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Original Article

Aberrant expression of MYH9 and E-cadherin in esophageal squamous cell carcinoma and their relationship to vasculogenic mimicry

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Abstract: Purpose: To investigate whether vasculogenic mimicry (VM) exists in esophageal squamous cell carcinoma (ESCC) and to elucidate the relationship among expression of MYH9, E-cadherin and VM. Methods: The expression of MYH9 (non-muscle myosin heavy chain 9), E-cadherin protein and VM in 120 specimens of esophageal squamous cell carcinoma (ESCC) and 120 specimens of normal esophageal mucosa were detected by using immunohistochemical and histochemical staining. Results: VM channels were identified in 58 (48.33%) of the 120 ESCC specimens and none of the normal esophageal mucosa was found to have VM. The rates of expression of MYH9 and E-cad in ESCC were 57.50% and 40.00%, while rates in the control group were 13.33% and 73.33%, respectively ($P<0.05$). VM and the expression levels of MYH9 and E-cad were significantly connected with lymph node metastasis, serosa invasion, pTNM staging and 5-year-survival rates of patients with ESCC ($P<0.05$). VM was positively correlated with MYH9, but negatively correlated with E-cad, and MYH9 was negatively significantly correlated with E-cad. The 5-year-survival rates of patients with ESCC were 6.89% (4/58) in the VM group and 67.74% (42/62) in the non-VM group, 8.00% (4/50) in high MYH9 expression group and 60.00% (42/70) in low MYH9 expression group. However, the 5-year-survival rate in high E-cad expression group was 86.95% (40/46) and that in low E-cad expression group was 8.11% (6/74) ($P<0.05$). Cox multifactorial regression analysis demonstrated that lymph node metastasis, pTNM stage, VM and expression levels of MYH9 and E-cad were independent risk factors in patients with ESCC ($P<0.05$). Conclusion: ESCC'patients with VM had a poor differentiation and a bad clinical prognosis; Combined detection of VM, MYH9 and E-cad may play an essential role in predicting the invasion, metastasis, and progression of patients with ESCC.

Keywords: Esophageal squamous cell carcinoma, VM, MYH9, E-cad, immunohistochemistry, prognosis

Introduction

Abnormal expression of genes is one of the important reasons for the development of esophageal cancer. Esophageal cancer (ES) including squamous cell carcinoma (SCC) and adenocarcinoma, is a serious malignancy with respect to prognosis and a fatal in most cases [1, 2]. Esophageal carcinoma affects more than 450,000 people worldwide and the incidence is rapidly increasing [3]. Currently, ESCC is the eighth most common incident cancer in the world because of its extreme aggressive ability and poor survival rate [4, 5]. MYH9 is a type II myosin, which is a component of the cytoskeleton that can be assembled into a bipolar polarity myosin filament after activation in the cells [6]. Its spherical head Mg^{2+}-ATPase can hydrolyze ATPase energy and move myofilament protein, which produces contractile force to mediate cell migration and division [7]. The occurrence of epithelial-mesenchymal transition is an important molecular mechanism for distant metastasis of tumors [8]. Cadherin is a transmembrane glycoprotein family that mediates adhesion between cells, cells and matrix, maintaining normal epithelial cell morphology, cell polarity, and tissue structural integrity [9-12].

Vasculogenic mimicry (VM) is a special blood supply channel found in malignant tumors in recent years [13]. It is a blood vessel-like tube surrounded by tumor cells in order to satisfy

their blood supply and remodeled by their own phenotype and extracellular matrix without the involvement of endothelial cells. It is a microcirculation pattern independent of vascular endothelial cells. Tumor tissue can be connected to the host vessel through VM, a new blood flow pathway to obtain nutrients, making the tumor quick to invade and metastasize, and at the same time, increasing the degree of malignancy of the tumor tissue [14-16]. There is adhesion between cancer cells, and between cancer cells and matrix. E-cadherin plays a key role in the formation of VM structure and is a key type of cellular adhesion molecule (CAMs). The expression of E-cad is directly related to the biologic behavior of the tumor cells [17, 18]. E-cad low expression level generally predicts lower intercellular adhesion and makes tumor cells more likely to invade and metastasize [19].

Overall, studies of VM, MYH9, or E-cad showed that these biomarkers might have great influence on the development of tumor. However, the associations among VM, MYH9. and E-cad in ESCC have not been widely reported. Hence we explored the correlation between their expression and clinicopathologic characteristics.

Materials and methods

Patients and specimens

We collected surgical resection of ESCC samples from patients who received surgery at the First Affiliated Hospital of Bengbu Medical College (Anhui Province, China) from January 2008 to December 2008. We collected 120 patients' characteristics after pathologic diagnosis as ESCC, including age, sex, tumor size, differentiation grade, location, lymphatic invasion, serosa invasion, gross type and pTNM (**Table 1**). These 120 patients were listed as the experimental group, and we set a control group as 120 normal esophageal mucosal tissues. All paraffin-embedded tissues received immunohistochemical staining. Informed consents were obtained from all patients and the study had been approved by the Ethical Committee of Bengbu Medical College. We excluded patients who had received preoperative chemotherapy or radiotherapy. Relevant clinical data were obtained by retrospective review of the patients' medical records. The overall survival time (OS) was defined as the time from the date of surgery to the date of death caused by ESCC or the last follow-up date. The follow-up time was 5-103 months until December 2008.

Reagents

Rabbit monoclonal antibodies against human MYH9 were bought from Abcam, USA and CD34 was bought from Lab Vision Company (USA). Elivison™ plus immunohistochemical kit and diaminobenzedine (DAB) chromogenic kit were purchased from Fuzhou Maixin Biology Technology Company (China). The Periodic Acid Schiff (PAS) reagents were prepared in Department of Clinicopathology in the First Affiliated Hospital of Bengbu Medical College.

Immunostaining and CD34+PAS double staining

All paraffin-embedded samples were fixed in 10% buffered formalin. All slides (4-μm thick) were de-paraffined, dehydrated with graded alcohol, and then washed in Phosphate-buffered Saline (PBS, PH 7.2) for 10 min. The endogenous peroxidase activity was blocked by incubation in methanol containing 3% H_2O_2 for 10 min at room temperature, then samples were boiled in 1.0% citrate (pH 6.0) for 2 minutes at high pressure to repair antigens. Slides were rinsed in PBS. The slides were blocked with goat serum for 20 min at room temperature, and incubated with rabbit monoclonal MYH9 (dilution: 1:500, ab138498, Abcam, USA), E-cad (dilution: 1:200, ab1416, Abcam, USA), and CD34 (dilution: 1:200, ab762, Abcam, USA) primary antibodies at 4°C overnight. Slides were treated with polymer enhancer for 30 minutes, then washed with PBS and treated with goat anti-mouse antibody for 30 minutes. All samples with CD34 primary antibodies were subjected to PAS staining to characterize the glycosylated basement membranes of endothelial cells of vessels as well as vessel-like (VM) channels [13]. Completely washed by PBS, the slides were colored in freshly-prepared 3,3'-diaminobenzidine (DAB) solution for 5 minutes, and then counter-stained with hematoxylin, dehydrated, air-dried and mounted.

Immunostaining evaluation

Each slice was reviewed by two independent experienced pathologists to evaluate the staining result of the protein. Scores were determined by combining the proportion of positively stained tumor cells and the intensity of stain-

Table 1. Association of VM, MYH9, and E-cad with clinicopathologic characteristics of patients with ESCC (n = 120)

Variable	VM		X^2	P	MYH9		X^2	P	E-cad		X^2	P
	N	P			N	P			N	P		
Tissue												
Con	120	0			96	24			32	88		
ESCC	62	58			51	69			72	48		
Gender			0.461	0.311			0.413	0.538			0.143	0.426
Male	38	32			30	40			41	29		
Female	24	26			21	29			31	19		
Age			1.334	0.166			0.112	0.44			0.204	0.396
<60	30	22			23	29			30	22		
≥60	32	36			28	40			42	26		
Anatomical location			0.677	0.42			1.206	0.812			0.404	0.584
Upper	2	3			3	2			3	2		
Middle	37	37			29	45			46	28		
Lower	23	18			19	22			23	18		
Gross type			0.367	0.596			3.621	0.318			3.345	0.283
Medullary	21	18			21	18			19	20		
Mushroom	30	28			20	38			39	19		
Ulcer type	9	9			8	10			11	7		
Sclerotic type	2	3	0.394	0.572	2	3	0.907	0.356	3	2	0.556	0.288
Diameter (cm)												
<5	31	29			27	33			31	20		
≥5	31	29			24	36			43	26		
Differentiation			1.714	0.315			1.384	0.301			0.706	0.410
Well	19	20			15	24			25	14		
Mediate	19	22			16	25			25	16		
Poor	24	24			20	20			22	18		
LNM			76.885	<0.001			75.328	<0.001			61.25	<0.001
Yes	7	53			2	58			57	3		
No	55	5			49	11			15	45		
Serosal invasion			59.118	<0.001			62.244	<0.001			54.511	<0.001
Yes	11	51			5	57			57	5		
No	51	7			46	12			15	43		
PTNM stage			35.319	<0.001			46.488	<0.001			48.027	<0.001
I-II	39	6			37	8			9	36		
III-IV	23	52			14	61			63	12		

ing. Tumor cell proportions were marked as follows: 0, no positive cells; 1, <11% positive cells; 2, 11%-50% positive cells; 3, 51%-75% positive cells and 4, >75% positive cells. Staining intensity was scored on a 4-point scale: 0, negative (no staining); 1, weak (light yellow); 2, moderate (yellow brown) and 3, strong (brown). The final score was determined by multiplying the intensity and the proportions of positivity scores, which ranged from 0 to 12. A score ≥3 was identified as positive expression.

Statistical analysis

Chi-square test, univariate analysis, and multivariate analysis were conducted to statistically evaluate the clinical significance of VM, MYH9, and E-cad in ESCC. The SPSS software package (version 24.0, IBM, USA) was used for the statistical analysis. The association between proteins and VM was certified by using Spearman correlation analysis. The association between the above factors and overall survival time of

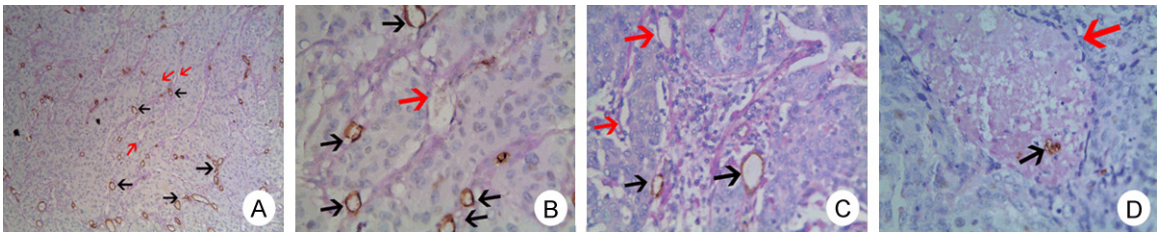

Figure 1. Positive staining of VM and microvessels in ESCC by CD34+PAS double staining. A: Positive staining of VM and microvessels in ESCC (100 magnification); B-D: Positive staining of VM and microvessels in ESCC (400 magnification). The red arrows represent VM structure and the black arrows microvessels.

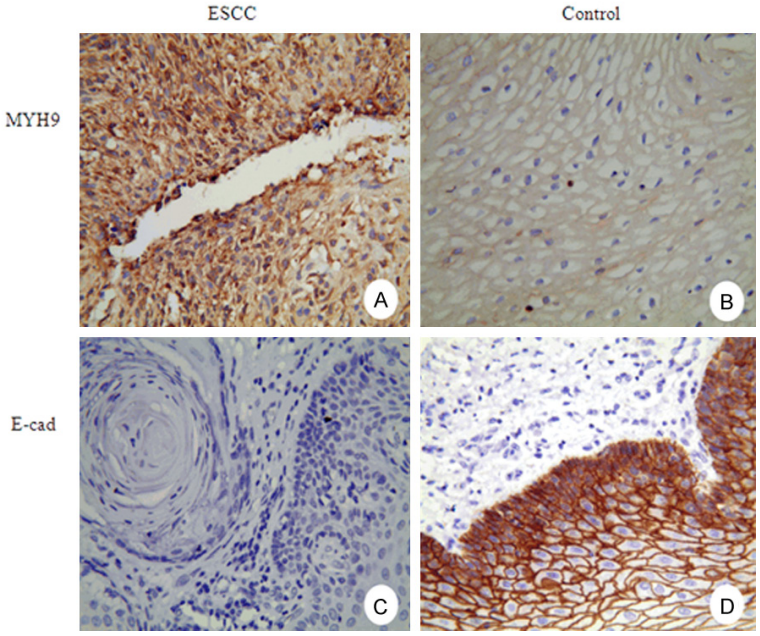

Figure 2. Immunostaining of MYH9 and E-cadherin in ESCC and the control tissues (400 magnification). A: Positive staining of MYH9 in membrane and cytoplasm of tumor cells. B: Negative staining of MYH9 in the control tissues. C: Negative staining of E-cadherin in membrane and cytoplasm of tumor cells. D: Positive staining of E-cadherin in the control tissues.

patients with ESCC was assessed by Cox multifactor regression analysis and Kaplan-Meier survival analysis by employing log-rank test. All the analyzes were performed with SPSS 22.0 software. A value of $P<0.05$ was set assignificant.

Results

VM in ESCC and control tissues

CD34 and PAS double staining showed that, in ESCC specimens, small vessel-like structures were surrounded by cancer cells (**Figure 1**). There were red blood cells in those channels and fewer necrotic tumor cells or inflammatory cells were found in the areas adjacent to the channels. Morphologically, the VM channels, positive for PAS but negative for CD34, took different forms, ranging from linear, tubular or branched patterns [5]. VM channels were identified in 58 (48.33%) of the 120 ESCC specimens. None of the normal esophageal mucosa was found to have VM.

The association between VM and clinicopathological factors in ESCC

In ESCC specimens, the positive rate of VM was found to be closely associated with lymph node metastasis, serosal invasion and pTNM stage ($P<0.05$). The positive rate of VM in the lymph node metastasis group was 88.33% but 8.33% in the non-lymph-node metastasis group. The rate was 82.26% in serosal invasion group but 12.07% in non-serosal invasion group. The rate was 13.33% at pTNM stage I-II and was 69.33% at stage III-IV, respectively ($P<0.05$). No significant associations were found between the positive rate of VM and gender, age, gross morphology, histologic type, diameter and differentiation of tumors (**Table 1**).

The association between MYH9 protein and clinicopathologic factors

High MYH9 protein expression was observed in 57.5% of ESCC specimens and 13.33% in controls, respectively ($P<0.05$) (**Table 1**). The positive staining for MYH9 protein was mainly found in the membrane and cytoplasm and fewer positive particles were seen in nuclei in both ESCC tumor cells and control cells (**Figures 2**, S1). The level of MYH9 expression was closely

Table 2. The correlation of MYH9, E-cad, and VM in ESCC (n = 120)

Variable	MYH9		r	P	E-cad		r	P
	Negative	Positive			Negative	Positive		
VM			0.798	<0.001			-0.633	<0.001
Negative	50	12			15	47		
Positive	1	57			58	2		
MYH9							-0.546	<0.001
Negative	-	-			7	44		
Positive	-	-			66	3		

associated with lymph node metastasis, serosal invasion and pTNM stage (P<0.05 for all). High MYH9 expression was found in 96.67% of the specimens in the lymph-node metastasis group and 18.33% in the non-metastasis group. The high expression rate was 91.93% in serosa invasion group and 20.69% in non-invasion group. The rate of high MYH9 expression was 17.78% in the stage I-II group and 81.33% in stage III-IV group, respectively (P<0.05). No significance was found between MYH9 expression and gender, age, gross morphology, histologic type, diameter and differentiation of tumors (**Table 1**).

The association between E-cad protein and clinicopathological factors

High E-cad protein expression was observed in 40.00% of ESCC specimens and in 73.33% of controls, respectively (P<0.05, **Table 1**). The staining for E-cad was mainly found in the membrane and cytoplasm in both ESCC tumor cells and control cells (**Figure 2**). The level of E-cad expression was closely associated with lymph node metastasis, serosal invasion and pTNM stage (P<0.05). High E-cad protein was found in 5.00% of the specimens in lymph node metastasis group and 75.00% in the non-lymph-node metastasis group. The high expression rate was 8.06% in serosa invasion group and 68.97% in non-invasion group. The rate of high E-cad protein expression was 8.00% in the pTNM stage I-II group and 16.00% in the stage III-IV group, respectively (P<0.05, for all). No significance was found between E-cad protein expression and gender, age, gross type, histologic type, diameter and differentiation of tumors (**Table 1**).

The correlation among VM, MYH9, and E-cad in ESCC

Spearman correlation analysis revealed a significant positive association between VM and MYH9 in ESCC specimens (r = 0.798, P<0.001). A significant negative association was found between E-cad and VM (r = -0.633). Similar association was seen between E-cad and MYH9 (r = -0.546, P<0.001). All these results are listed in **Table 2**.

Prognosis analysis by Kaplan-Meier method

Overall survival (OS) time was defined as the period from the first time when the surgery was operated to death of the ESCC patients or last follow-up. Kaplan-Meier survival analysis exhibited that the median OS (31.668) and overall 5-year-survival rate [5.17% (3/58)] in the VM-positive group were significantly lower than the OS (61.200) and the rate [64.51% (40/62)] in the VM-negative group (Log-rank = 51.733, P<0.001). The median OS (31.644) and overall 5-year-survival rate [6.00% (3/50)] in the high MYH9 expression group were significantly lower than OS (67.624) and the rate [57.97% (40/69)] in the low MYH9 expression group (Log-rank = 69.208, P<0.001). On the other hand, an opposite trend in the median OS and overall 5-year-survival rate was observed in both high and low E-cad protein expression groups. In the high E-cad protein expression group, the OS and rate were 67.051 and 83.33% (40/48), respectively; in the low E-cad protein expression group they were 33.122 and 4.17% (3/72), respectively (Log-rank = 55.712, P<0.001) (**Figure 3**).

Univariate and multivariate analyzes

Cox multifactor regression analysis showed that, among VM, MYH9, and E-cad proteins and the clinicopathological factors, MYH9 and E-cad expression levels, VM, lymph node metastasis and pTNM stage were independent prognostic factors of ESCC (P<0.05, **Table 3**).

Discussion

Tumor metastasis plays an extremely important role in decreasing the survival rate of patients with ESCC. Esophageal cancer is one of the most common malignant tumors and the main cause of death is infiltration and metastasis. The current study has reported that the non-muscle myosin heavy chain IIA (MYH9) can be involved in the development of tumors as a

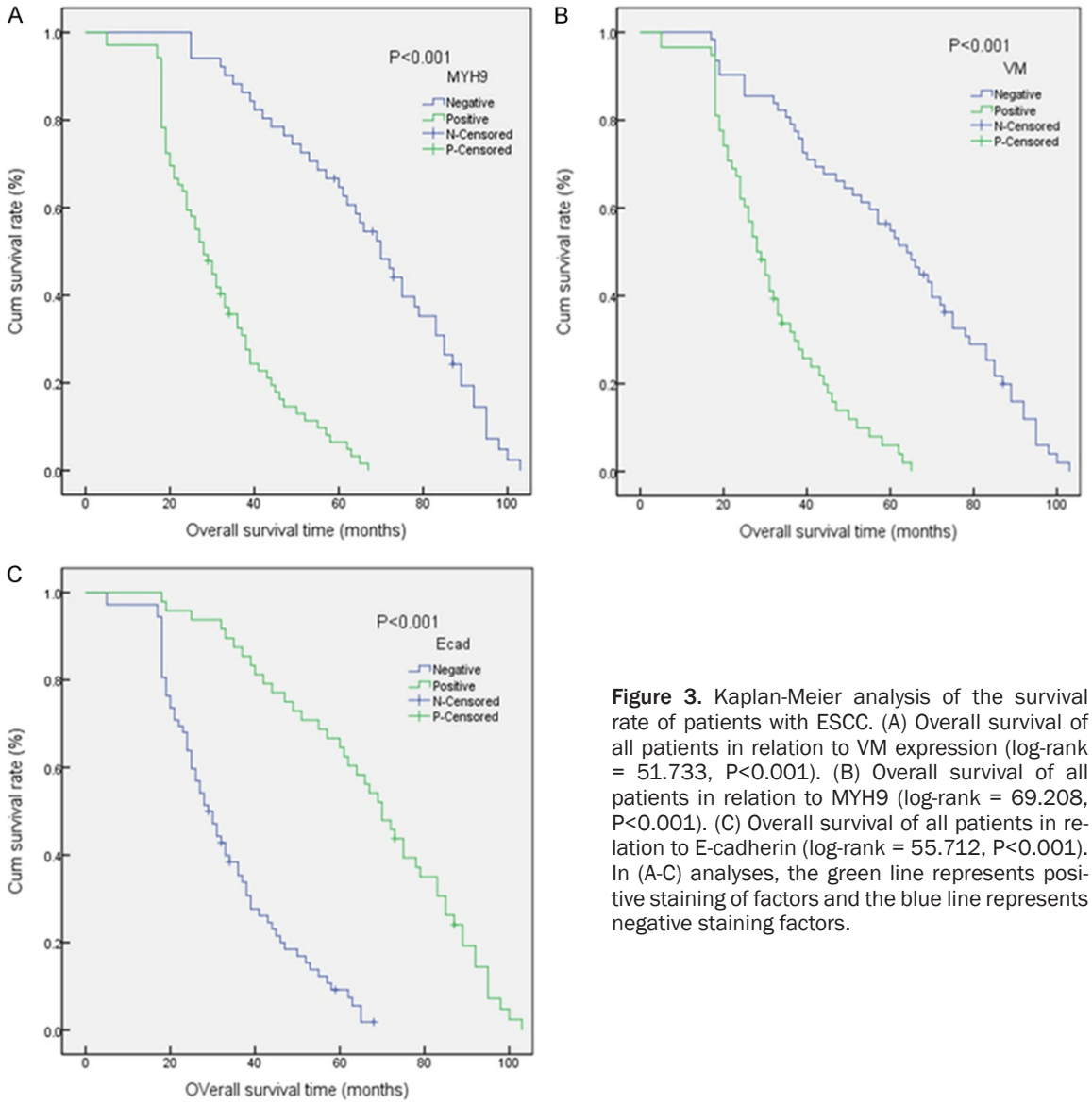

Figure 3. Kaplan-Meier analysis of the survival rate of patients with ESCC. (A) Overall survival of all patients in relation to VM expression (log-rank = 51.733, P<0.001). (B) Overall survival of all patients in relation to MYH9 (log-rank = 69.208, P<0.001). (C) Overall survival of all patients in relation to E-cadherin (log-rank = 55.712, P<0.001). In (A-C) analyses, the green line represents positive staining of factors and the blue line represents negative staining factors.

proto-oncogene in lung cancer, breast cancer, liver cancer, and gastric cancer [20-22]. MYH9 gene is localized in human chromosome 22q13.1 that encodes a 227 KD protein [23]. MYH9 a plays an important role in the cytoskeleton as shown in the experiment of DAMMWELK [24]. MYH9 protein has many important physiologic functions, not only participating in the formation of a cytoplasmic contraction ring but also modulating invasion and metastasis of cancer cells [25-29]. In this study, we found that MYH9 expression was high in 57.50% of ESCC subjects and 13.33% of controls, respectively. Furthermore, the high MYH9 expression was associated with lymph node metastasis, serosal invasion, pTNM stage, and OS in ESCC patients. MYH9 expression was significantly higher in the lymph node metastasis/serosal invasion groups than in non-lymph node metastasis/serosal invasion groups. Patients with high MYH9 expression tended to be at higher pTNM stage and had lower 5-year-survival rates than those with low MYH9 expression.

Invasion and metastasis of human cancers are affected by a variety of factors, including E-cadherin which is a 120 KD a transmembrane glycoprotein belonging to the super-family of Ca^{2+}-dependent cell adhesion molecules. E-cad has been considered as a tumor suppressor, and it is weakly expressed in many cancers, including lung, gastric, breast and bladder cancers [30-

Table 3. Multivariate survival analysis of patients with ESCC (n = 120)

Variable	B	SE	Wald	df	Sig.	Exp (B)	95.0% CI	
							Down	Up
Gender	0.038	0.215	0.032	1	0.859	1.039	0.682	1.584
Age	-0.163	0.222	0.539	1	0.463	0.850	0.550	1.313
Site	0.068	0.191	0.126	1	0.722	1.070	0.736	1.557
Gross morphology	0.115	0.133	0.742	1	0.389	1.122	0.864	1.457
Size	-0.166	0.230	0.518	1	0.472	0.847	0.539	1.331
Differentiation	-0.057	0.140	0.168	1	0.682	0.944	0.718	1.242
LNM	0.212	0.657	0.104	1	0.047	1.236	0.341	4.480
Serosal invasion	-0.377	0.537	0.494	1	0.082	0.686	0.239	1.964
PTNM	-0.032	0.320	0.010	1	0.020	0.968	0.517	1.813
VM	1.777	0.513	12.026	1	0.001	5.914	2.166	16.149
MYH9	1.222	0.501	5.944	1	0.015	3.392	1.271	9.057
E-cad	-1.708	0.383	19.870	1	0.024	0.469	0.156	1.913

LNM: lymph node metastasis; ESCC: esophageal squamous cell carcinoma.

34]. Our study showed that E-cad expression was very low-expressed in 40.00% of ESCC patients as compared with the controls (73.33%) ($P<0.05$). E-cad expression was negatively correlated with lymph node metastasis, likelihood of serosal invasion, pTNM stage, and OS of patients with ESCC. That is to say, ESCC patients with high E-cad expression for the most part were at lower pTNM stage, indicating no lymph-node metastasis/serosal invasion and better prognosis. These results indicated that the low-expressed E-cad may play an indispensable role in the invasion, metastasis, and progression of ESCC. These results were consistent with the results of other studies [30, 35, 36]. E-cad inhibits the invasion and metastasis of tumor cells by forming an E-cad/β-catenin complex and binding with β-catenin competitively and then reduces the intracellular episomal β-catenin level to block the Wnt-β-catenin signaling pathway, which is activated in carcinogenesis [37, 38]. Abnormality in either E-cad or its other components in the E-cad/β-catenin complex will result in diffusion, invasion, and metastasis of tumor cells by weakening the adhesion between tumor cells and enhancing locomotivity of tumor cells [39].

Tumor growth and metastasis are closely related to the formation of new blood vessels. Previously, endothelial-dependent blood vessels were considered as the source of blood circulation for tumor cells. Since Maniotis and his team-workers have described the concept of VM in their study about highly aggressive uveal melanomas in 1999 [40], VM has been discovered in a variety of malignancies, such as breast cancer, liver cancer, glioma, ovarian cancer, non-small cell lung cancer (NSCLC) and prostate cancer [41-45]. VM channels refers to vascular channel-like structures formed by tumor cells instead of endothelial cells for delivering blood and nutrients. In this study, upon PAS and CD34 double staining, VM channels have straight, branched or curved forms, with closed or open loops and network patterns and were PAS-positive but CD34-negative [40]. Zhang et al proposed a "three-stage" theory, which divides tumor vascularization into three stages: VM channels, mosaic blood vessels, and endothelium-dependent blood vessels [46, 47]. The molecular mechanism of VM is not well understood, and therapeutic strategies targeting endothelial cells have no effect on tumor cells with VM channels. Negative associations were observed among E-cad, MYH9, and VM suggesting that reduced adhesion and anchorage of tumor cells, together with elevated MYH9 expression, may deform tumor cells into non-epithelial-dependent blood vessels and thus result in the formation of VM channels in ESCC [40]. Multivariate analysis showed that lymph node metastasis, serosal invasion, pTNM stage, VM, MYH9, and E-cad were independent prognostic factors in ESCC. Lymph node metastasis, serosal invasion, and pTNM stage have been generally believed to be prognosis-related events in clinical practice, but they are not specific or sensitive enough as accurate prognostic indicators for ESCC. Hence it is of importance to find novel molecular markers for biologic behavior and indicators for the prognosis of ESCC.

In summary, as a type II myosin, MYH9 is an important component of cytoskeletal proteins which can be assembled into a bipolar polarity myosin filament after activation in the cell, and it has many important physiologic functions such as participating in the composition of the cytoplasm and promoting cell migration [48]. MYH9 can also be involved in tumorigenesis

and development by affecting cell cycle apoptosis and migration. The study found that MYH9 protein was highly expressed in cancer tissues and correlated positively with its pathologic features. MYH9 can regulate the proliferation and migration of esophageal cancer cells, epithelial-mesenchymal transition (EMT) process, and biologic behavior of tumor stem cells [49]. EMT is an important biologic process that influences tumor metastasis by regulating the expression of transcription factors, such as E-cad, ZO-1, and vimentin. These conclusions are in line with previous studies [49-51]. It was indicated that EMT also played an important role during the development of ESCC [51]. The low expression of E-cad protein not only suggests the intercellular adhesion is decreased, but also predicts the cytoskeleton has the ability to form an intercellular space which is owing to lack of adhesion formed by adhesion between adjacent cells. Simultaneously all these factors promote the formation of VM [52]. Hence EMT plays an indispensable role in the formation process of VM structures [52]. Our results implied that MYH9 affects ESCC evolution and combined detection of VM, MYH9, and E-cad are of importance for metastasis and prognosis in ESCC. However, what causes the low-expression of E-cad and the high-expression of MYH9 in ESCC? Is the decline in adhesion the "cause" or "result" of VM structure? The process of VM involves the biologic activity of the tumor cells themselves, and is it an active deformation ability or a passive adaptive ability? Further research aims at taking on tumor growth metastasis and various influencing factors. In addition, mimicking blood vessels have blood supply functions, which are inevitably linked to endothelial blood vessels. What is the accurate structure connecting the VM with blood vessels? Is VM present in all poorly differentiated, highly aggressive tumors? In order to improve the treatment effect of esophageal cancer patients, exploring the relationship between VM and clinicopathologic features of ESCC plays an important role in understanding the treatment of esophageal cancer patients. More research is needed on the mechanism of VM.

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Disclosure of conflict of interest

None.

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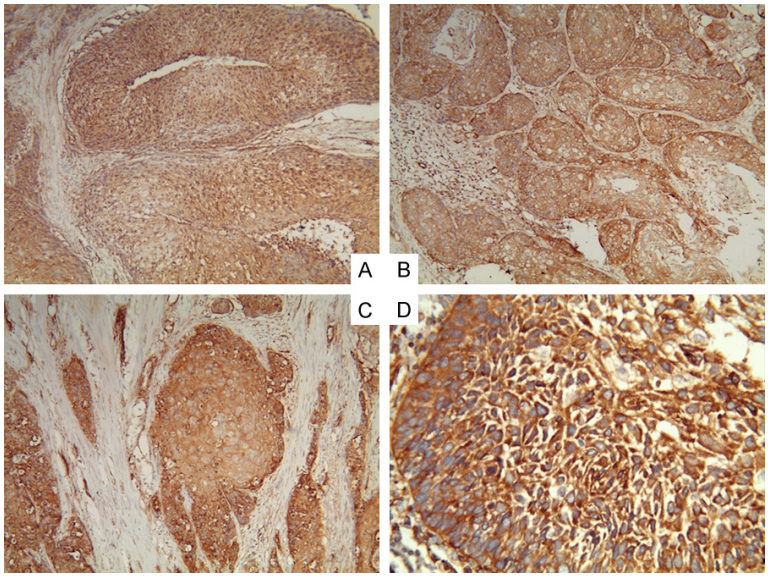

Figure S1. Positive staining of MYH9 in membrane and cytoplasm of tumor cells.