclassified. And among them, 9 cases belonged to clinical stage I, 18 cases belonged to stage II, 14 cases belonged to stage III, 7 cases belonged to stage IV. Specimens were fixed in 10% formalin, embedded in paraffin, cut into 5um thick serial sections for HE staining and immunohistochemistry detection.

2.2 Reagents

Polyclonal anti-ES antibody was product of Santa-Cruz Company of the United States. VEGF antibody, CD34 antibody, tissue antigens, EDTA solution, and SP immunohistochemistry kit were purchased from Maixin Company of Fuzhou China.

2.3 Immunohistochemistry Method

Immunohistochemistry is a widely used method to detect specific antibody according to the principle of reaction between antibody and antigen.

Method: Conventional hydration was conducted for the paraffin wax sections. Tissue antigens were repaired by EDTA bath following the instructions provided by the Maixin Company and incubated 10 min at room temperature with peroxidase inhibitor; after 10 min of addition of normal serum, liquid was discarded, primary antibody was then added and incubated at 37ºC for 1h in a wet box and then second antibody biotin-labeled and streptavidin - peroxidase solution were added incubating for 10 min in succession; sections were washed 3 times with TBS (0.01 mol / L, pH 7.4) between every two steps as above and were stained with demonstration solution A and B (DAB), after the antibody reaction , regarding brown as a terminal point before wash away. It was worthy mentioned that keeping all the terminal points consistent was significant. Subsequently, the sections were stained with hematoxylin before sealing. Multiple independent examiners made the histologic analysis and radiographic observation and there was a calibration among the examiners as followed.

Grade determination: Appearance of brown particles in cytoplasm was determined as positive staining of ES and VEGF (Fig. 1). Then quantitatively determine grades by general staining intensity and number of positive cells: sort out the strongest expressed site, name the basically no staining and similar to background as 0 point; name the light staining, slightly stronger than the background as 1 point; name the medium staining, significantly stronger than background as 2 points; name the strong staining, colored dark brown as 3 points. Name positive cells <10% as 0 point, 11% to 25% as 1 point, 26% to 50% as 2 points, 50% to 75% as 3 points, > 75% as 4 points. Four grades were obtained after adding these two items together: 0 to 1 was decided as (-), 2 as (+), 3 to 4 as (++), over 5 as (+++). As a control of recording reliability, the procedure was randomly repeated 15 days later. The two successive determinations differed in value by less than 5%. Results were expressed as means±standard deviations (SD). All results were considered to be significant at P=0.05 and 0.01 (critical level). Statistical calculations were carried out using SPSS 16.0 software package. Rank sum test (Mann-Whitney test) was used for intergroup comparison; Mann-Whitney U method was used between two groups; Kruskal Wallis Test was used among multiple groups. Correlations were analyzed with Spearmam rank.

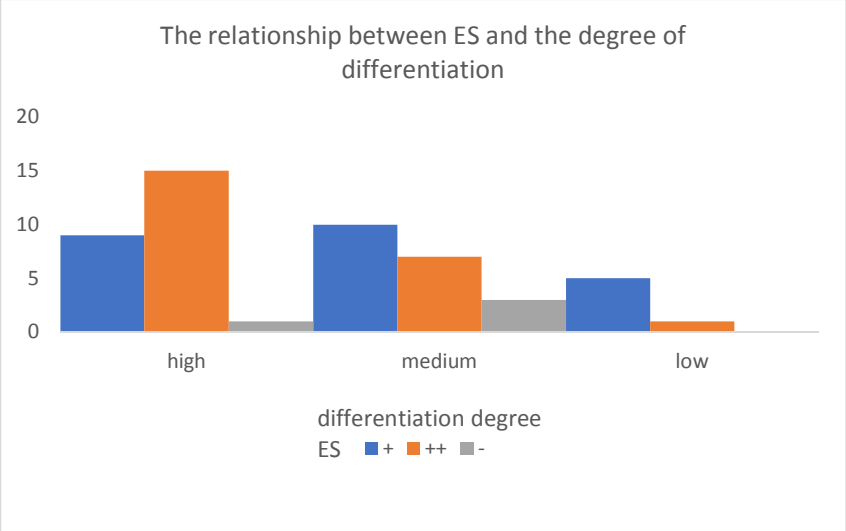

Fig. 1. The relationship between ES and the degree of differentiation

2.4 MVD Determination Method

Select the highest distribution area of microvessels under the 100 times light microscope, slice selection in the area, count 5 perspective views of the blood vessels stained brown by CD34 under the 200 times view observation, decide the average value as the Microvessel Density. Each microvascular endothelial cell or endothelial cell cluster that clearly separated with neighbor microvessel and positively stained was treated as a separate microvessel [12].

2.5 Statistical Analysis

Apply SPSS 16.0 statistical software to data analysis, use rank sum test (Mann-Whitney test) for intergroup comparison; use Mann-Whitney U method between two groups; use Kruskal Wallis Test among multiple groups. Analyze correlation with Spearmam rank. P <0.05 was considered as statistically significant.

3. RESULTS

3.1 Immunohistochemical Expression

ES protein was mainly expressed in tumor cytoplasm (Fig. 1) (Table 2). There was no significant difference of ES expression distribution between different genders, age groups, tumor differentiations, lymph node metastases and clinical stages (P> 0.05).

VEGF was expressed in tumor cytoplasm as brown granules (Fig. 2) (Table 3). Positive VEGF staining rate of tumor cells was 50.0% (26/52). No significant difference was found in different genders, age groups and differentiations (p>0,05); but significant difference was found between staining expression and lymph node metastasis as well as clinical stages (P <0.05), with a positive staining rate of 92.31% (12/13) with lymph nodes metastasis, higher than those without metastasis (34.38%, 11/32). In the other hand, there was a significant increase of the positive rate of staining expression in Phases III and IV clinical stage, in terms of that positive rate of expression was 33.33% (3 / 9) in stage I, 27.78% (5 / 18) in stage II, 71.43% (10/14) in stage III and 100.0% (7 / 7) in stage IV.

3.2 Relationship between VEGF Expression and MVD

The OSCC group was divided into two sub-groups according to the expression of VEGF (Table 4): VEGF-positive group, 26 cases with a MVD value of 22.84 ± 11.27, VEGF negative group, 26 cases with a MVD value of 15.65 ± 7.34. Difference between the two sub-groups was significant (P <0.01), meanwhile, correlation analysis showed that the correlation coefficient was 0.45 (p <0.05), indicating that VEGF expression correlated with MVD.

3.3 Intimate Correlation of MVD Values with Tumor Differentiation and Lymph Node Metastasis

MVD value presented highest in poorly differentiated group and higher in lymph node

metastasis group (22.28 ± 12.34) than in those without lymph node metastasis (17.99 ± 9.27), the difference was statistically significant ($p < 0.05$) (Fig. 3).

3.4 No Correlation of ES with VEGF, MVD, and Clinical Parameters

Correlation coefficient was less than 0.5 ($p > 0.05$).

4. DISCUSSION

The expression level of VEGF is low in the normal mucosa, while high in some pathological situations. Consistent to the previous scholars' indication, in our studies, VEGF did not express in the normal oral mucosa, and only was weakly positive in the perivascular, still only moderately expressed in oral precancerous lesions, meanwhile strongly expressed in OSCC. This suggests that VEGF participates in the pathological process of oral mucosa from normal tissues to precancerous lesions and then to OSCC, indicating that VEGF can be an important indicator of biological behavior of OSCC. With progressing of TNM stage, size of tumor increases, thus leading to the relative hypoxia of the tumor, which further stimulates the secretion of VEGF and promote angiogenesis. MVD of OSCC is related to the degree of differentiation. The worse the differentiation was, the higher

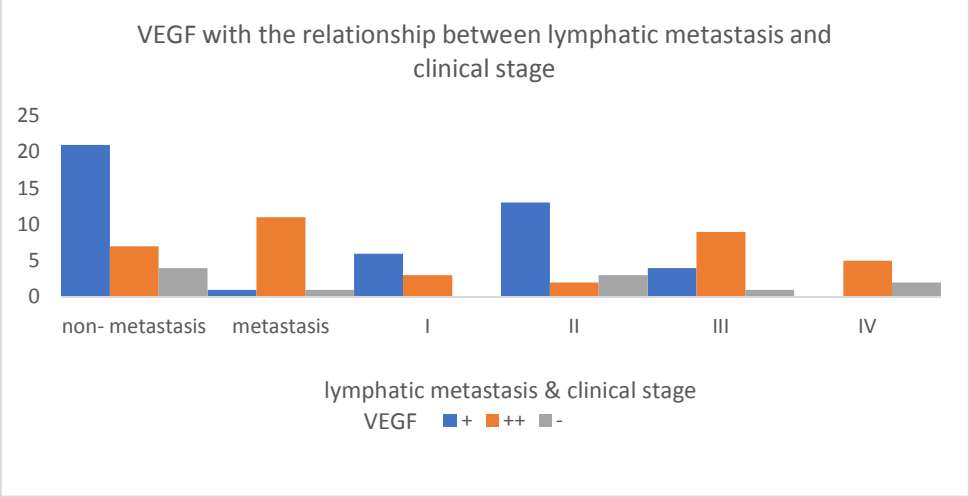

Fig. 2. VEGF with the relationship between lymphatic metastasis and clinical stage

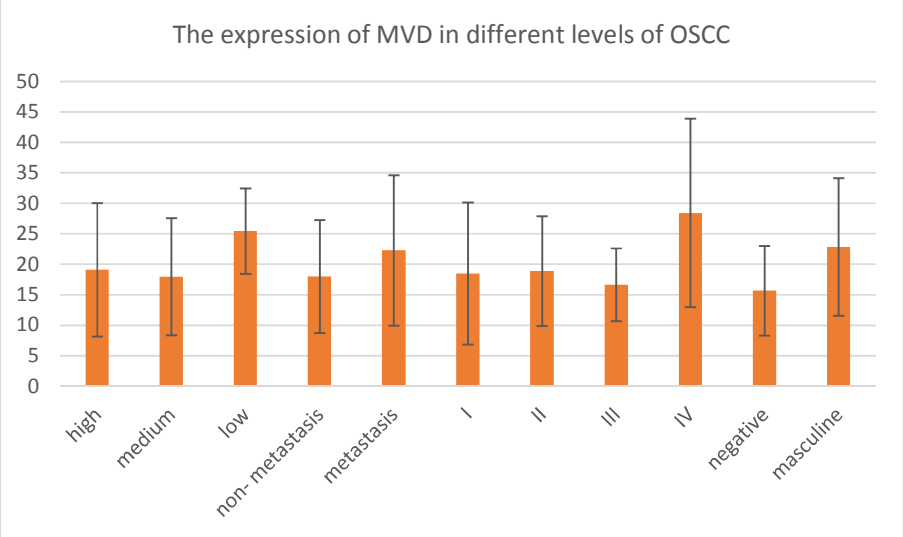

Fig. 3. The expression of MVD in different levels of OSCC

Table 1. ES, VEGF and MVD relationships between expression and clinical parameters in OSSS group

Clincial factor	Number	ES				VEGF				MVD	
		-	+	+ +	p	-	+	+ +	p	($\bar{\chi}$ ±S)	p
Sex											
Male	33	14	15	4		16	11	6		17.31±6.68	
Female	19	11	8	0	0.171	10	8	1	0.510	22.61±13.80	0.264
Age											
< 60	22	12	7	3		11	9	2		21.66±12.13	
≥ 60	30	13	16	1	0.733	15	10	5	0.799	17.48±8.05	0.163
Differentiated level											
High	25	9	15	1		13	10	2		19.08±10.95	
Medium	20	10	7	3		10	7	3		17.96±9.60	
Low	6	5	1	0	0.156	2	2	2	0.485	25.44±7.00	0.048
Lymphatic metastasis											
Non-metastasis	32	16	14	2		21	7	4		17.99±9.27	
Metastasis	13	6	7	0	0.977	1	11	1	0.005	22.28±12.34	0.037
Clinical stage											
I	9	4	4	1		6	3	0		18.48±11.66	
I I	18	8	8	2		13	2	3		18.87±9.01	
I I I	14	6	7	1		4	9	1		16.64±5.96	
I V	7	5	2	0	0.571	0	5	2	0.010	28.43±15.45	0.106
Expression level of VEGF											
Negative	26	11	13	2						15.65±7.34	
Masculine	26	14	10	2	0.462					22.84±11.27	0.000

the MVD value was. MVD for cases with lymph node metastasis is significantly higher than those without lymph node metastasis. This indicates that MVD could be exploited to be an important indicator of the degree of the malignancy of OSCC.

The phenomenon as we found that capillaries positively labeled with monoclonal antibody CD34 mainly distributed surrounding the tumor, and around the cancer cells which were positive with VEGF staining, indicated the coherence of VEGF protein expression and angiogenesis. In this study, it was confirmed that MVD in VEGF-positive group was significantly higher than that in VEGF-negative group. And the results that MVD and the expression of VEGF were positively correlated indicated that VEGF expression in OSCC was closely linked with MVD, which was consistent to Denhart and Michailidou's research results [14,15].

When it came to ES, it increased with the malignancy degree, and decreased with the descending of the differentiation degree of OSCC, increasing of the clinical stage, and the metastasis of lymph node. This showed that the increasing expression or ES absence is a dynamicly protective response; it is involved in the growth and metastasis of OSCC.

Evidently, compared with conventional chemotherapy, anti-tumor angiogenesis therapy is not directly targeted to the tumor cells, but to vascular endothelial cells. Because the tumor

Table 2. The relationship between ES and the degree of differentiation

The degree of differentiation	ES			P
	+	++	-	
High	9	15	1	0.156
Medium	10	7	3	
Low	5	1	0	

Table 3. VEGF with the relationship between lymphatic metastasis and clinical stage

	VEGF			P
	+	++	-	
Lymphatic metastasis				
Non- metastasis	21	7	4	0.005
Metastasis	1	11	1	
Clinical stage				
I	6	3	0	0.010
II	13	2	3	
III	4	9	1	
IV	0	5	2	

Table 4. The expression of MVD in different levels of OSCC

	MVD($\bar{\chi}$ ±S)	P
The degree of differentiation		
High	19.08±10.95	0.048
Medium	17.96±9.60	
Low	25.44±7.00	
Lymphatic metastasis		
Non- metastasis	17.99±9.27	0.037
Metastasis	22.28±12.34	
Clinical stage		
I	18.48±11.66	0.106
II	18.87±9.01	
III	16.64±5.96	
IV	28.43±15.45	
Expression level of VEGF		
Negative	15.65±7.34	0.000
Masculine	22.84±11.27	

cells are instable, the chromosome is changeable, and prone to chromosomal deletion, rearrangement, translocation, mutation, especially vulnerable to cause drug resistance; while, the chromosomes group of endothelial cells are primarily normal, relatively stable and hard to induce drug resistance, thus anti-angiogenic therapy has its own characteristics. Scholars [16-19] have confirmed via animal experiments and clinical observations, that Endostatin inhibits tumor growth by inhibiting angiogenesis and the effect is amazing. Thus the study of anti-tumor angiogenesis through Endostatin gene therapy is greatly concerned. Although, the United States have begun clinical trials of Endostatin protein since the end of 1999, the clinical application of Endostatin protein is facing many obstacles: (1) Because of the unstability of the Endostatin protein, it's difficult to prepare large quantities of biologically active protein; (2) The process of Endostatin protein production technology is complex; (3) Endostatin's hemiperiod is very short, so it needs to be repeated several times a day-dosing to maintain effective drug concentrations; (4) The application of Endostatin to tumors requires a so long course of treatment that patients are subjected to enormous economic burden. Therefore, gene therapy, which makes a body into an Endostatin protein "factory", is currently the best way to solve above problems. It has obvious advantages, firstly, it can produce highly active endogenous protein; secondly, one treatment leads to a long period of stable expression, which reduces the cost and simplifies the treatment, and easy to be accepted by patients; finally, gene therapy maintains Endostatin protein at a relatively stable level in body, and its pharmacokinetics is more reasonable. Although this research is far from gene therapy, it provides a preliminary support for later anti-angiogenesis therapy.

5. CONCLUSION

This clinical and preliminary data among endostatin, VEGF and microvessel density from cases provides certain light into anti-angiogenesis therapy strategy for related diseases.

CONSENT AND ETHICAL APPROVAL

The study was carried out in accordance with the Helsinki Declaration of 1975, as revised in 2000, and was reviewed and approved by the local ethical committee (No.20150012). All the patients gave their written informed consent for participation in the study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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