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ORIGINAL ARTICLE

Association of clinicopathologic parameters with the expression of inducible nitric oxide synthase and vascular endothelial growth factor in mucoepidermoid carcinoma

Ou Yang Ke-xiong[1]*, Liang Jun[2]*, Huang Zhi-quan[3]

[1]*Department of Oral and Maxillofacial Surgery,Stomatological Hospital of Guangzhou Medical College, Guangzhou;* [2]*Department of Oral and Maxillofacial Surgery, The Fifth Affiliated Hospital, Sun Yet-sen University, Zhuhai;* [3]*China Department of Oral and Maxillofacial Surgery, The Second Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China*

BACKGROUND: The roles that inducible nitric oxide synthase (iNOS) and vascular endothelial growth factor (VEGF) play in tumorigenesis have been given special attention. In many tumors, their expression is upregulated. In addition, iNOS can stimulate the expression of VEGF. This study was carried out to investigate the expression of iNOS and VEGF as well as their relationship with angiogenesis and the clinicopathological characteristics of mucoepidermoid carcinoma (MEC).

METHOD: The expression of iNOS and VEGF was detected by Streptavidin–peroxidase immunohistochemistry, and microvessel density (MVD) was determined by anti-CD34 antibody staining in 70 MEC cases and 40 normal salivary gland tissues (NSG). Follow-up was performed on the 70 patients with MEC. Non-parametric tests were performed for the comparison of iNOS and VEGF expression.

RESULTS: The positive expression rates of iNOS and VEGF were successively enhanced in NSG, well-differentiated and poorly differentiated MEC ($P < 0.05$). MVD counts were positively correlated with the expression levels of iNOS and VEGF in MEC ($P < 0.05$). The expression of iNOS was positively correlated with the expression of VEGF ($P < 0.05$). iNOS and VEGF expression were significantly associated with tumor differentiation, size metastasis, and relapse ($P < 0.05$) but were not correlated lymph node metastasis and metastasis.

CONCLUSION: Inducible nitric oxide synthase can stimulate the expression of VEGF, and their expression status may help assess tumor malignancy and patient prognosis.

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Correspondence: Dr Jun Liang, Department of Oral and Maxillofacial Surgery, The Fifth Affiliated Hospital, Sun Yet-sen University, Mei Hua Dong lu 52# zhuhai519000, China. Tel: +86 75 6252 8203, Fax: +86 75 6252 8203, E-mail: liangjunzhuhai@hotmail.com
*These authors contributed equally to this article.
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Introduction

Mucoepidermoid carcinoma (MEC) is the most common malignant tumor of the salivary glands. Current opinion still diverges between a 2-tier system that classifies MEC into low- and high grade and a 3-tier grading system that incorporates an intermediate level(Goode *et al*, 1998; Brandwein *et al*, 2001).Owing to different invasive capacities and biological characteristics, MECs with different degrees of differentiation are linked to different prognoses and require different treatments(Bradley, 2001; Brandwein *et al*, 2001; Regis De Brito Santos *et al*, 2001).

Angiogenesis is the formation of new blood vessels from existing ones. It is a key component of many pathological and physiological processes such as embryogenesis, wound repair, inflammation, and tumorigenesis (Folkman and Shing, 1992). Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic growth factors known. Among angiogenesis factors, it is characterized by its dual angiogenic and tumorigenic roles that affect the growth and spread of malignant neoplasms (Podar *et al*, 2002; Podar and Anderson, 2005). Previous studies demonstrated VEGF as an important regulator of cellular growth, survival, and migration in tumor cells (Kaneko *et al*, 2007), including oral squamous cell carcinoma (OSCC) (Brennan *et al*, 2002; Lan *et al*, 2009).Nitric oxide (NO) is produced by three different isoforms of NO synthase (NOS): the neural and the endothelial NOSs are constitutively expressed, whereas the inducible isoform or inducible NOS (iNOS) can be expressed in response to proinflammatory agents (Nathan and Xie, 1994). iNOS generates high levels of NO in tissues. Increased iNOS expression has been demonstrated in a number of carcinomas, including head and neck SCC. Increased iNOS expression and the generation of high NO levels

might have a role in oral OSCC development (Chen *et al*, 2002; Connelly *et al*, 2005). iNOS might be implicated in the VEGF-associated angiogenic process and stimulate the generation of VEGF.

Several questions with respect to the roles of both iNOS and VEGF in MEC have not yet been addressed in the literature: How does their expression affect the development of MEC? What is their relationship with each other in MEC? What is the specific mechanism responsible for these effects? This study examined the expression iNOS and VEGF MEC using immunohistochemistry staining techniques and then analyzed the data, which may help to assess tumor malignancy and patient prognosis.

Materials and methods

Patients

Paraffin-embedded specimens were collected from 70 patients with MEC who had undergone radical surgeries from 1995 to 2005 in the Department of Oral and Maxillofacial Surgery at the Second and Fifth Affiliated Hospitals, Sun Yat-sen University. Forty patients are women, and 30 are men; their age ranged from 21 $\sim$ 62Y with an average of 34.9 $\pm$ 3.7Y; nine patients have distant metastasis. The diagnosis for each specimen was confirmed by a histopathological examination. None of the patients received any prior therapy, such as radiotherapy or chemotherapy. The follow-up of the 70 patients with MEC ranged from 1 to 60 months, with an average of 29.9 months. This retrospective study utilized tissue specimens from the 70 consecutive patients and follow-up data from a local pathology repository. There were 48 tumors from the parotid gland and 22 from the submandibular gland. All tumors were categorized according to the World Health Organization histological classification (Hompson, 2007) and staged according to the International Union Against Cancer system. The control group comprised 40 normal salivary gland tissues (NSG), 22 from women and 18 from men; 35 parotid gland tissues; and five submandibular gland tissues.

Immunohistochemical Staining

Immunohistochemical staining used the rabbit anti-human iNOS polyclonal antibody (NeoMarkers, Fremont, California), the anti-VEGF rabbit polyclonal antibody (Santa Cruz Biotech Inc, California), and mouse anti-human CD34 polyclonal antibody (Dako, Carpinteria, California). Staining used the general ultrasensitive streptavidin peroxidase immunohistochemistry system (Zhongshan Biotechnology, Beijing, China), a ready-to-use streptavidin peroxidase kit and a DAB chromogenesis kit (Lab Vision/NeoMarkers, Fremont, California). Paraffin blocks were cut at a thickness of 4 μm. The sections were deparaffinized with xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked by incubating sections with 3% H_2O_2 in methanol for 10 min. Primary antibodies were added at a dilution of 1:70. The sections were stained with anti-iNOS, anti-VEGF, and anti-CD34 antibodies and incubated at 4°C overnight. The general biotin-linked secondary antibody was added dropwise, followed by incubation of the sections at 37°C for 30 min. The streptavidin peroxidase solution was added in a similar fashion, and the same incubation conditions were used. The diaminobenzidine stain was applied for 5 min to detect chromogenesis. After counterstaining the cell nuclei with hematoxylin, the sections were mounted. The negative control used phosphate-buffered saline instead of the primary antibody. Mammary carcinoma, which expresses all three proteins (iNOS, VEGF, and CD34), was used as the positive control tissue.

Analysis of results

A positive result for iNOS and VEGF was indicated by the development of yellow-brown granules in the cytosol, which were detected under a light microscope with high-power objective illumination. The resultant sections were first examined at low magnifications (×40 and ×100) to identify the areas without folding and edge effect. Within this area, five random × 400 visual fields were examined. The proportion of positively staining cells and the strength of staining were combined to produce a semi-quantitative scoring standard for immunohistochemistry. Cytoplasmic stains for both iNOS and VEGF were scored according to the presence and intensity of staining on the section, where 0 = unstained, 1 = lightyellow, 2 = yellowish brown, and 3 = dark brown. In addition, the percentages of stained tumoral cells to the total number of tumoral cells on the section were scored as follows: 1 = less than 25%, 2 = 25–75%, and 3 = more than 75%. A cumulative score for each case was determined by multiplying the presence intensity score by the ratio score. The cumulative scores were then categorized as negative (j) = 0, positive (+) = 1 to 4, or strongly positive (++) = more than 4(Chen *et al*, 2009). The Weidner method (Weidner, 1995) was used to determine capillary microvessel densities (MVDs). Under a low-power objective lens, the entire section was examined to search for regions of high capillary density, referred to as hot spots. A 200× objective lens was then used to count the clusters of vascular endothelial cells with brown staining. The final result was the average of three such counts.

Statistical analysis

Homogeneity of variance was used to compare means, and enumerated data were analyzed with the chi-squared test and the Spearman rank-order correlation analysis The patient's outcome was compared with survival analysis (log-rank test). All statistical analyses were performed by the SPSS WIN program package 13.0 (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant for a P-value < 0.05.

Results

iNOS and VEGF expression

Inducible NO synthase expression was positive in 10 (25.0%) of the NSG, 34 (75.6%)of well-differentiated, and 22 (88.0%)of poorly differentiated MEC. This difference between the three groups was significant

Table 1 Expression of inducible nitric oxide synthase (iNOS) and vascular endothelial growth factor (VEGF) and microvessel density (MVD) counts

		iNOS expression			*VEGF expression*			
Groups	n	–	+	++	–	+	++	*MVD (Mean ± s.d.)*
NSG	40	30	7	3	27	9	4	16.32 ± 1.21
MEC	70	14	31	25	15	25	30	
Well	45	11	21	13	11	19	15	31.35 ± 2.15
Poorly	25	3	10	12	4	6	15	38.04 ± 2.74
x^2 or *F*-value			31.352			25.928		28.301
P-value			0.000			0.000		0.000

MEC, mucoepidermoid carcinoma; NSG, normal salivary gland tissues.

($P < 0.05$) (Table 1) (Figures 1–3). VEGF expression was positive in 13 (32.5%) of the NSG, 34 (75.6%) of well-differentiated, and 21 (84.0%) of poorly differentiated MEC. This difference between the three groups was significant ($P < 0.05$) (Table 1) (Figures 4–6).

MVD count

The MVD count in the NSG group, well-differentiated, and poorly differentiated MEC was 16.32 ± 1.21, 31.35 ± 2.15, 38.04 ± 2.74, respectively. This difference between the three groups was significant ($P < 0.05$) (Table 1) (Figures 7–9).

Relationship between iNOS and VEGF expression and MVD

The MVD count was 16.43 ± 1.83 in the MEC samples that were negative for iNOS expression ($n = 14$). The MVD count was 17.98 ± 1.83 in the MEC samples that were positive for iNOS expression ($n = 31$). The MVD count was 37.72 ± 2.66 in the MEC samples that were strongly positive for iNOS expression ($n = 25$). A comparison between the groups revealed an *F* value of 27.754 and a statistically significant difference ($P < 0.05$) (Table 2).

The MVD count was 16.21 ± 1.91 in the MEC samples that were negative for VEGF expression ($n = 15$). The MVD count was 24.74 ± 2.42 in the MEC samples that were positive for VEGF expression ($n = 25$). The MVD count was 32.92 ± 3.15 in the MEC samples that were strongly positive for VEGF expression ($n = 30$). A comparison between the groups revealed an *F* value of 7.171 and a statistically significant difference ($P < 0.05$) (Table 2).

Table 2 Correlation of the expression levels of inducible nitric oxide synthase (iNOS) and vascular endothelial growth factor (VEGF) with the number of microvessel densities (MVDs) in mucoepidermoid carcinoma

Groups	N	*MVD (Mean ± s.d.)*	F-*value*	P-*value*
iNOS				
(–)	14	16.43 ± 1.83	27.754	0.000
(+)	31	17.98 ± 1.83		
(++)	25	37.72 ± 2.66		
VEGF				
(–)	15	16.21 ± 1.91	7.171	0.002
(+)	25	24.74 ± 2.42		
(++)	30	32.92 ± 3.15		

Table 3 Correlation between the expression levels of inducible nitric oxide synthase (iNOS) and vascular endothelial growth factor (VEGF) in mucoepidermoid carcinoma

	VEGF expression				
iNOS expression	(–)	(+)	(++)	x^2	P-*value*
(–)	7	4	3	0.406	0.008
(+)	5	15	11		
(++)	3	6	16		

Correlation between the expression of iNOS and VEGF

Correlation analysis of iNOS and VEGF expression in MEC indicated that $x^2 = 0.406$ ($P < 0.05$), demonstrating that the two factors were positively correlated (Table 3).

Association of clinicopathologic parameters with the expression of iNOS and VEGF

The correlation of iNOS and VEGF expression with the clinicopathological parameters of the MEC tumor is summarized in (Table 4). The expression levels of iNOS and VEGF in MEC were significantly related to tumor differentiation, size, and relapse ($P < 0.05$), but were not correlated lymph node metastasis and metastasis. At the end of the five year follow-up, 92.8% (13/14) of patients with negative iNOS expression survived without relapse; in contrast, only 60.7% (34/56) of patients with positive iNOS expression survived without relapse (Figure 10, $x^2 = 4.657$, $P = 0.031$); 93.3% (14/15) of patients with negative VEGF expression survived without relapse, and in contrast, only 60.0% (33/55) of patients with positive VEGF expression survived without relapse (Figure 11, $x^2 = 5.337$, $P = 0.021$).

Discussion

The angiogenesis plays an important role in many physiologic and pathologic processes. The tumor growth and metastatic potential of a solid neoplasm seem to depend on angiogenesis. It is a multistep process, including basement membrane degradation, endothelial cell migration, and sprouting into interstitial space,

Table 4 Correlation of the expression levels of inducible nitric oxide synthase (iNOS) and vascular endothelial growth factor (VEGF) with the clinicopathological characteristics of mucoepidermoid carcinoma patients

	iNOS expression				*VEGF expression*			
Clinicopathology	*Negative (%)*	*Positive (%)*	x^2	P-*value*	*Negative (%)*	*Positive (%)*	x^2	P-*value*
TMN stage								
T stage								
T_{1+2}	9	18	4.884	0.027	10	17	6.360	0.012
T_{3+4}	5	38			5	38		
N stage								
N0	10	50	2.917	0.088	11	49	2.390	0.122
N1	4	6			4	6		
M stage								
M0	13	48	0.072	0.786	14	47	0.139	0.709
M1	1	8			1	8		
Relaps								
NO	13	34	3.889	0.049	14	33	4.521	0.033
YES	1	22			1	22		

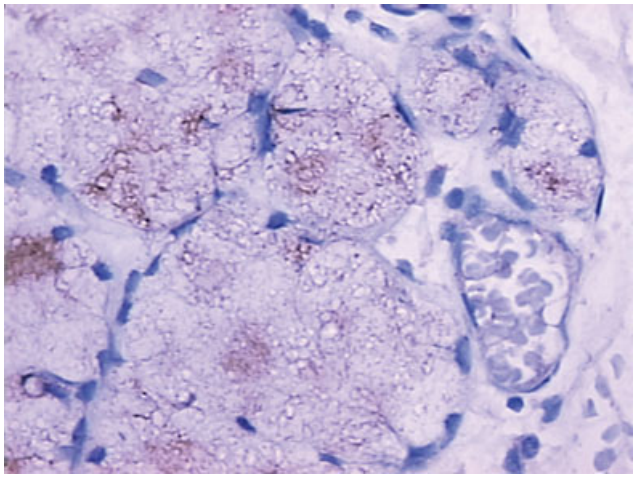

Figure 1 Negative expression of inducible nitric oxide synthase in normal salivary gland tissue 200×

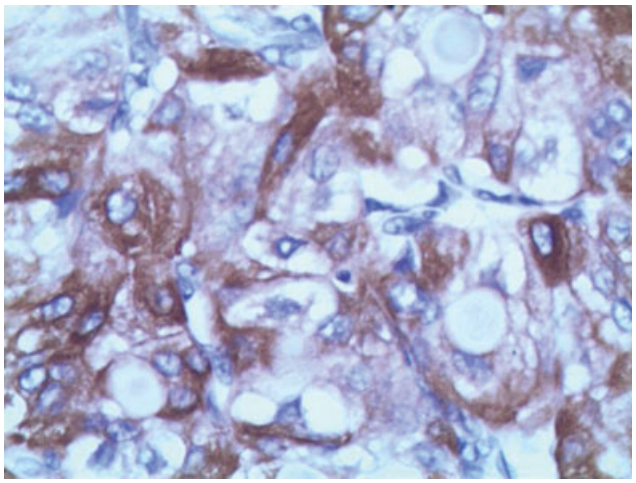

Figure 3 Positive expression of inducible nitric oxide synthase in poorly differentiated mucoepidermoid carcinoma 200×

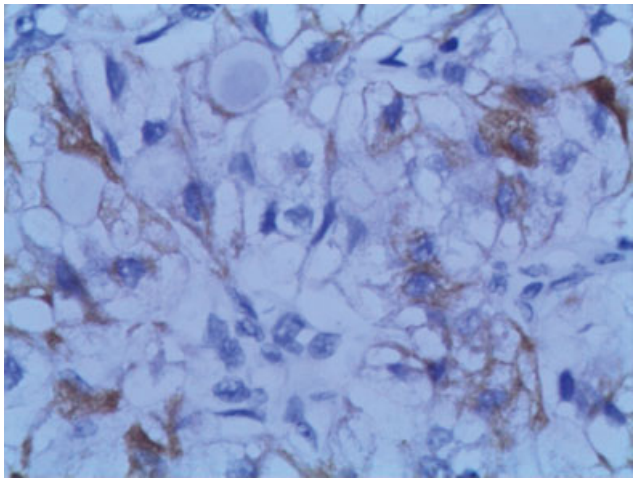

Figure 2 Positive expression of inducible nitric oxide synthase in well-differentiated mucoepidermoid carcinoma 200×

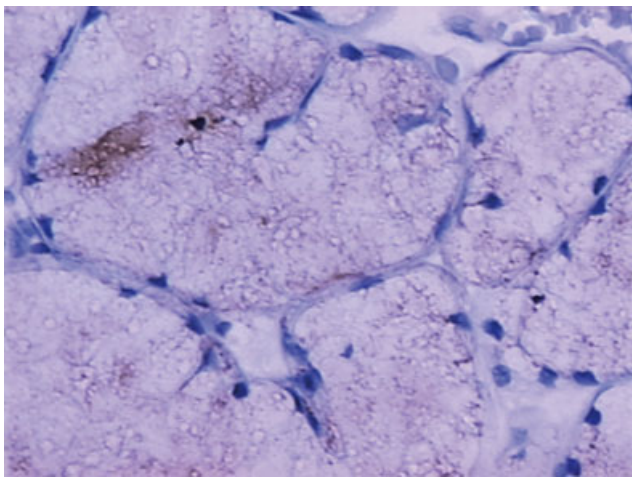

Figure 4 Negative expression of vascular endothelial growth factor in normal salivary gland tissue 200×

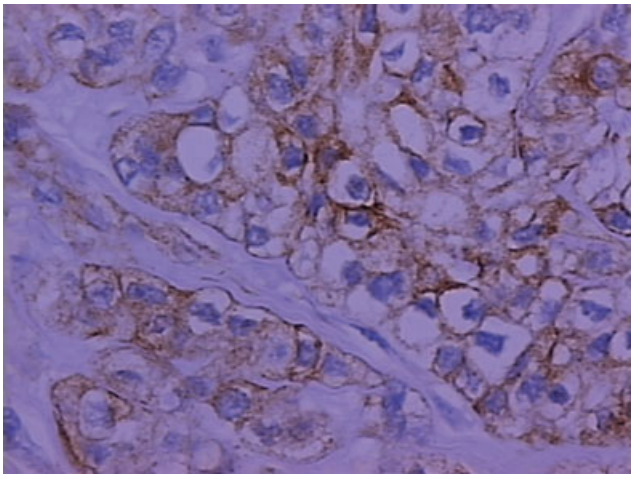

Figure 5 Positive expression of vascular endothelial growth factor in well-differentiated mucoepidermoid carcinoma 200×

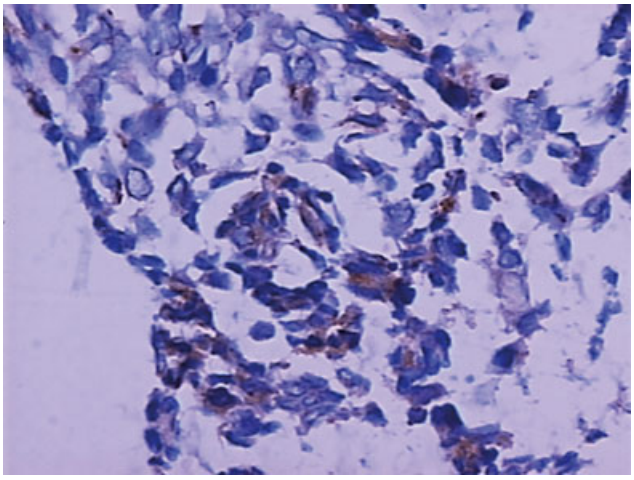

Figure 6 Positive expression of vascular endothelial growth factor in poorly differentiated mucoepidermoid carcinoma 200×

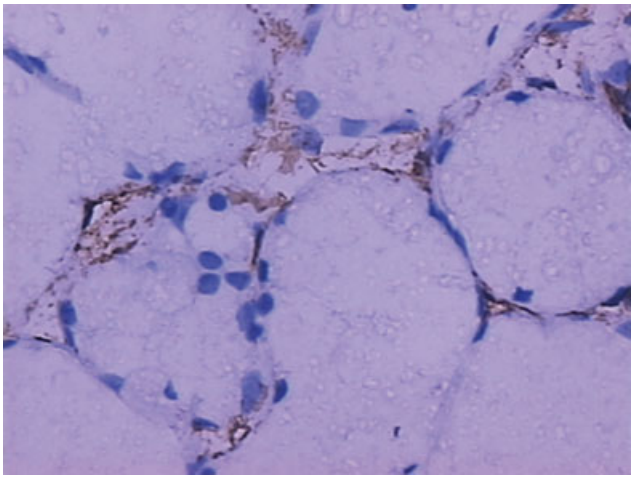

Figure 7 Expression of CD34 in normal salivary gland tissue 200×

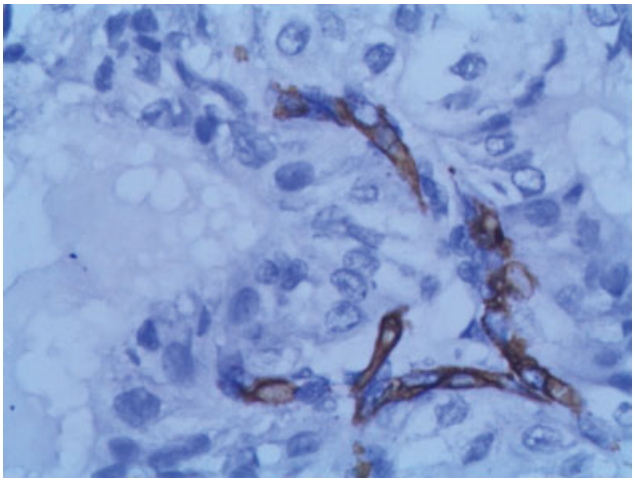

Figure 8 Expression of CD34 in well-differentiated mucoepidermoid carcinoma 200×

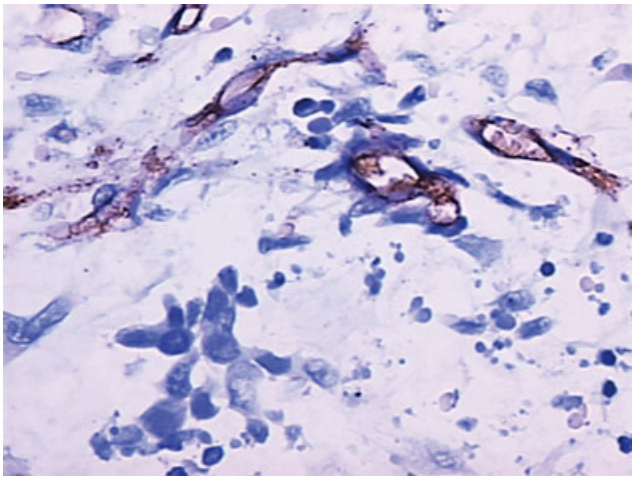

Figure 9 Expression of CD34 in poorly differentiated mucoepidermoid carcinoma 200×

endothelial cell proliferation, lumen formation, and new basement membrane and anastomosis formation(Bussolino *et al*, 1997).Research has shown that there is an interaction between NO and VEGF and that they collaboratively promote angiogenesis. NO is involved in every step of VEGF angiogenesis promotion (Ziche *et al*, 1997).The present research aimed to study the expression of iNOS and VEGF and their relationships with malignancy of MEC and patient prognosis. Increased iNOS and VEGF expression is significantly associated with the degree of tumor differentiation, size, and relapse, suggesting that examination of the expression status of these two proteins in MEC may help to assess the tumor-related risk to patients and guide further therapy.

Vascular endothelial growth factor and its receptor are critical factors in angiogenesis. Their overexpression is closely related to tumoral growth, invasion, and metastasis (Lee *et al*, 2000). VEGF promotes tumor growth by means of two mechanisms. The first is by

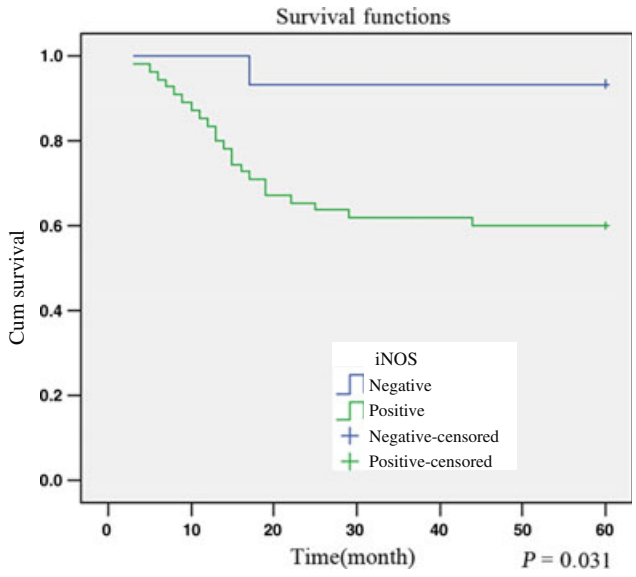

Figure 10 The relationship between inducible nitric oxide synthase expression and patient's outcome

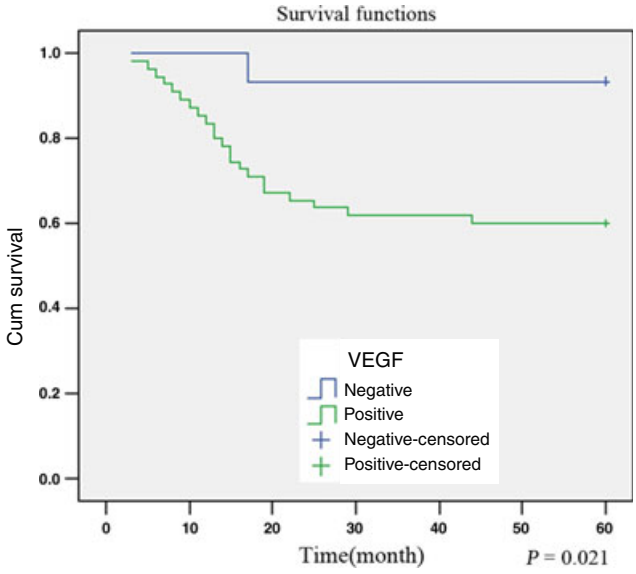

Figure 11 The relationship between vascular endothelial growth factor expression and patient's outcome

increasing the permeability of blood vessels and causing them to leak serum proteins (mainly fibrinogen) to provide suitable substrates for tumoral cell growth and new capillary formation. The second is by acting on its receptor and expressing its endothelial cell-specific mitogenic activity. VEGF stimulates the proliferation of vascular endothelial cells and induces the formation of blood vessels, further promoting the formation of capillary networks and providing adequate nutrients for tumoral growth. The prognostic significance of MVD correlated with increased VEGF levels(Ria *et al*, 2003; Ribas *et al*, 2004) is still questionable(Kroll and Waltenberger, 1998; Rajkumar and Kyle, 2001; Nieves *et al*, 2009). Our results clearly indicate a difference in the mutual induction of VEGF and iNOS directly correlate the individual parameters of tumor cell immunoreactivity with tumor tissue MVD, which demonstrates the difference in the MVD of MEC as compared with NSG.

There is a correlation between iNOS and VEGF related to tumor microvascular density (Franchi *et al*, 2006; Vakkala *et al*, 2006). NO plays an important role in the VEGF-induced proliferation and migration of endothelial cells and involved in VEGF-stimulated vascular permeability. iNOS-produced NO increases VEGF expression and synthesis mainly by increasing the activity of the promoter for the VEGF gene (Kimura *et al*, 2000). In addition, NO plays an important role at all steps of VEGF-induced blood vessel formation (Garcia-Cardena and Folkman, 1998) by participating (with VEGF) in increasing blood vessel permeability, promoting endothelial migration, and at the same time, inducing the mitogenic function of VEGF on vascular endothelial cells. Current research has shown a close relationship between iNOS and VEGF expression, and both iNOS and VEGF are closely related to tumor angiogenesis. Yin *et al*, 2005 studied colorectal cancer using immunohistochemistry and found that the expressions of iNOS and VEGF were significantly higher in colorectal cancer than in the control group. There is an intimate relationship between iNOS and VEGF. The expression of Inos and VEGF is related to microvascular density. Zhang *et al*, 2005 used immunohistochemical studies on tissues obtained from 80 patients with adenoid cystic carcinoma and salivary gland tissues obtained from 20 normal individuals. They found a significant correlation between microvascular density and the expression of iNOS and VEGF, and the expression of iNOS and VEGF was higher *in vivo* than *in vitro*.

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Author contributions

Ou Yang Ke-xiong contribute to statistic.; Liang Jun contribute to article design; Huang Zhi-quan contribute to draft and image.

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