Correlation of SOX4, SOX17, VE-cadherin and vasculogenic mimicry with prognosis in esophageal squamous cell carcinoma

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Abstract

Background: This study to explore the relationships between SOX4, SOX17, vascular endothelial (VE)-cadherin and VM with the occurrence and development of ESCC.

Methods: SOX4, SOX17, and VE-cadherin expression as well as VM in tissues were determined by immunohistochemistry (IHC). The cell invasion, migration, and proliferation were determined after silencing of VE-cadherin. SOX4, SOX17, and VE-cadherin protein and mRNA expression were quantified by Western blotting and qRT-PCR analyses, respectively.

Results: The expression of SOX17, SOX4, and VE-cadherin were significantly correlated with clinical characteristics of ESCC. After VE-cadherin silencing, the cell invasion, migration, and proliferation were decreased, while SOX17 levels were increased and SOX4 levels were decreased.

Conclusions: SOX17, SOX4, and VE-cadherin are involved in the development of ESCC.

Background

The incidence of esophageal cancer (EC) has been steadily increasing year after year, making EC the sixth most common cause of cancer-related death in the world [1]. The incidence and mortality of EC in China continue to remain high [2] with 90% of cases being esophageal squamous cell carcinoma (ESCC) [3]. Despite being a common cancer, the process of tumorigenesis in EC remains unclear.

The growth of a solid tumor depends upon its vascularity. Anti-angiogenic therapies are designed to target vascular endothelial cells and prevent formation of tumor blood vessels [4]. Vasculogenic mimicry (VM) is a recently identified tumor micro-circulation model that is independent of the organism's endothelial cells, and its growth model is completely different from the classical tumor vascular growth model [5]. Vascular endothelial cadherin (VE-cadherin) is a specific transmembrane adhesion protein found on the surface of vascular endothelial cells. It maintains the integrity of vessels and promotes adhesion between adjacent endothelial cells [6,7]. Recent studies have shown that overexpression of VE-cadherin may be an important regulatory mechanism for VM [8,9].

In the early 1990s, the discovery of the sex-determining region on Y (SRY) led to the identification of the SRY-related box (SOX) transcription factors [10,11]. These factors often have pleiotropic functions that can lead to the activation of alternate transcriptional programs [12-14]. SOX17, first cloned from cDNA libraries of mouse testicular tissue, was found to have a stage-specific function in spermatogenesis [15]. Later, SOX17 was found to have anti-proliferative effects in endometrial cancer by suppressing the transcription of Notch effector MAML3, a co-activator of β-catenin [16]. In a colonic carcinoma model, SOX17 antagonizes β-catenin signaling by redirecting β-catenin away from WNT target genes and by depleting its protein levels via the GSK3-independent promotion of its proteasomal degradation [17]. On structure function analysis, SOX17 was found to be inactivated in colon cancer [18], lung cancer [19], and hepatocellular carcinoma [20].

SOX4 is a member of the SOX family that contains a highly conservative and migratory DNA-binding domain. The translated proteins are 47-kDa transcription factors involved in development [20]. Previous studies have found that SOX4 is one of the 64 "tumor characteristic" genes, and its expression is up-regulated in a variety of tumor tissues, such as bladder cancer, liver cancer, prostate cancer, and acute myelogenous leukemia [21, 22]. Over-expression of SOX4 promotes the invasion and migration of cancer cells, and SOX4 binds to the p53 promoter to inhibit the pro-apoptotic effect of p53, thereby reducing the therapeutic effect of radiotherapy [23].

We previously reported high expression of SOX4 in ESCC and the involvement of VE-cadherin in VM formation. SOX4 over-expression promotes the development of epithelial–mesenchymal transition (EMT) which allows the tumor cells to remodel. Also, the tumor cells with VM structure were found to be separated from the lumen by only one layer of PAS-positive substance [24]. However, the exact mechanistic roles of SOX4 and 17 in ESCC remain unclear. Whether SOX4 and SOX17 interact together to participate in the formation of VM in ESCC is not yet known. This experimental study intended to observe the effect of VE-cadherin silencing via siRNA interference on the expression of SOX4 and SOX17 in ESCC and the corresponding impacts on the invasion and metastasis of ESCC.

Materials And Methods

In this study, tissue samples from 210 patients with ESCC and 60 patients with normal esophageal mucosa were collected from the First Affiliated Hospital of Bengbu Medical College from January 2014 to December 2015. The tissue samples of ESCC were obtained from surgical specimens. None of the patients with ESCC included in this study had received chemotherapy or radiotherapy before the surgery. Detailed clinicopathological data of the study participants are presented in Table 1. Additionally, fresh saline tissue samples of ESCC and adjacent normal tissues were collected from 10 patients between August 2019 and December 2019 from the First Affiliated Hospital of Bengbu Medical College. These samples were placed in liquid nitrogen immediately for later use in western blot analysis. For detection of mRNA levels, the fresh tissues were immersed in RNA store solution (TIANGEN, Beijing, China) in the ratio of 1:10 and stored in liquid nitrogen at 4°C overnight.

Immunohistochemistry (IHC)

The paraffin-embedded samples were sectioned into 3-μm-thick slices. After dewaxing and debenzenization, limonic acid high-pressure antigen repair, anti-SOX17 antibody (dilution ratio 1:150, AB224637, Abcam, USA), anti-SOX4 antibody (dilution ratio 1:150, AB236557, Abcam, USA), anti-VE-cadherin antibody (dilution ratio 1:200, AF6265, Affinity Biosciences, USA), and CD34 (dilution ratio 1:250, AB110643, Abcam, USA) were added, separately. 3, 3-N-DiaminobenzidineTetrahydrochloride (DAB) color was added to the treated slices.

In SOX17- and SOX4-positive cells, flaky or granular brownish yellow staining was seen in the nucleus or cytoplasm, while VE-cadherin−positive cells showed flaky or granular brownish yellow staining of the cell membrane and cytoplasm. The staining results included the proportion of positive cells and staining intensity [25]. The proportion of positive cells refers to the percentage of positive cells among the total observed cells of the same species: 0 (≤10%); 1 point (11~25%); 2 points (26%~50%); 3 points (51%~75%); and 4 points (> 75%). Staining intensity was graded as 0, 1, 2 and 3 points for no staining, light yellow, brownish yellow and tan yellow staining, respectively. The points for percentage of positive cells and staining intensity were multiplied, and the mean value was calculated to decide the staining results as follows: 0~3 was considered negative, and 4~12 was taken as positive.

For all CD34-stained immunohistochemical sections, DAB color development was performed, and the color development reaction was stopped by washing with flowing water for 1 min. The cells were rinsed with water for 2 min and then stained with periodic acid-Schiff (PAS) for 15~30 min. The cells were again rinsed with distilled water thrice for 1 min each. VM was detected by the presence of tumor cells around the PAS-positive and CD34-negative tubes with few necrotic tumor cells and inflammatory cells infiltrating the surrounding tissues, and absence of red blood cells in the lumen of the tubes. Endothelium-lined normal vessels were identified by the presence of CD34-positive endothelial cells in their wall.

Cell lines and cell culture

The human ESCC cell lines EC9706 and Eca109 were grown in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, USA) in 5% CO_2 at 37℃. The cell lines were divided into three groups: control group, control siRNA group, and VE-cadherin siRNA group. In the control group, EC9706 and Eca109 cells were not treated. In the control siRNA group, EC9706 and Eca109 cells were infected with the empty plasmid. In the VE-cadherin siRNA group, EC9706 and Eca109 cells were infected with a lentivirus encoding precursor VE-cadherin or vector and treated with puromycin for 2 weeks to obtain stably transfected cells.

siRNA transfection

EC9706 and Eca109 cells were seeded into six-well plates and transfected with VE-cadherin siRNA and a negative control (NC, GenePharma, China) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. The siRNA sequence was: CCAUUGUGCAAGUCCACACAUTT (forward, 5'-3') and AUGUGGACUUGCACAAUGGTT (reverse, 5'-3'). The cells were subjected to analysis as described in the Results section.

Western blotting

An appropriate amount (250~500 mg) of fresh tissue or properly preserved tissue was immersed in 1 ml of strong radio-immunoprecipitation assay (RIPA) buffer containing phenylmethylsulfonyl fluoride (PMFS) was added. An electric homogenizer was used to produce the homogenate. The samples were collected after addition of lysis buffer and placed on ice for cracking for 20~30 min. Then centrifugation was performed at 12000 rpm for 10 min. ESCC cells were lysed in RIPA buffer with proteinase inhibitors. Protein concentrations were quantified using a BCA assay kit (Beyotime Biotechnology, China). Subsequently, the proteins were isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Membranes were incubated in 5% non-fat milk and immunoblotted with the following antibodies: anti-SOX17 antibody (diluted 1:500, AB224637, Abcam, USA), anti-SOX4 antibody (diluted 1:500, AF2610, Affinity Biosciences, USA), anti-VE-cadherin antibody (diluted 1:1000, AF6265, Affinity Biosciences), and anti-β-actin antibody (Cell Signaling Technology, USA).

Quantitative real-time PCR

Trizol (Invitrogen, USA) was used to extract mRNA from ESCC tissues or cells, and the extracted mRNA samples were reversely transcribed into cDNA templates. Quantitative real-time PCR was performed using an ABI 7900 System with SYBR green (TaKaRa, China). The primers for SOX4 were as follows: AGCAAGAAGGCGAGTTAGTT (forward, 5-3') and TGACCAAGA AGCAAAATAAAA (reverse, 5'-3'). The primers for SOX17 were as follows: GGTTTTTGTTGCTGTTG (forward, 5'-3') and AACTTGGAAATAGGGTTTTGAC (reverse, 5'-3'). The primers for VE-cadherin were as follows:

TACCAGCCAAGTTGTGA (forward, 5'-3') and GCCGTGTTATCGTGATTATCC (reverse, 5'-3'). The transcript levels were quantified by normalization to GAPDH expression (5'-CTGGGCTACACTGAGCACC, forward, 5'-3' and AAGTGGTCGTTGAGGGCAATG, reverse, 5'-3') as an internal standard.

Wound healing assays

Two cell lines were seeded overnight in six-well plates followed by transfection with VE-cadherin siRNA or NC siRNA. When cells reached greater than 90% confluency, the tip of a pipette was used to make a wound, and the detached cells were rinsed away with phosphate-buffered saline (PBS). Images of the wounds were taken at 0 h and 24 h.

MTT assay

ESCC cell lines were seeded overnight into 96-well plates at 5×10^3 cells per well. Subsequently, the cells were transfected with VE-cadherin siRNA for 72 h. Cell viability was measured by MTT assay as described previously [25].

Transwell migration and invasion assay

Cell migration and invasion were evaluated by Transwell assay as previously described [26]. Briefly, transfected ESCC cells were seeded in 24-well plates with 8-μm-pore-size chamber inserts (Corning, USA). The upper chambers were coated with Matrigel (BD Biosciences, USA) before cell seeding. After incubation for 48 h, invading and migrating cells on the bottom surface of each chamber were stained with Giemsa solution and photographed. The migrating cells were then counted in five random fields for quantification.

Statistical analyses

The continuous and categorical data are presented as mean ± standard deviation (SD) and frequency (percentage), respectively. Comparisons of quantitative data between two and multiple groups were conducted by Student's t test and one-way analysis of variance (ANOVA), respectively, using GraphPad Prism 8.0. Kaplan–Meier curves with log-rank tests were used for univariate overall survival (OS) analysis. Cox regression models were used for multivariate OS analysis. Differences were considered to be statistically significant if $P<0.05$.

Results

SOX17, SOX4, and VE-cadherin expression and VM are associated with clinical characteristics of ESCC

The positivity rates for SOX17 expression in the normal esophageal mucosa and ESCC samples were 83.3% and 41.4%, respectively, and the difference was statistically significant ($P<0.05$). There was no significant correlation between SOX17 protein expression and clinicopathological characteristics such as gender, age or tumor location ($P>0.05$). SOX17 protein expression showed inverse correlations with tumor size, grade of differentiation, depth of invasion, and pathological tumor node metastasis (pTNM) stage. The rate of SOX17 expression was lower among cases with lymph node metastasis (LNM) than among those without LNM ($P<0.05$). The rate of SOX4 protein expression in the normal esophageal mucosa was significantly higher than in ESCC (30% vs. 60%, $P<0.05$). The SOX4 expression rate showed positive correlations with the grade of differentiation, depth of infiltration, LNM, and pTNM stage of ESCC ($P<0.05$). The rate of VE-cadherin protein expression in ESCC was significantly higher than in the normal esophageal mucosa (51.9% vs. 3.3%, $P<0.05$). Moreover, VE-cadherin protein expression showed no correlation with patient age and gender. VE-cadherin protein expression was positively correlated with tumor size, grade of differentiation, LNM, pTNM stage, and depth of infiltration of ESCC ($P<0.05$). The positive rate of VM in ESCC was 50% (105/210), while no VM was found in the test group (Figure 1). VM positivity was not correlated with gender, age, tumor location, or histological grade ($P<0.05$), but did correlate with tumor size, gross type, infiltration depth, LNM, and pTNM stage ($P<0.05$). The above results are summarized in Table 1 and Fig. 1A–H.

Correlation analysis

Spearman correlation analysis revealed that SOX17 expression in ESCC was negatively correlated with SOX4 expression (r = -0.418), VE-cadherin expression (r = 0.487), and VM (r = -0.609, all $P<0.001$; Table 2). On the other hand, SOX4 expression was positively correlated with VE-cadherin expression (r = -0.576) and VM (r = -0.447, $P<0.001$; Table 2). VE-cadherin expression also showed a positive correlation with VM (r = -0.715, $P<0.001$; Table 2).

Survival analysis

The 5-year OS rate in the ESCC group was 37.1% (78/210). The OS of patients with SOX17 expression was significantly better than that of patients without SOX17 expression ($P<0.001$; Table 3, Figure 2A). On the other hand, the OS of patients with SOX4 expression, VE-cadherin expression, and VM was significantly lower than that of patients negative for these factors ($P<0.001$; Table 3, Figure 2B-D).

On Cox regression model analysis, various factors such as gender, age, tumor type, tumor location, tumor diameter, histological grade, LNM, depth of invasion, pTNM stage, VM, SOX17 expression, SOX4 expression, and VE-cadherin expression were identified as prognostic factors. It was found that the expression of SOX17, SOX4, and VE-cadherin as well as VM were independent risk factors affecting the long-term prognosis of ESCC patients (Table 4).

Comparison of SOX17, SOX4, and VE-cadherin protein levels and mRNA levels in fresh ESCC and adjacent tissue samples

In fresh samples from 10 ESCC patients, the mean SOX17 protein (0.816±0.337 vs. 1.338±0.435, $P<0.05$) and mRNA (0.223±0.373 vs. 1.611±1.978, $P<0.05$) expression levels in ESCC tissues were significantly lower than the corresponding levels in the adjacent tissues (>5 cm away from the tumor). However, the mean SOX4 protein (1.315±0.598 vs. 0.554±0.393, $P<0.05$) and mRNA (2.221±1.933 vs. 0.402±0.403, $P<0.05$) expression levels in ESCC tissues were higher than the corresponding levels in the adjacent tissues. The mean VE-cadherin protein (0.721±0.171 vs. 0.494±0.175, $P<0.05$) and mRNA (0.350±0.293 vs. 0.092±0.071, $P<0.05$) expression levels were also significantly higher in ESCC tissues than in adjacent tissues. The results are shown in Figure 3.

Silencing VE-cadherin inhibits the invasion and migration of EC cells

Transwell experiments demonstrated that the migration and invasion abilities of the EC9706 and Eca109 cell lines were significantly lower in the VE-cadherin siRNA group than in the corresponding control groups ($P<0.05$). No difference was observed between the control group and the control siRNA group (Figure 4A–D).

Additionally, the wound healing speed of cells in the VE-cadherin siRNA group was significantly slower than those in the corresponding control groups (Figure 4E-G).

Silencing VE-cadherin reduced EC cell proliferation

The proliferative abilities of EC9706 and Eca109 cells were significantly weakened after silencing of VE-cadherin ($P<0.05$, Fig. 4H). No significant difference was observed between the control group and the control siRNA group (Fig. 4H).

Silencing VE-cadherin increased SOX17 expression and decreased SOX4 expression

After transfection, the VE-cadherin protein expression was significantly lower in the VE-cadherin siRNA group than in the control groups ($P<0.05$). Moreover, SOX17 protein expression was significantly upregulated and SOX4 protein expression was downregulated in the VE-cadherin siRNA group (Fig. 5).

Discussion

The formation of VM can provide blood supply for the rapid proliferation of tumors, relieve the ischemic and hypoxic micro-environment around the tumors, and further accelerate the invasion and metastasis of tumors, which influences the clinical stage and long-term prognosis of cancer patients [5, 27]. In this study, we confirmed the existence of VM in ESCC by IHC analysis of tumor tissues. At the same time, we found the VM was closely associated with the depth of invasion, pTNM stage, and LNM. The above findings are consistent with those of previous studies [28]. On survival analysis, the presence of VM was an independent poor prognostic factor in ESCC patients.

VE-cadherin, as an adhesion protein, can mediate the adhesion of cells to each other and maintain the further formation of tumor blood vessels. In the present study, VE-cadherin was found to be highly expressed in ESCC, and its positive expression was directly related to the depth of invasion, occurrence of LNM, and pTNM stage in ESCC. VE-cadherin expression was found to be an independent poor prognostic factor for ESCC patients. *In vitro* experiments suggested that high expression of VE-cadherin can accelerate ESCC invasion and metastasis, similar to the findings of previous reports [28]. Notably, after siRNA-mediated interference of bcl-2 expression in EC9706 cells under hypoxic conditions, the expression of VM-related molecules, such as VE-cadherin and matrix metalloproteinase 2 (MMP2), was significantly inhibited and VM generation was significantly reduced [29]. Moreover, VE-cadherin downregulation in melanoma is associated with the loss of VM formation [30]. Heinolainen et al. [31] and Han et al. [32] speculated that VE-cadherin might be an important determinant of VM in EC. Based on the findings of the present study, we firmly believe that VE-cadherin promotes the formation of VM in ESCC.

The SOX17 transcription factor has been known to have tumor suppressive function in ESCC [35-37]. SOX17 overexpression suppresses colony formation and cell migration/invasion in ESCC cell lines. In addition, SOX17 overexpression was found to inhibit tumor growth and metastasis in an ESCC xenograft model [33-35]. In the present study, we found significantly higher SOX17 expression in ESCC compared to the normal esophageal epithelium, confirming the tumor suppressive function of SOX17. A previous study demonstrated that hypermethylation of the promoter of the SOX17 gene leads to silencing of SOX17 protein

expression in ~50% ESCC patients [33]. In the present study, the low expression of SOX17 was significantly correlated with tumor differentiation, depth of invasion, LNM, and pTNM stage, suggesting that SOX17 acts as a tumor suppressor gene in ESCC. Moreover, SOX17 expression was an independent predictor of prognosis in this study.

The present study found that SOX4 protein and mRNA expression levels were significantly increased in ESCC. Moreover, the high expression of SOX4 promoted the invasion of ESCC. Schilham et al. also found that SOX4 overexpression is closely associated with ESCC metastasis [36]. SOX4 has been found to be involved in tumorigenesis and tumor invasion in different types of cancers through the activated Wnt, transforming growth factor (TGF)-β, Hedgehog and Notch pathways or the regulation of microRNA expression [30]. Zilong et al. speculated that miR-212-3p targets SOX4 to inhibit the Wnt/β-catenin signaling pathway and promote apoptosis in ESCC [37].

VM is closely related to tumor growth, invasion, metastasis, and long-term prognosis of cancer patients [38-40]. In the present study, we confirmed that VM formation in ESCC was positively correlated with high SOX4 expression and low SOX17 expression. These findings indicate that VE-cadherin may promote the formation of VM in ESCC by affecting the expression levels of SOX4 and SOX17. In a previous study, we confirmed that SOX4 may promote the formation of VM by promoting EMT in ESCC [23]. Studies have shown that in a hypoxic environment, hypoxia-inducible factor (HIF)-2α, a VM-initiating factor, is activated, which increases VE-cadherin transcription. VE-cadherin, in turn, induces repositioning of EphA2 to the cell membrane. Furthermore, PI3K is activated by VE-cadherin and EphA2 simultaneously. The activated PI3K regulates the activation of the pre-gene of membrane type 1 matrix metalloproteinase (MT1-MMP). The combination of MT1-MMP and MMP2 promotes the fragmentation of laminin 5γ25γ2 (LN-5γ2) chains into fragments (5γ2 and 5γ2χ), and increased levels of these two fragments in the extracellular micro-environment eventually leads to the formation of a VM net-like structure [41].

In conclusion, the present study found low SOX17 expression, high SOX4 expression, and high VE-cadherin expression in ESCC. Moreover, the expression of these proteins was closely associated with VM in ESCC. We believe that development of targeted therapies to suppress SOX4 expression or enhance SOX17 expression may impair the formation of VM, thereby prolonging the survival of ESCC patients. Future studies are required to determine the exact pathophysiological mechanism linking SOX4, SOX17, VE-cadherin, and VM.

Abbreviations

ESCC: esophageal squamous cell carcinoma; VM: vasculogenic mimicry; EMT: epithelial-mesenchymal transition; EC: esophageal cancer; LNM: lymph node metastasis; pTNM: pathology tumor node metastasis; VE-cadherin: vascular endothelial cadherin; SRY: sex determining region on Y; HMG box: high mobility group box; SOX17: Sex determining region of Y chromosome box 17; SOX4: Sex determining region of Y chromosome box 4; PBS: phosphate-buffered saline; AVONA: one-way analysis of variance; OS: overall survival; FAK: focal adhesion kinase; ERK1/2: extracelluar signal regulated kinase 1 and 2; DAB: 3,3-N-diaminobenzidine tetrahydrochloride; FBS: fetal bovine serum; RIPA: radio-immunoprecipitation assay; PMFS: phenylmethylsulfonyl fluoride; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF: polyvinylidene fluoride.

Declarations

Author contributions: Yanzi Qin, Wenjun Zhao and Zhaogeng Cai contributed equally to this work. Yanzi Qin and Li Ma participated in the research design. Yanzi Qin, Wenjun Zhao, Qi Wang, Jin Gao, Ningning Yang and Hongfei Ci performed the research and wrote the manuscript. Zhaogeng Cai, Li Ma and Zhenzhong Feng contributed towards critically revising the manuscript.

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Competing interests: The authors declare that they have no competing interests.

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Tables

Table 1 Relationships of SOX17 expression, SOX4 expression, VE-cadherin expression, and VM with the clinicopathological characteristics of ESCC

Variable	SOX17		χ^2	P	SOX4		χ^2	P	VE-cadherin		χ^2	P	VM
	Positive	Negative			Positive	Negative			Positive	Negative			Positive
Gender			0.507	0.477			0.323	0.570			0.632	0.427	
Male	68	101			103	66			90	79			87
Female	19	22			23	18			19	22			18
Age (years)			0.001	0.971			0.013	0.909			0.798	0.372	
<65	37	52			53	36			43	46			42
≥65	50	71			73	48			66	55			63
Gross type			8.605	0.035			9.185	0.027			10.932	0.012	
Ulcerative	31	55			55	31			53	33			51
Medullary	35	54			58	31			46	43			43
Mushroom	11	11			8	14			6	16			7
Constrictive	10	3			5	8			4	9			4
Location			0.796	0.672			4.178	0.124			8.207	0.017	
Upper	9	10			13	6			9	10			10
Middle	42	55			51	46			41	56			43
Lower	36	58			62	32			59	35			52
Differentiation			7.473	0.024			2.151	0.341			5.284	0.071	
Well	16	12			16	12			9	19			11
Moderate	60	79			80	59			75	64			72
Poor	11	32			30	13			25	18			22
Diameter (cm)			26.719	0.000			17.898	0.000			11.188	0.001	
<3.5	64	46			51	59			45	65			42
≥3.5	23	77			75	25			64	36			63
Serous infiltration			9.598	0.002			36.768	0.000			27.920	0.000	
Yes	44	88			100	32			87	45			77
No	43	35			26	52			22	56			28
Lymph node metastasis			37.199	0.000			44.507	0.000			90.646	0.000	
Yes	12	68			71	9			75	5			67
No	75	55			55	75			34	96			38
pTNM stage			23.297	0.000			37.135	0.000			56.959	0.000	
I+II	76	69			67	78			50	95			52
Ⅲ+Ⅳ	11	54			59	6			59	6			53

Table 2 Correlation of SOX17, SOX4, and VE-cadherin expression and VM in ESCC (n=210)

Variable	SOX17		r	P	SOX4		r	P	VE-cadherin		r	P
	Positive	Negative			Positive	Negative			Positive	Negative		
SOX4			-0.418	0.000								
Positive	31	95										
Negative	56	28										
VE-cadherin			-0.487	0.000			0.576	0.000				
Positive	20	89			95	14						
Negative	67	34			31	70						
VM			-0.609	0.000			0.447	0.000			0.715	0.000
Positive	12	93			86	40			96	13		
Negative	75	30			19	65			17	88		

Table 3 Mean and median overall survival (OS) (n=210)

	Mean[a]				Median				Log rank (Mantel-Cox)	p
	Estimate	Std. Error	95% Confidence interval		Estimate	Std. error	95% Confidence interval			
			Lower bound	Upper bound			Lower bound	Upper bound		
SOX17									61.200	0.000
Positive	62.621	2.533	57.657	67.584						
Negative	34.432	1.913	30.683	38.181	27.000	2.773	21.566	32.434		
Overall	46.174	1.816	42.616	49.732	37.000	3.150	30.827	43.173		
SOX4									70.109	0.000
Positive	33.937	1.807	30.395	37.478	27.000	2.640	21.826	32.174		
Negative	64.339	2.551	59.340	69.339						
Overall	46.174	1.816	42.616	49.732	37.000	3.150	30.827	43.173		
VE-cadherin									111.011	0.000
Negative	28.927	1.541	25.906	31.947	25.000	1.115	22.815	27.185		
Positive	64.518	2.194	60.219	68.818						
Overall	46.174	1.816	42.616	49.732	37.000	3.150	30.827	43.173		
VM									92.811	0.000
Negative	29.581	1.719	26.212	32.950	25.000	1.394	22.267	27.733		
Positive	62.422	2.213	58.084	66.760						
Overall	46.174	1.816	42.616	49.732	37.000	3.150	30.827	43.173		

Table 4 Results of multivariate logistic regression analyses of OS (n=210)

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
Age	0.938	0.192	23.878	1	0.000	2.554	1.754	3.721
Sex	-0.161	0.235	0.469	1	0.493	0.851	0.537	1.350
General type	0.025	0.118	0.046	1	0.829	1.026	0.815	1.291
Location	0.138	0.156	0.779	1	0.377	1.148	0.845	1.558
Diameter (cm)	-0.261	0.200	1.703	1	0.192	0.771	0.521	1.140
Differentiation	0.141	0.154	0.845	1	0.358	1.152	0.852	1.558
Infiltration depth	-0.441	0.252	3.063	1	0.080	0.644	0.393	1.054
Lymphatic metastasis	-0.496	0.351	1.997	1	0.158	0.609	0.306	1.212
pTNM	-0.258	0.319	0.654	1	0.419	0.773	.413	1.444
SOX17	0.579	0.290	3.976	1	0.046	1.784	1.010	3.150
SOX4	-0.802	0.293	7.525	1	0.006	0.448	0.253	0.795
VE-cadherin	-0.784	0.353	4.936	1	0.026	0.456	0.229	0.912
VM	-0.640	0.298	4.607	1	0.032	0.527	0.294	0.946

Figures
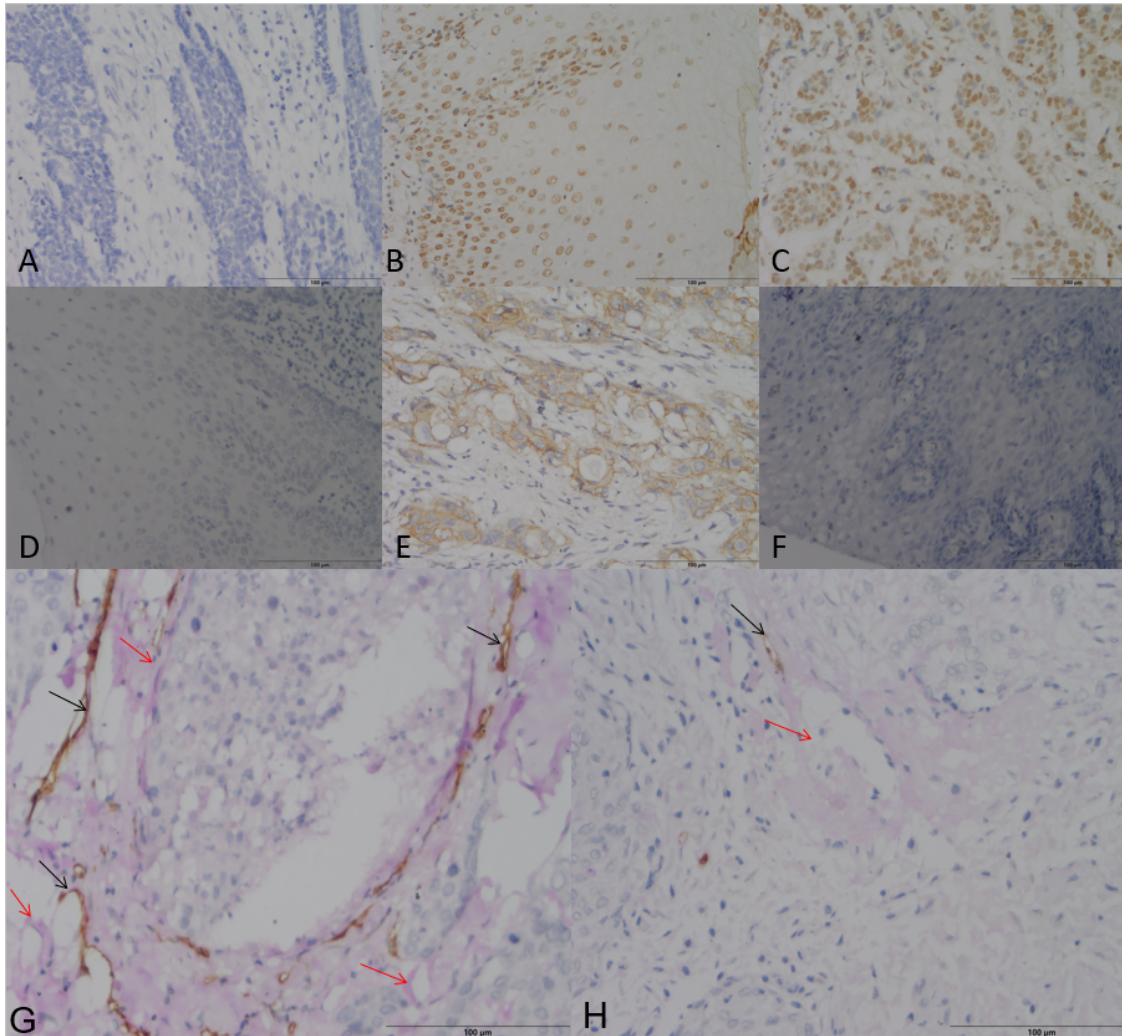
Figure 1

Immunohistochemical analysis of SOX17, SOX4, and VE-cadherin expression as well as VM in ESCC and normal esophageal tissues (×400 magnification): negative staining of SOX17 in ESCC (A), positive staining of SOX17 in the nucleus of normal esophageal tissue (B), positive staining of SOX4 in the nucleus of ESCC (C), negative staining of SOX4 in the normal esophageal tissue (D), positive staining of VE-cadherin in the cell membrane and plasma of ESCC (E), negative staining of VE-cadherin in the normal esophageal tissue (F), VM in ESCC (G, red arrow), positive staining of normal blood vessels with CD34 (H, black arrow).
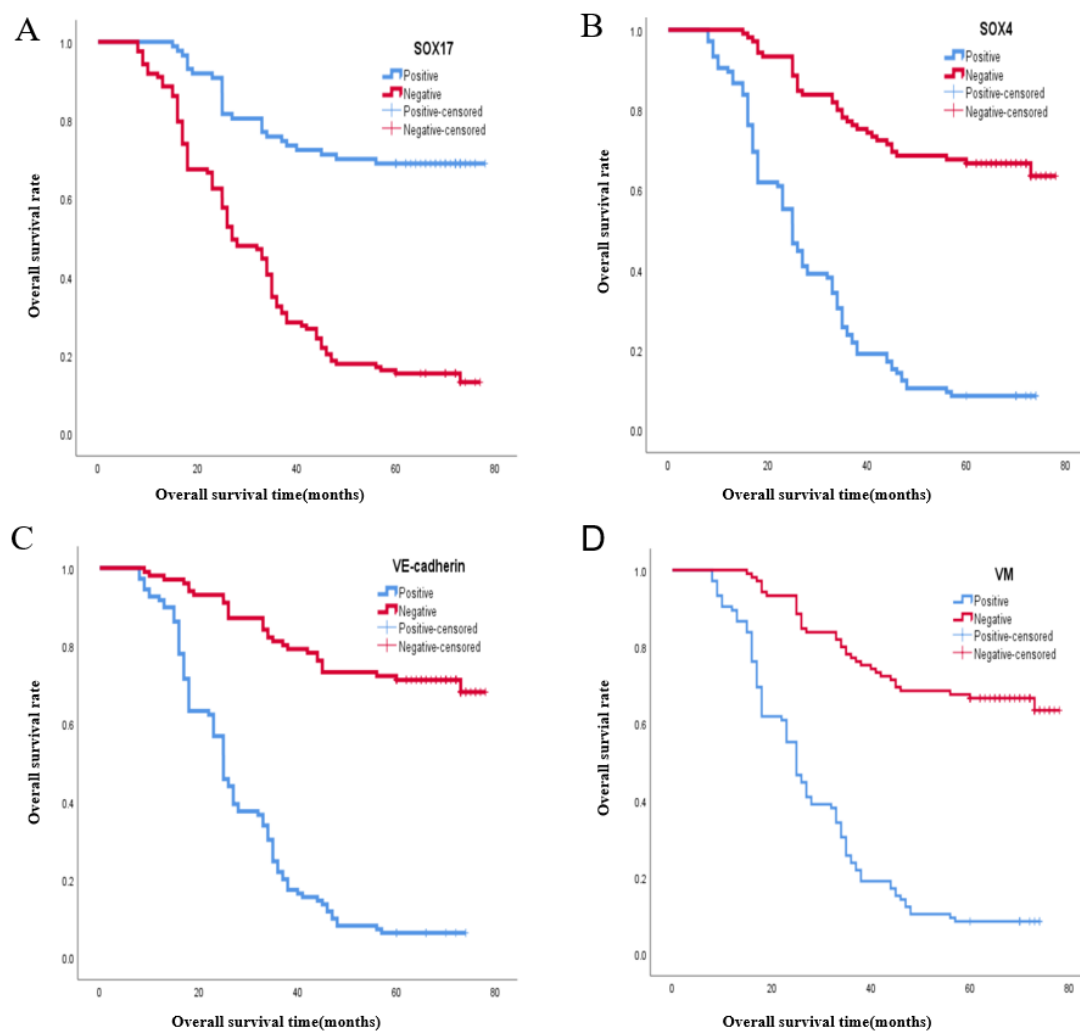
Figure 2

Kaplan–Meier analysis of the survival rates of patients with ESCC: higher overall survival of patients with positive SOX17 expression (I, log-rank =61.200, P<0.001), lower overall survival of patients with positive SOX4 expression (J, log-rank = 70.109, P<0.001), overall survival of patients in relation to VE-cadherin (K, log-rank =32.174, P<0.001), lower overall survival of patients with presence of VM (L, log-rank =92.811, P<0.001). Blue lines: cases positive for SOX17, and SOX4, VE-cadherin expression and VM; red line: cases negative for SOX17, SOX4, and VE-cadherin expression and VM.
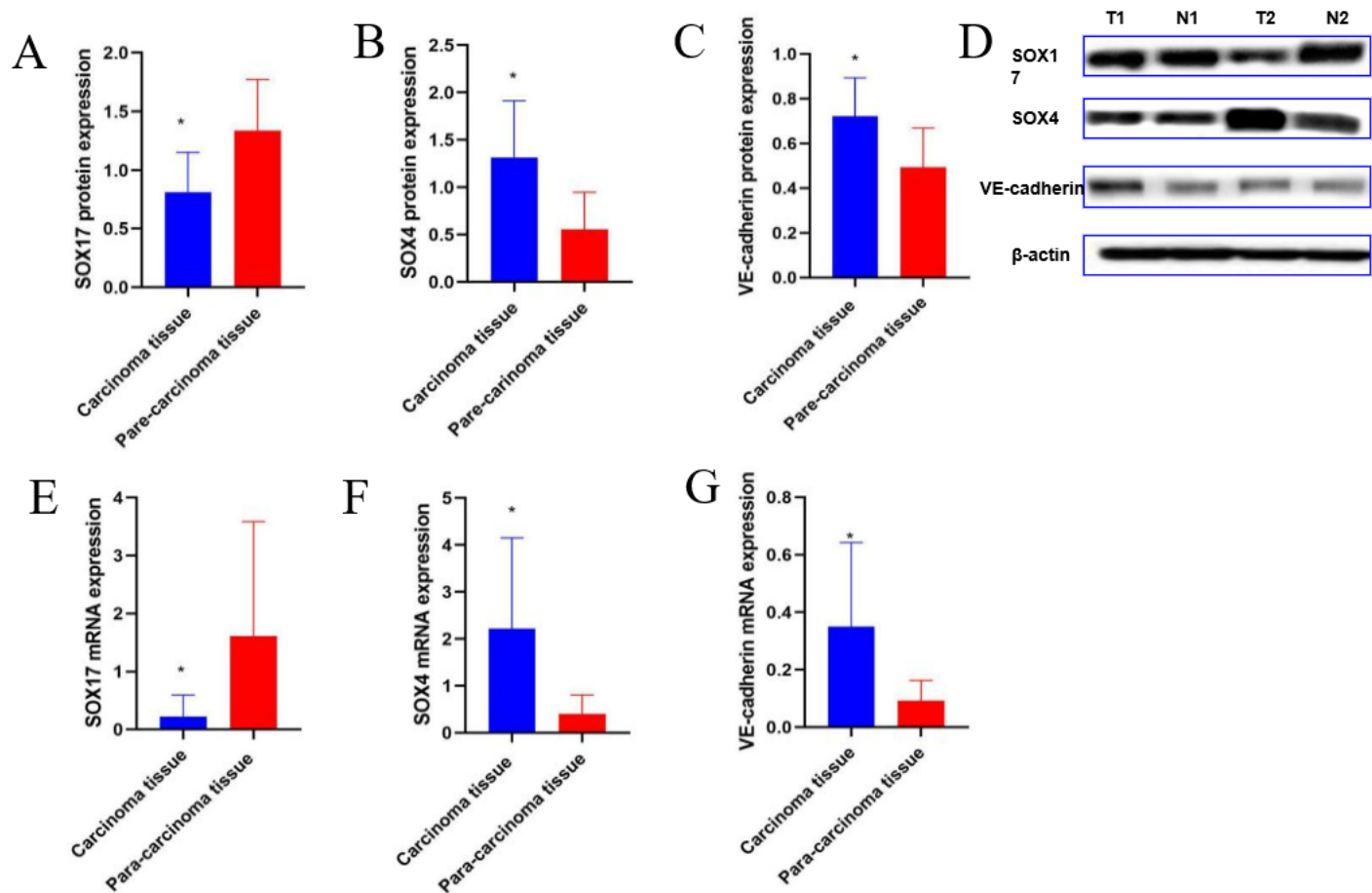
Figure 3

SOX17, SOX4, and VE-cadherin protein and mRNA expression. A, B, C. Graphical representation of SOX17, SOX4, and VE-cadherin protein expression, respectively (*P<0.05). D. Western blot analysis of SOX17, SOX4, and VE-cadherin protein levels in ESCC tissues and non-tumor tissues (T1, T2 correspond to ESCC tissues; N1, N2 correspond to normal mucosal tissues). E, F, G. Graphical representation of SOX17, SOX4, and VE-cadherin mRNA expression, respectively (*P<0.05).
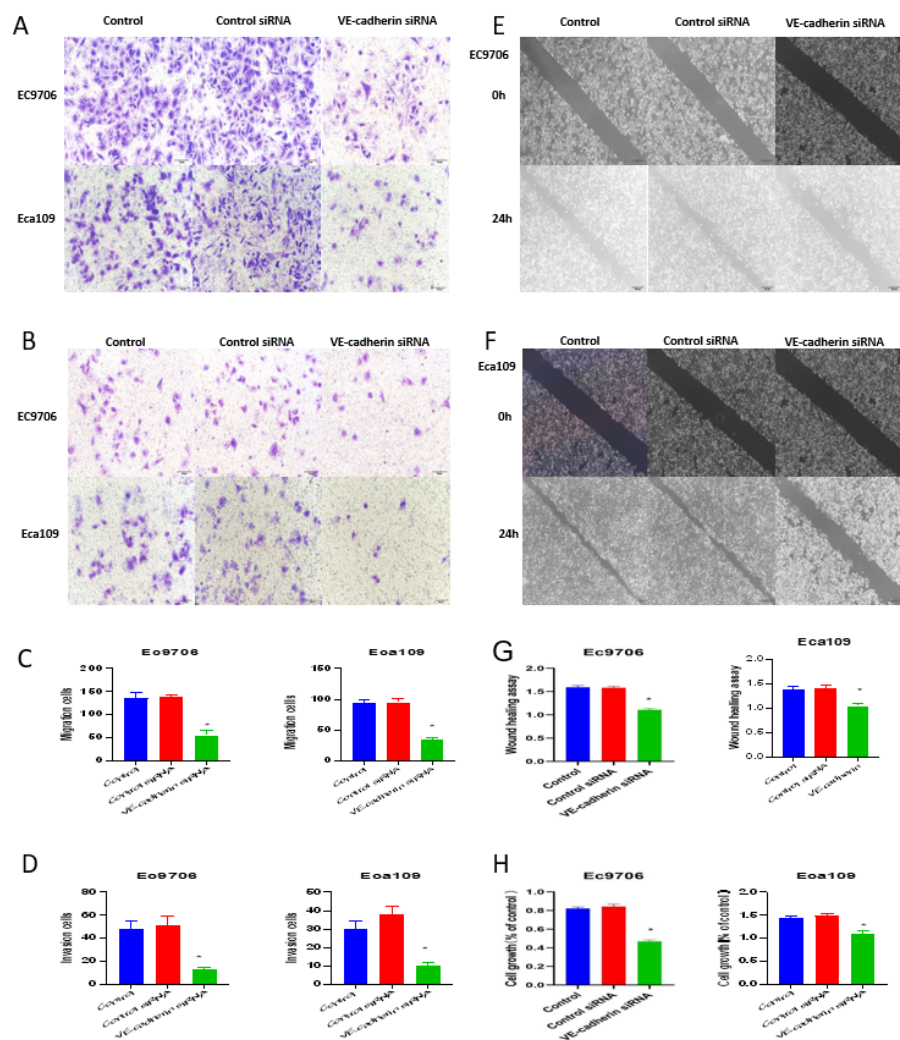
Figure 4

A~D. Migration and invasion abilities of EC cells after VE-cadherin silencing. Transwell assay showing the migration of the EC9706 and Eca109 cells (A); Transwell assay showing the invasion of EC9706 and Eca109 cells (B); Graphical representation of migration and invasion among EC9706 and Eca109 cells (C, D). The wound healing experiment showed the effect of silencing VE-cadherin on the migration and healing ability of EC9706 and Eca109 cells (E, F, G). Comparison of in vitro proliferation of EC9706 and Eca109 cells (H). *P<0.05 vs control or control siRNA.
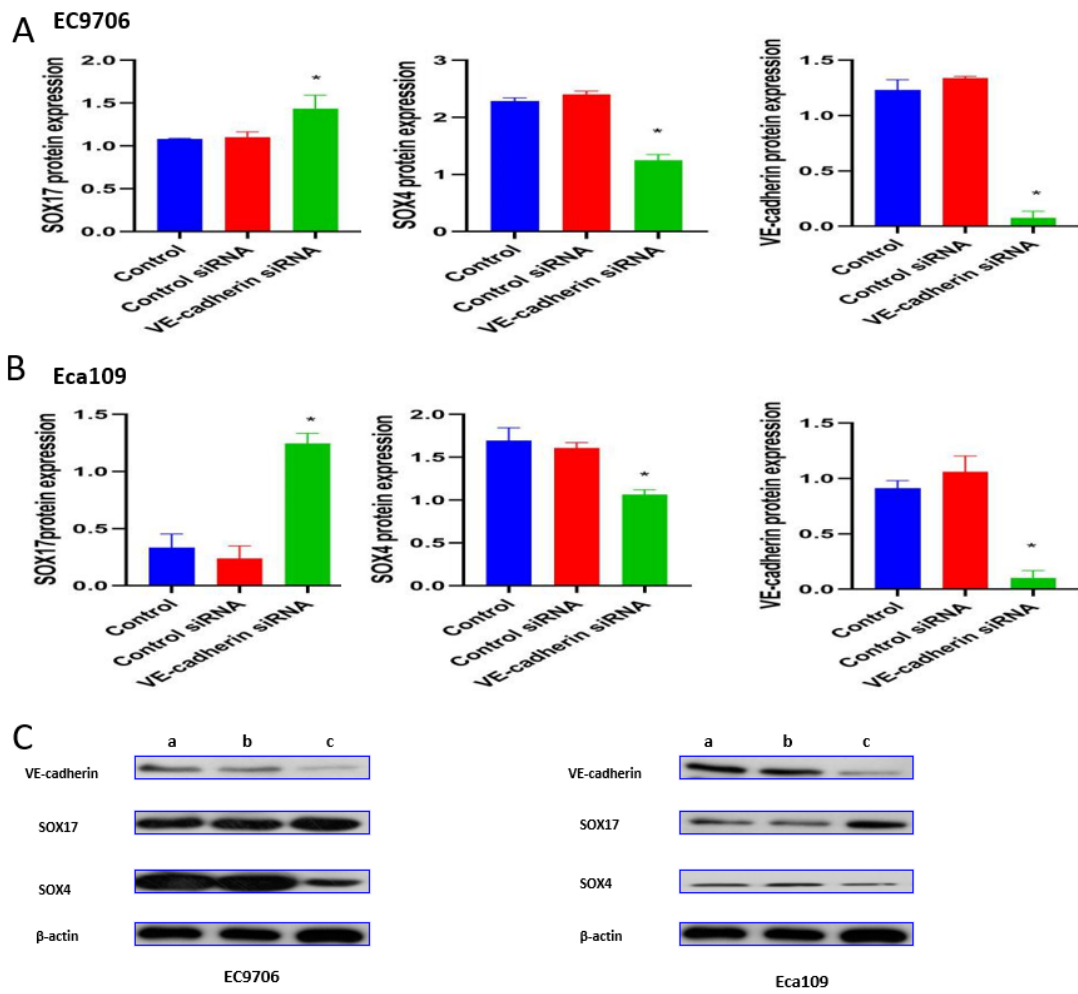
Figure 5

Top panel: Western blot analysis to detect the expression of SOX17, SOX4 and VE-cadherin in the three groups of EC9706 and Eca109 cells after VE-cadherin silencing (A, B). Quantitative western blot data (C, D). (a indicates the control group; b indicates the control siRNA group; and c indicates the VE-cadherin siRNA group). *P<0.05 vs control or control siRNA