Med Oncol (2015) 32:20
DOI 10.1007/s12032-014-0480-z

ORIGINAL PAPER

N-cadherin participated in invasion and metastasis of human esophageal squamous cell carcinoma via taking part in the formation of vasculogenic mimicry

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Received: 5 December 2014 / Accepted: 20 December 2014 / Published online: 10 January 2015
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Abstract Vasculogenic mimicry (VM) refers to the unique ability of highly aggressive tumor cells to mimic the pattern of embryonic vasculogenic networks, and the presence of VM correlates to an increased risk of metastasis and poor clinical outcome of cancers. Several key molecules, including N-cadherin, have been implicated in VM. However, the role of N-cadherin in the formation of VM in esophageal squamous cell carcinoma (ESCC) had not been elucidated. In this study, firstly we aimed to identify VM patterns in ESCC tissues and to explore their clinical significance. VM was present in 12 out of 56 samples, and ESCC with lymph node metastasis had a higher incidence of VM than that without lymph node metastasis. More importantly, VM channels were associated with the expression of N-cadherin in ESCC tissues. In order to further explore the role of N-cadherin in VM formation and invasion and metastasis in ESCC, secondly, we silenced the expression of N-cadherin with small hairpin RNA in ESCC cell line KYSE-70; herein, we showed that KYSE-70 cells with N-cadherin silencing lost not only the capacity to form tube-like structures on collagen (VM) but also the invasion, metastasis and proliferation ability in KYSE-70 cells in vitro. Taken together, antivascular therapies targeting tumor cell VM may be an effective approach to the treatment of patients with highly metastatic ESCC.

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Keywords N-cadherin · Vasculogenic mimicry · Invasion and metastasis · Esophageal squamous cell carcinoma

Introduction

Advances in diagnostic and therapeutic approaches have led to excellent expectations for long-term survival for early esophageal squamous cell carcinoma (ESCC), while the prognosis for advanced ESCC with extensive invasion and metastasis remains poor [1, 2]. Tumor invasion and metastasis requires a blood supply for growth and hematogenous metastasis. For many years, angiogenesis via the sprouting of new vessels from existing ones was considered to be the exclusive method of tumor vascularization [3]. In 1999, Maniotis et al. [4] first reported that highly aggressive and metastatic melanoma cells are able to form highly patterned vascular channels lined externally by tumor cells, without the existence of endothelial cells. This process was termed as vasculogenic mimicry (VM), which is independent of angiogenesis, and is composed of tumor cells and a basement membrane [5]. Subsequently, VM has been observed in numerous types of aggressive tumors, such as colorectal cancer [6], head and neck squamous cell carcinoma [7, 8], glioblastoma [9], breast cancer [10], ovarian carcinoma [11]. The presence of VM was associated with a high tumor grade, invasion and metastasis, and short survival [12]. However, the detailed mechanism of tumor VM remains to be further elucidated.

Cadherins are a family of transmembrane proteins that mediate calcium-dependent homophilic cell–cell contacts. Among them, it has been indicated that VE-cadherin plays a critical role in the formation of VM [13, 14]. Down-regulation of VE-cadherin expression in the aggressive melanoma cells abrogated their ability to form vasculogenic

networks [15]. N-cadherin, another one of the cadherins family, has been extensively studied about their biological activities and associations with cancer cell invasion [16]. Recent studies on prostate cancer and breast cancer proved that the up-regulated N-cadherin plays an important role in cell progression and metastasis [17, 18]. However, the association of N-cadherin expression with the VM formation of the ESCC is unknown.

In the current study, we identified VM patterns in ESCC tissues, explored their clinical significance and analyzed the expression of N-cadherin in the ESCC tissues with VM formation. In addition, we compared the ability of human ESCC cells expressing low levels of N-cadherin to form vascular channels on three-dimensional matrigel cultures and to assess the effect on invasion, metastasis and proliferation ability of KYSE-70 cells in vitro.

Materials and methods

Ethics statement

This study was performed under a protocol approved by the Institutional Review Boards of the First Affiliated Hospital of Zhengzhou Medical University, and all examinations were performed after obtaining written informed consents.

Patients and samples

The immunohistochemical study was performed on samples from 56 patients with ESCC who underwent potentially curative surgery without preoperative therapy at the First Affiliated Hospital of Zhengzhou University, between 2009 and 2013. The diagnoses of these ESCC samples were verified by pathologists. The age of the patients ranged from 36 to 79 years, and the median age was 60.3 years. Thirty-one were males, and 25 were females. Among the 56 cases, histologically, all the cases were confirmed to be ESCC. The histological grade included grade I (15 cases), grade II (24 cases) and grade III (17 cases). 31 cases were accompanied with lymph node metastasis, and 25 cases had no lymph node metastasis. The invasion depth was divided into two groups consisting of 16 cases with invasion of the superficial muscularis and 40 with invasion of the deep muscularis or fibrous membrane.

CD34–PAS dual staining

Vasculogenic mimicry was detected by CD34–PAS dual staining as described previously [19]. Briefly, standard immunohistochemical staining was performed on 5-μ formalin-fixed paraffin-embedded tumor sections, for CD34 (1:200, polyclonal antibody; Santa Cruz Biotechnology), followed by immunodetection using the EnVision™ + System (Peroxidase Kit; Santa Cruz Biotechnology). The slides were then rinsed with distilled water for 5 min, incubated with periodic acid–Schiff (PAS) for 15 min, counterstained with Mayer's hematoxylin for 1 min and viewed under a light microscope to detect CD34 and PAS signals. The whole section was examined for the presence of VM (CD34-negative and PAS-positive vessels) by three independent observers without knowledge of patient outcome. Adult esophageal mucosa samples were used as controls.

Immunohistochemical studies

Formalin-fixed paraffin-embedded specimens were analyzed by immunohistochemistry with antibodies of N-cadherin (1:200; Santa Cruz Biotechnology) according to manufactory instruction. Immunodetection was performed using the EnVision™ + System (Peroxidase Kit; Santa Cruz Biotechnology). The slides were rinsed with distilled water for 5 min, counterstained with Mayer's hematoxylin for 1 min, dehydrated through an alcohol gradient and cover-slipped. Tissues from esophageal mucosa samples served as controls. Membrane or nucleus staining for N-cadherin was considered positive. The degree of immunostaining was scored separately by two independent investigators, who were blinded to the histopathologic features. Tumor scoring based on percent positivity was classified as 0 for negative, 1 for $\leq$10 %, 2 for 10–25 % and 3 for $\geq$25 % staining. The intensity of staining was graded as 0 for absent immunoreactivity, 1 for weak, 2 for moderate and 3 for intense positivity. The staining intensity index of N-cadherin was determined by multiplying the proportion of positively stained tumor cells and the intensity of staining.

Cell culture and transient transfection

ESCC cell line (KYSE-70) was obtained from the Cell Bank of the Chinese Academy of Medical Sciences (Beijing, China). KYSE-70 cells maintained in an RPMI 1640 medium supplemented with 10 % fetal bovine serum at 37 °C under 5 % CO_2. Cells were subcultured when they reached 70–80 % confluence by 0.25 % trypsin digestion. When the newly plated cells reached the exponential growth phase, they were used for experiments. KYSE-70 was transfected with 100 nM specific N-cadherin siRNA or nonsense siRNA for 48 h, using Lipofectamine™ reagent in serum-free RPMI 1640 medium according to the manufacture's instruction. Untreated KYSE-70 cells were used as a negative control. Efficacy of transfection was tested in KYSE-70 cells by real-time RT-PCR and Western blot.

RNA preparation and reverse transcription PCR (RT-PCR)

Total RNA in each group was isolated by TRIzol reagents. According to the manufacturer's protocol, 5 μg of total RNA was used to generate the first strand of DNA using the High-Capacity cDNA Reverse Transcription Kit. One-tenth of the cDNA mixture was used as the template for the subsequent PCR amplification. Reverse transcription reaction were optimized using TaqDNA polymerase for the linear range of cDNA amplification. Linear amplification of a 269-bp fragment of N-cadherin was achieved at 30 cycles of replication (94 °C 4 min, 94 °C 30 s, 52 °C 40 s, 72 °C 60 s, 72 °C 10 min) with the primers of 5′-CCTTTC ACTGCGGATACGTG-3′ and 5′-ATCCCTCAGGAACT GTCCCA-3′, and of 487-bp fragment of GAPDH at 30 cycles of replication with the primers of 5′-GGTGCATGT GGACAGCATTT-3′ and 5′-CTGGTGCCAGGAAAGCA CAT-3′. All analyses were performed in triplicate.

Western blot analysis

Western blot was carried out according to the previous publication. Briefly, KYSE-70 cells in each group were harvested and lysed using the entire protein extraction reagent (keyGEN, China) according to the manufacturer's protocols. Protein concentrations were determined with standard BCA method. Equal amounts of protein (60 μg/lane) for each sample were separated by SDS-PAGE gel electrophoresis and then transferred onto a PVDF membrane (N-cadherin: 20 V, 25 min; β-actin: 20 V, 60 min). After blocking with 0.1 % Tween 20 and 5 % nonfat dry milk in Tris-buffered saline at 4 °C overnight, the membrane was incubated with primary antibody (N-cadherin: 1:1,000) at room temperature for 2 h and then incubated with horseradish peroxidase-conjugated secondary antibody (1:4,000) for 1 h. Protein bands were detected (the colored membranes) with the enhanced chemiluminescence (ECL) system and exposed to X-ray film. All analyses were performed in triplicate.

Three-dimensional cultures

Matrigel (Collaborative Biomedical) was thawed at 4 °C, and 200 μL was quickly added to each well of a 24-well plate and allowed to solidify for an hour at room temperature and then for 30 min at 37 °C in humidified 5 % CO_2 incubator. KYSE-70 cells in each group seeded onto the 3D matrix in complete medium and incubated at 37 °C for 48 h. The samples were observed under inverted microscope and images were taken.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Cells proliferation ability was determined by an MTT assay. KYSE-70 cells in each group were seeded into 96-well plates (approximately 5,000 cells/well) and incubated for 0, 24, 48, and 72 h, respectively. At different time intervals, MTT reagent (Sigma, USA) was added to each well at 5 mg/ml in 20 μl and cells were incubated for another 4 h. Then the cells were incubated at 37 °C and dissolved in 100 μl DMSO after 4 h incubation. The absorbance of the cell lysates in DMSO solution was read at 490 nm by a microplate reader (Bio-Rad, Hercules, CA) for OD values. Experiments were performed in triplicate for each experimental condition.

Cell invasion assays

KYSE-70 cells (1×10^5) in each group were added to the upper compartment of the invasion chamber. The lower compartment was filled with 10 % FBS in RPMI 1640. All cells were incubated at 37 °C for 6 h in 5 % CO_2. After incubation, the noninvading cells were removed from the upper surface of the membrane by scrubbing with a cotton swab. The cells those invaded through the membrane and adhered to the lower surface were fixed in 95 % alcohol, stained with HE and counted under a microscope in five fields of vision at 400 magnification. Experiments were performed in triplicate for each experimental condition. The values obtained were calculated by averaging the total number of cells from three membranes.

Wound healing assay

KYSE-70 cells in different groups were seeded into 6-well plates and allowed to grow to 70 % confluency in 1640 medium. An artificial homogenous wound was created onto the monolayer with a sterile 10-μl tip. After scratching, the cells were washed with serum-free medium. Images of cells migrating into the wound were captured after 24 h by inverted microscope.

Statistical analysis

The statistical analysis was performed with SPSS version 13.0 for Windows. Associations between VM and clinical data were determined by the Pearson's χ^2 test. All p values were two-sided, and statistical significance was set at $p = 0.05$.

Results

Vasculogenic mimicry was correlated with lymph node metastasis and tumor grade

CD34/PAS double staining was used to identify VM in ESCC tissue. CD34-negative and PAS-positive vascular-like patterns containing red blood cells, which were formed by ESCC cells, were considered as VM. VM was found in 12 of 56 cases (21.5 %) examined (Fig. 1). The cells composing the channels were negative for CD34 indicating that they were not endothelial or endothelial progenitor cells.

In order to determine the clinical significance of VM in ESCC, we compared the presence of VM with clinicopathological characteristics of the patients. VM was detected preferentially in ESCC with lymph node metastasis, 33.7 %, while fewer VM (9.4 %) was found in ESCC without lymph node metastasis. ESCC with lymph node metastasis had a higher incidence of VM than that without lymph node metastasis ($p = 0.000$). Furthermore, VM was also correlated with tumor grade. VM was detected preferentially in high-grade gliomas: 7 of 17 grade III (41.2 %), and 4 of 24 grade II (16.7 %), while fewer VM was found in grade I ESCC tissues examined (1 of 15 grade I, 6.7 %) ($p = 0.000$). No associations were found between the existence of VM and the invasion depth, sex and age ($p > 0.05$).

Expression of N-cadherin was associated with VM in ESCC tissue

Significant association was found between the existence of VM and N-cadherin expression. Tumor specimens that expressed high levels of N-cadherin proteins had VM (82.37 ± 34.79), whereas tumors (generally ESCC without lymph node metastasis) with low or no N-cadherin expression had fewer evidence of VM (33.65 ± 11.89). Significant association was found between the existence of VM and N-cadherin expression ($p < 0.01$).

Down-regulation of N-cadherin abrogated VM in ESCC cells

In order to further test the hypothesis that N-cadherin was critical to the formation of VM in ESCC, N-cadherin was knocked down by siRNA in ESCC cell line KYSE-70 to further explore the effects of N-cadherin on VM.

The results of RT-PCR and Western blot showed that N-cadherin siRNA could inhibit the mRNA and protein expression of N-cadherin efficiently ($p < 0.01$) (Fig. 2a, b).

Then, we examined the effect of N-cadherin on the VM of KYSE-70 cells. Previously, tube formation ability was always assessed using a Matrigel basement membrane model; in the present study, we used a well-established in vitro model of three-dimensional cultures for detecting VM formation to elucidate whether knockdown of N-cadherin abrogated VM formation in KYSE-70 cells. KYSE-70 cells transfected with negative siRNA or blank KYSE-70 cells grown in a 3D type-I collagen matrix culture after 48 h showed a modest level of conversion to "tube" formations. These cells partially formed long spindle shapes. Cell protrusions were obvious, and the cells formed connective structures resembling blood vessels. The VM formation level was extremely low in cells transfected with N-cadherin siRNA and less than the control cells ($p < 0.05$) (Fig. 2c). Our results provided further support for the possible role of N-cadherin in promoting VM formation.

Down-regulation of N-cadherin inhibited invasion and metastasis ability of ESCC cells in vitro

VM formation was associated with cell invasion and migration. Then, we detected the effect of N-cadherin

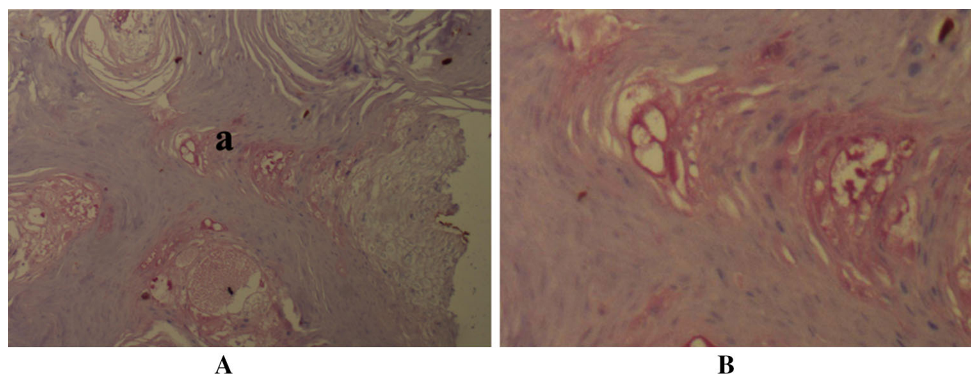

Fig. 1 Representative micrographs of VM in ESCC. **a** PAS-positive (*purple red*) zone without CD34-positive cells indicates VM; some angiogenesis vessels are seen with CD34-stained (*brown*) cells in the internal lumen of vessels. **b** A magnification of image (*a*); *erythrocyte shadows* are seen in the channels

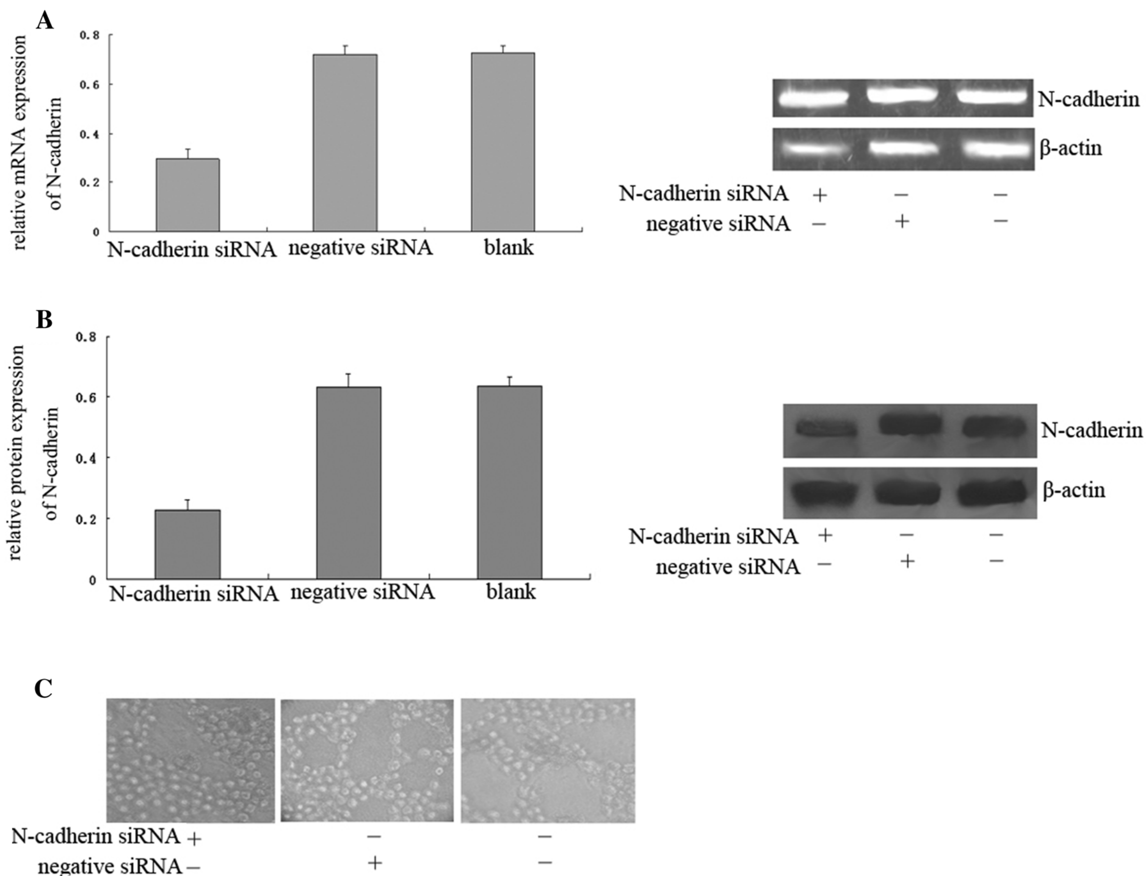

Fig. 2 Effect of N-cadherin on VM formation in KYSE-70 cells. **a**, **b** RT-PCR and Western blot analysis of N-cadherin mRNA and protein expression in KYSE-70 cells transfected with N-cadherin siRNA, negative siRNA or blank KYSE-70 cells. N-cadherin siRNA could inhibit the expression of N-cadherin in KYSE-70 cells significantly compared to that in KYSE-70 cells transfected with negative siRNA or blank KYSE-70 cells ($p < 0.01$). A representative experiment is shown. The relative amounts of the mRNA and protein were quantified and normalized to the corresponding β-actin amounts. **c** Three-dimensional cultures of KYSE-70 cells transfected with N-cadherin siRNA, negative siRNA or blank KYSE-70 cells. KYSE-70 cells transfected with N-cadherin siRNA formed fewer VM patterns compared to that in KYSE-70 cells transfected with negative siRNA or blank KYSE-70 cells ($p < 0.05$)

siRNA on the invasion and metastasis ability of KYSE-70 cells. The transwell plates were used to measure the in vitro ability of KYSE-70 cells to invade a basement membrane matrix. We found that KYSE-70 cells in control groups passed more of the transwell membrane and had more invasive capability than KYSE-70 cells transfected with N-cadherin siRNA in vitro (Fig. 3a); the number of passing membrane cells, i.e., invaded tumor cells in KYSE-70 cells transfected with N-cadherin siRNA group, markedly decreased ($p < 0.05$). Thus, N-cadherin inhibited significantly the invasion of KYSE-70 cells in vitro. The wound healing assay was used to determine the effect of N-cadherin siRNA on migration ability of KYSE-70 cells in vitro. As shown in Fig. 3b, the wound healing ability in KYSE-70 cells in control group was higher than KYSE-70 cells transfected with N-cadherin siRNA. It was showed that knockdown of N-cadherin expression inhibited significantly the migration of KYSE-70 cells in vitro.

Down-regulation of N-cadherin inhibited proliferation ability of ESCC cells in vitro

MTT assay was used to determine the effect of knockdown of N-cadherin expression on proliferation ability of KYSE-70 cells. And the results showed the proliferation ability of KYSE-70 cells after transfected with N-cadherin siRNA decreased significantly compared with those in control groups (Fig. 4). Furthermore, N-cadherin siRNA inhibited markedly the proliferation ability of KYSE-70 cells in a time-dependent manner.

Discussion

Invasion and metastasis are the major cause of tumor mortality. Tumor growth and invasion are dependent on a persistent blood supply; therefore, the capability of generating neovessels through diverse mechanisms is associated with

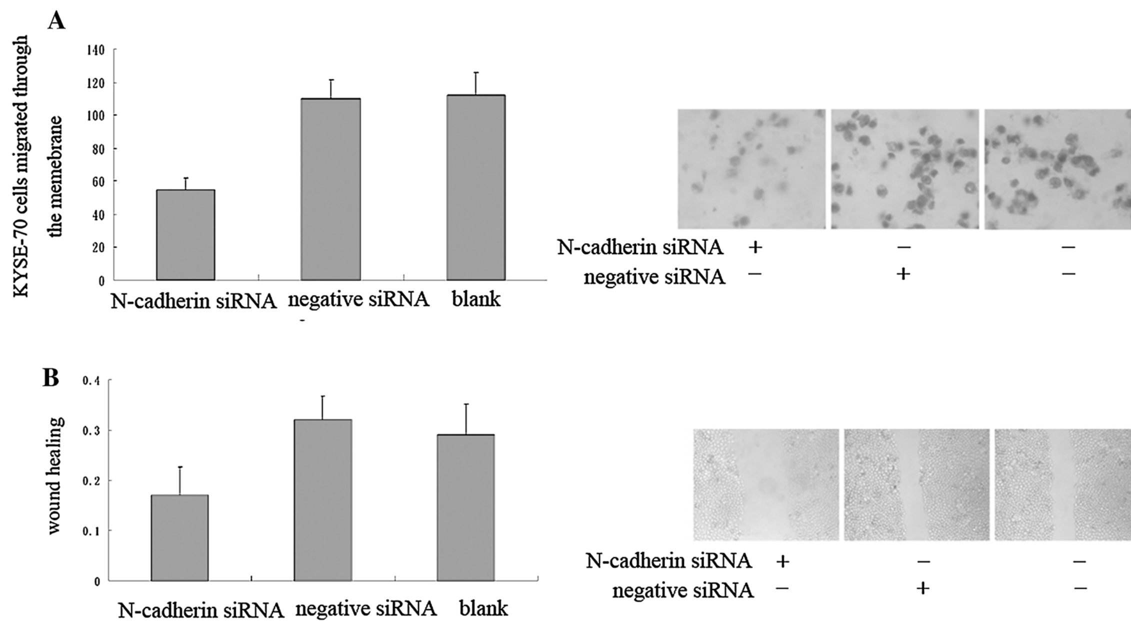

Fig. 3 Effect of N-cadherin on invasion and metastasis ability of KYSE-70 cells. **a** Invasion assay showed the effect of N-cadherin siRNA on the invasion abilities of KYSE-70 cells. The cells transfected with N-cadherin siRNA had lower invasion abilities than the control group. **b** Wound healing assay. Quantitative analysis showed a significant difference between the wound healing rates of tumor cells transfected with N-cadherin siRNA and those transfected with negative siRNA or blank cells ($p < 0.05$)

its malignant potential in tumor [20]. VM is a type of blood supplement formed by highly invasive and genetically dysregulated tumor cells with a pluripotent embryonic-like genotype. Such tumor cells contributed to the plasticity and gain the ability to participate in the processes of neovascularization and ultimately constructing a fluid-conducting, matrix-rich meshwork [21, 22]. Tumors exhibiting in VM related to more aggressive tumor biology and increased tumor-related mortality. The current study investigated the prevalence and clinical significance of VM in ESCC. CD34-negative and PAS-positive channels were detected in 21.5 % ESCC tissues examined. The VM channels were arranged in arcs, loops and networks. In addition, VM was detected predominantly in ESCC with lymph node metastasis, and a significant association was found between VM and lymph node metastasis.

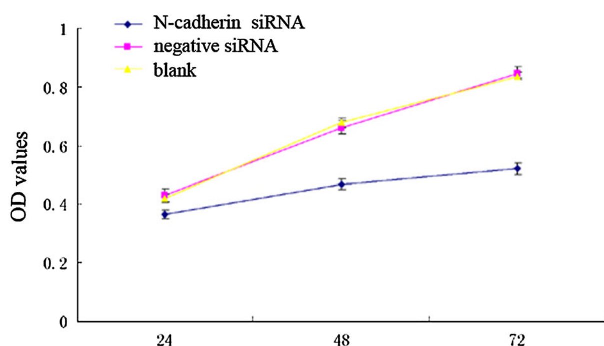

Fig. 4 Effect of N-cadherin on proliferation ability of KYSE-70 cells. MTT assay showed the effect of N-cadherin siRNA on the proliferation abilities of KYSE-70 cells. The proliferation ability of KYSE-70 cells transfected with N-cadherin siRNA decreased greatly compared with those in control groups

N-cadherin is a major adhesion molecule involved in the development and plasticity of the nervous system. N-cadherin-mediated cell adhesion regulates neuroepithelial cell polarity, neuronal precursor migration, growth cone migration and synaptic plasticity [23, 24]. In various cancer cells, the abnormal expression of N-cadherin correlates with the induction of cell motility. For example, the expression of N-cadherin induces cell migration in breast cancer cells [25], melanoma [26], prostate cancer [27] and squamous carcinoma [28].

To our data, there are no reports about correlation between N-cadherin expression and VM in ESCC. In this study, we evaluated N-cadherin expression in ESCC by immunohistochemistry and correlated the results with VM. A significant association was observed between VM and N-cadherin expression, suggesting that N-cadherin could contribute to VM formation in ESCC. In order to further elucidate the role of N-cadherin in the formation of ESCC, we knocked down the expression of N-cadherin by RNAi and got an obvious inhibitory effect on tube formation in KYSE-70 cells. Our results provided further support for the possible role of N-cadherin in promoting VM formation. It was suggested that vascular channels in general, including

VM channels, play a role not only in supplying oxygen and nutrients required for tumor growth, but also in enhancing tumor metastasis. So, we compared the invasion and metastasis ability of KYSE-70 cells transfected with N-cadherin siRNA and KYSE-70 cells transfected with negative siRNA or blank KYSE-70 cells. The results showed that down-regulating the expression of N-cadherin could efficiently decrease not only the invasion and metastasis ability but also the proliferation ability of KYSE-70 cells. Therefore, it was proposed that N-cadherin expression was associated with invasion and metastasis and proliferation ability of KYSE-70 cells via participating VM formation.

In conclusion, the expression of N-cadherin was associated with VM formation in ESCC and may correlate with the aggressive and invasive nature of ESCC via the formation VM.

Acknowledgments This study was supported by a Grant from Henan Province Basic and Frontier Technology Research Projects (No. 132300410127).

Conflict of interest The authors declare that there is no conflict of interests. The authors have no commercial interest in any materials discussed in this paper.

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