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Original Article

Tumor suppressor TSLC1 inhibits growth, proliferation, invasiveness and angiogenesis in nude mice xenografted tumor of Eca109 cells

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Abstract: Tumor suppressor in lung cancer 1 (TSLC1) is a novel tumor suppressor gene whose inactivation is implicated in the occurrence, invasion, metastasis and prognosis of esophageal cancer. TSLC1 was studied by comparing the tumor formation of TSLC1 transfectant and control cells in nude mice. Compared with blank group and mock group, tumor size and infiltrating range of transfected group was less, differentiation of tumor tissue was slightly better, and differences of tumor angiogenesis was worse. There was no obvious difference between blank group and mock group. We have shown TSLC1 gene inhibited the growth proliferation, infiltration and angiogenesis of Eca109 cells.

Keywords: Tumor suppressor gene, tumor suppressor in lung cancer -1 (TSLC1), esophageal cancer, tumorigenicity, nude mice

Introduction

The esophageal cancer ranks the eighth of the commonest malignant tumors in the world. Nearly 400,000 new cases emerged annually in the world and about 80% of them in developing countries [1]. The incidence is high in Iran, China, Japan and some countries in South Asia. Recently it has been reported that approximate 60% of the world's esophageal cancer was occurred in China, where the cancer contributes to the fourth highest death caused by cancers, greatly affecting people's health especially in rural areas. The early diagnosis of esophageal cancer has been proved to be difficult, and unfavorable therapeutic response has been noted. Up to now, the molecular mechanisms of esophageal carcinogenesis have been studied, and several responsible genes, including tumor suppressor genes which contribute to the genesis of esophageal cancer in a loss-of-function manner have been identified, including BLU/ZMYND10 on chromosomal region 3p21 [2], and tumor suppressor gene tumor suppressor in lung cancer 1 (TSLC1) on region 11q22-23. Loss or low expression of tumor suppressor gene TSLC1 has been found in specimens and cell line derived from many types of human tumors; and the lack of TSLC1 expression was associated with the occurrence and development of these cancers. The genetic or epigenetic aberrations affecting TSLC1 have been documented in tumor tissues of a variety of origins, including the esophagus, stomach, liver and pancreatic cancers as well as other digestive tumors, laryngeal cancer [3], canine meningiomas [4], breast cancer [5] nasopharyngeal cancer [6], hepatocellular carcinoma [7], lung cancer [8] adult acute lymphoblastic cell leukemia (ALCL) [9], and colon cancer [10]. Studies have revealed that loss expression of TSLC1 gene was associated with the prognosis of patients

Table 1. Volume change of tumor in nude mice after inoculation

Group (n)	Growth Volume: $\bar{x} \pm s$ (mm^3)			
	10 d	17 d	24 d	30 d
Transfected group 10	1.18 ± 0.11	8.83 ± 0.88	54.85 ± 4.20	162.07 ± 8.34
mock group 10	12.33 ± 2.76	55.24 ± 7.0	151.95 ± 12.16	256.34 ± 19.19
Blank group 10	12.87 ± 3.17	57.11 ± 7.13	153.23 ± 11.56	272.42 ± 18.66

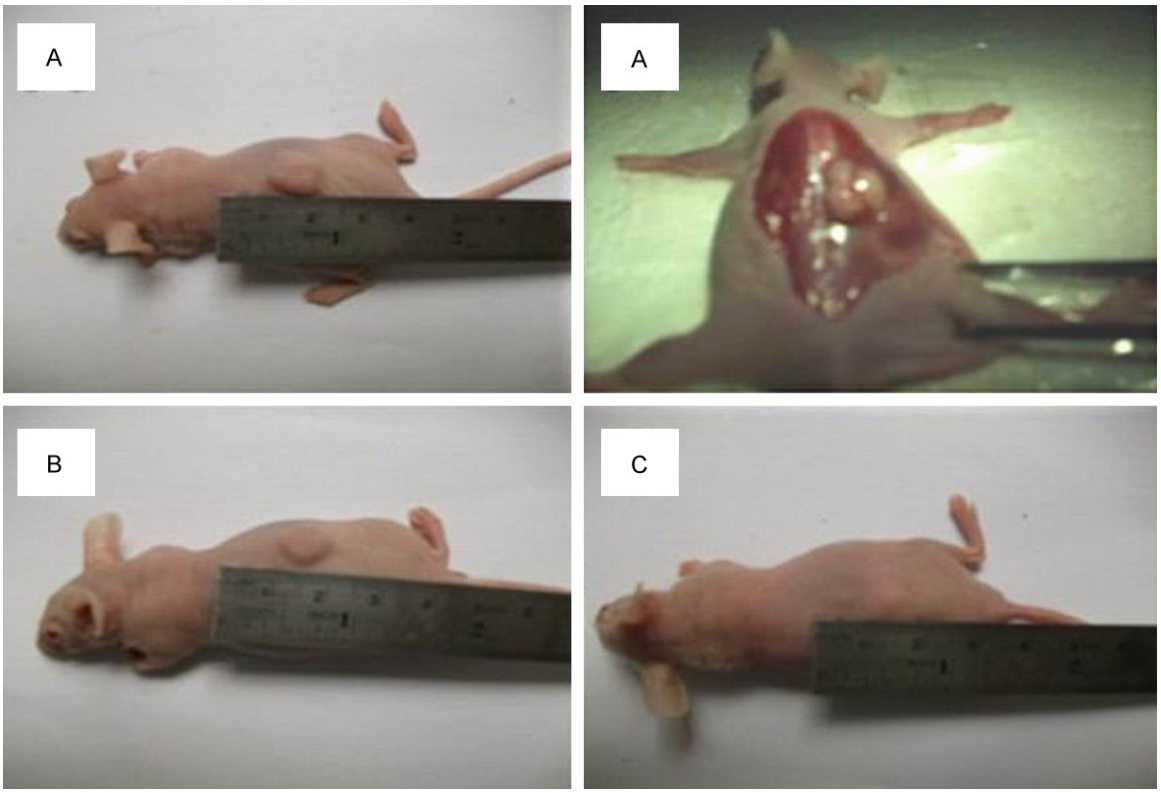

Figure 1. Subcutaneous tumor in each group of nude mice. A: Subcutaneous tumor in blank group of nude mice. B: Subcutaneous tumor in mock group of nude mice. C: Subcutaneous tumor in transfected group of nude mice.

with esophageal squamous cell carcinoma and could be used as prognostic factors [11]. Liang has proposed that TSLC1 represents a promising biomarker for cancer diagnosis and a potential target for cancer therapy through analyzing a large number of literatures [12]. But so far, the biological functions of TSLC1 are still unclear. Available data suggested that TSLC1 function to trigger apoptosis, and regulate cell cycle, but its tumor suppressive potential has not been studied in nude mice xenograft model of human cancers. In the present study, the in vivo tumor inhibitory effect of TSLC1 in esophageal cancer is investigated in a xenograft model by inoculation of Eca109 cells to nude mice.

Material and methods

Cell line and reagents

Human esophageal cancer cell line Eca109 was purchased from the Cell Bank of Institute of Life Science, Chinese Academy of Science, Shanghai, China, and stored in liquid nitrogen in our laboratory, and they were unfrozen before the experiments. TRIZOL kit and Lipofectamine 2000 Liposomal transfection kit were purchased from Invitrogen (Carlsbad, CA, USA). DL2000 DNA Marker, λ-Hind III DNA Marker, High Fidelity PrimeScript™ RT-PCR Kit, LB Liquid/solid culture medium, BglII/EcoRI Res triction endonuclease were purchased from

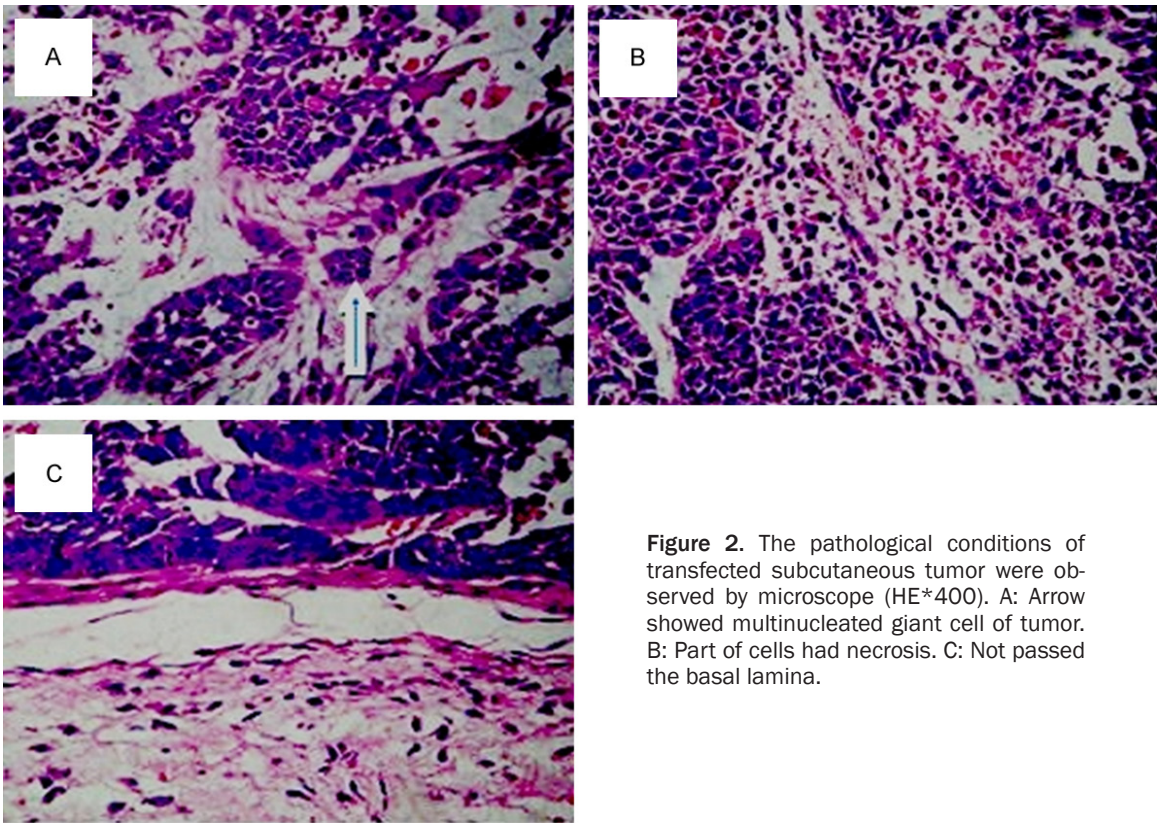

Figure 2. The pathological conditions of transfected subcutaneous tumor were observed by microscope (HE*400). A: Arrow showed multinucleated giant cell of tumor. B: Part of cells had necrosis. C: Not passed the basal lamina.

Takara Biotechnology Co, Ltd (Dalian, China). Fetal bovine serum (FBS), RPMI-1640 Cell culture medium, PBS, 0.25% trypsin and 0.02% EDTA, DMSO, Green-chain double resistance and MEM medium were purchased from Tianhang Biological Technology Co, Ltd (Zhejiang, China). Mini- plasmid extraction kits and RNase A were purchased from Beyotime Animal Pharmaceutical Co, Ltd (Haimen, Zhejiang, China). Our experiments have been approved by Animal Experiments Ethics Committee of the Guangdong Medical College.

Cell culture and creation of human esophageal carcinoma cell line Eca109 transfectant expressing TSLC1 gen

Eca109 cells stably expressing TSLC1 were obtained after transfection and antibiotic selection, and described in reference [13]. Briefly, the cells were transfected with TSLC1 expression vector by lipofectamine, and the resultant cell were cultured for two weeks with G418 and the visible colonies were transferred to culture plates. They were allowed to grow to sufficient number before test of TSLC1 with Western blotting or RT-PCR.

Nude mice model and tumor inhibition

Sixty SPF grade BALB/c nude mice were obtained from the Laboratory Animal Center of Guangdong Medical College and were divided into three groups, each group then divided into two subgroups, male and female in half. Each subgroup was separately manipulated: Subcutaneous tumors, lung and liver metastases by intravenous injection in nude mice. The experimental lung and liver metastases were created by intravenous injection in nude mice. The experimental group was transfected the cell containing pIRES2-EGFP-TSLC1. The group with transfected cell containing pIRES2-EGFP was called control group. The blank group was inoculated with parental Eca109 line of esophageal cancer. Experimental group, control group, as well as a blank set of cell suspension made of Eca109 esophageal cancer cells called T1E, M1E and B1E. T1E, M1E and mice of B1E group were injected with Eca109 cells into dorsum subcutaneously to form the subcutaneous tumors, each group composed by 10 nude mice. For other two groups the cells were injected into caudal vein of nude mice to form the

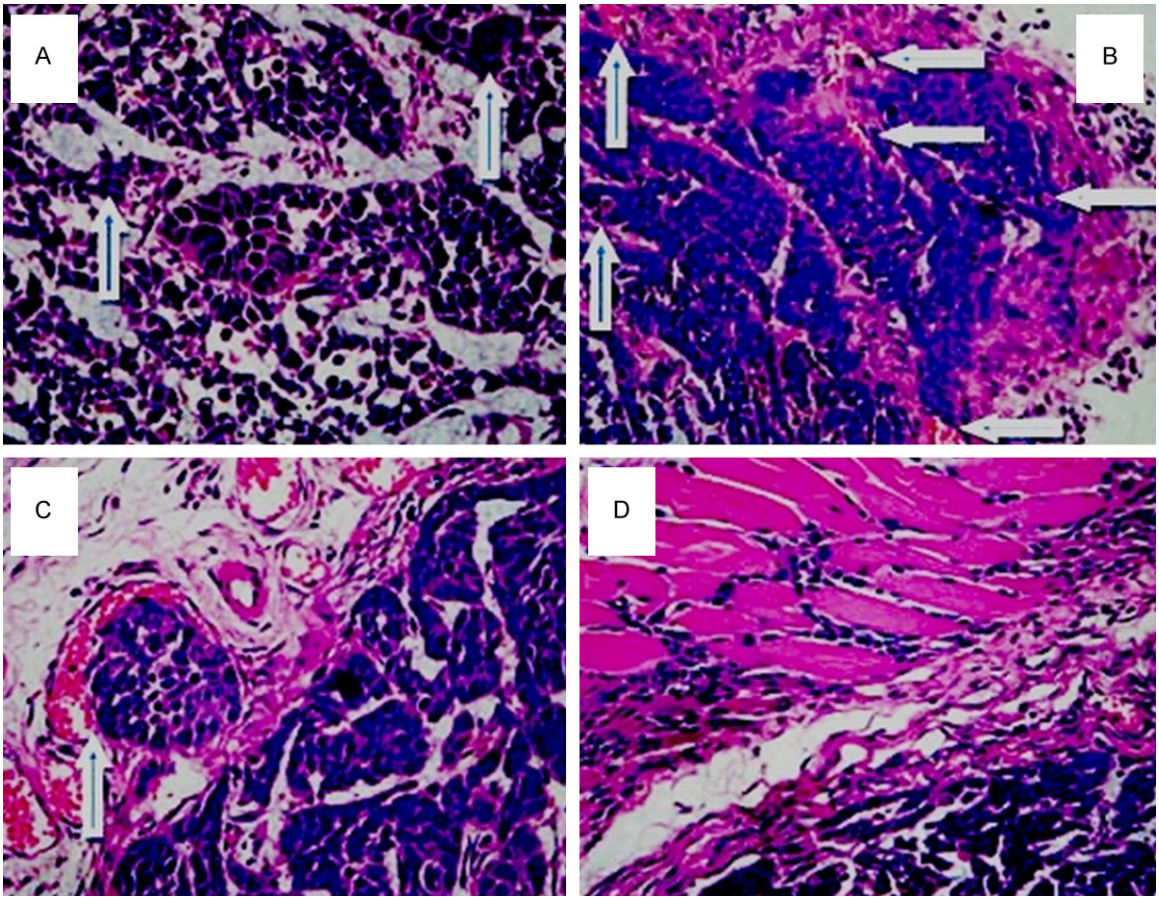

Figure 3. The pathological conditions of mock subcutaneous tumor were observed by microscope (HE*400). A: Arrows show multinucleated giant cell of tumor. B: Arrows show vessels of tumor. C: Arrow indicates oncocytes have infiltrated into vascular cavity. D: Oncocytes have infiltrated the muscular layer.

visceral metastases, each group was composed of 10 nude mice.

Evaluation of the xenografted tumor growth

The morphologic changes were observed by inverted microscope on three group cells. The expressions of TSLC1 were detected by RT-PCR. General conditions of nude mice were recorded every day. The growth of tumors: The long and short diameters and thickness were measured with vernier gauges of subcutaneous tumors every other day. The three lengths were perpendicular. Rate of tumor suppression was calculated as (the average volume of blank group tumors - the average volume of transfected group tumors)/the average volume of blank group tumors × 100%. Pathology examination: The size of subcutaneous and visceral metastases in nude mice, extent of infiltration, differentiation degrees of tumor tissue, as differences of tumor angiogenesis.

Statistical analysis

All statistical analyses were conducted using the SPSS 17.0 software package. The data variables were presented by mean ± standard deviation ($\bar{x}$ ± SD). If the variance was aligned, ANOVA q test was used for comparison between three groups. If variances are not aligned, using rank sum test. All tests were two-sided. The *P* values less than 0.05 were considered statistically different.

Results

General condition of nude mouse

The life condition of the group of nude mouse carrying xenograft with ECa09 transfectant with TSLC1 was normal all the time, voracious and active. The blank group and the group carrying xenograft with mock Eca109 transfectant

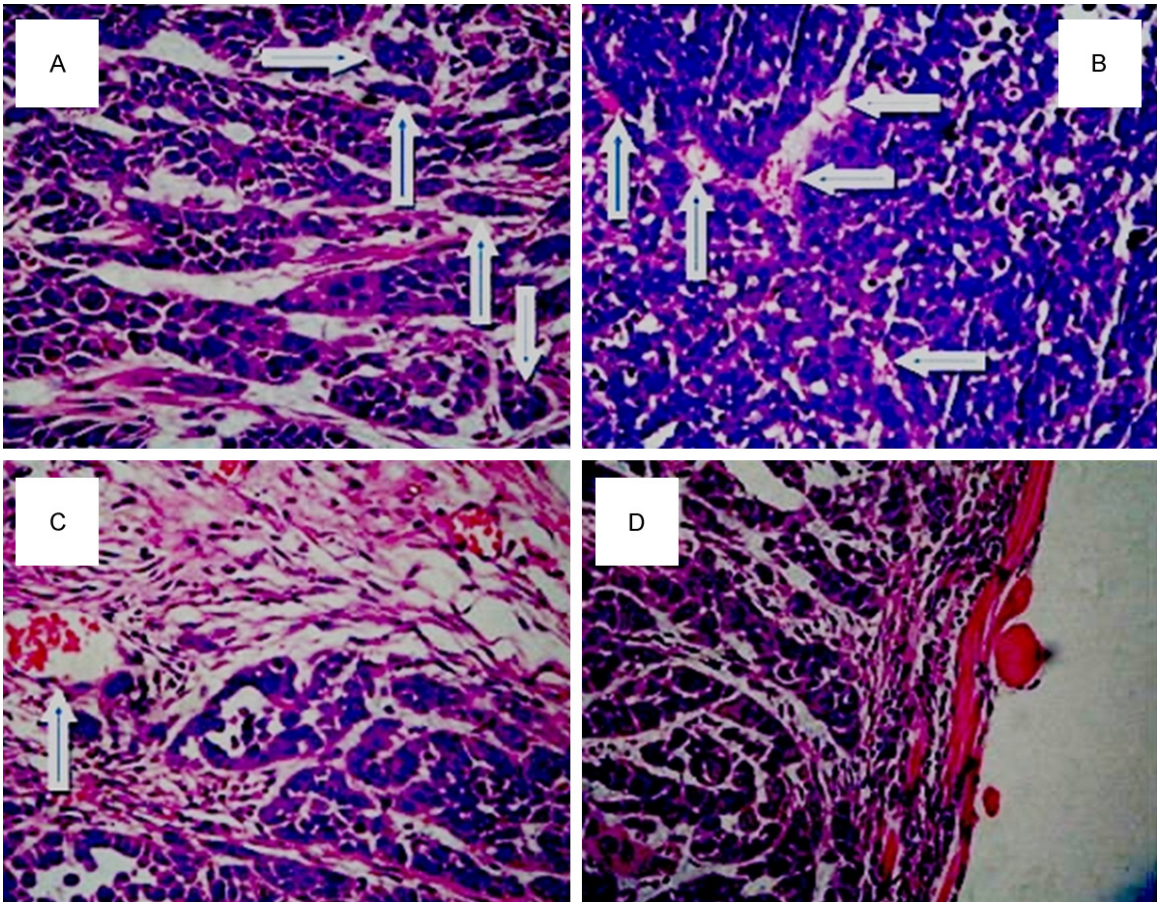

Figure 4. The pathological conditions of blank subcutaneous tumor (HE*400). A: Arrows show multinucleated giant cell of tumor. B: Vessels of tumor (arrow). C: Arrow indicates oncocytes have infiltrated into vascular cavity. D: Oncocytes have infiltrated the muscular layer.

lost appetite gradually, listlessness and oscitancy.

The growth of tumors

Palpable tumors were identified in blank group injected with parental Eca109 cells in five days after inoculation. The average volume of tumor was 272.42 mm^3. The tumors also began to appear in mock group xenografted with mock Eca109 in five days after inoculation. The average volume of tumor was 256.34 mm^3. The tumors began to appear in group xenografted with TSLC1 transfectant of Eca109 in ten days after inoculation. The average volume of tumor is 162.07 mm^3 (**Table 1** and the **Figure 1**). ANOVA q test was used to compare the tumor volumes of the three groups of at different times. After 10 days, the difference between experimental group and blank group (transfectant vs non-transfectant Eca109) was $p = 0.02$, experimental group and control group (transfectant vs mocked Eca109) was $P = 0.03$, controlled group and blank group was $P = 0.878$; after 17 days, the difference between experimental group and blank group was $p < 0.0001$, experimental group and control group was $p < 0.0001$, controlled group and blank group was $P = 0.822$. After 24 days, the difference between experimental group and blank group was $p < 0.0001$, experimental group and control group was $p < 0.0001$, controlled group and blank group was $P = 0.822$. After 30 days, the difference between experimental group and blank group was $p < 0.0001$, experimental group and control group was $p < 0.0001$, controlled group and blank group was $P = 0.488$. The values of *P* less than 0.05 were considered statistically significant. The differences between experimental group and blank group, experimental group and control group had statistically different tumor volumes at the same

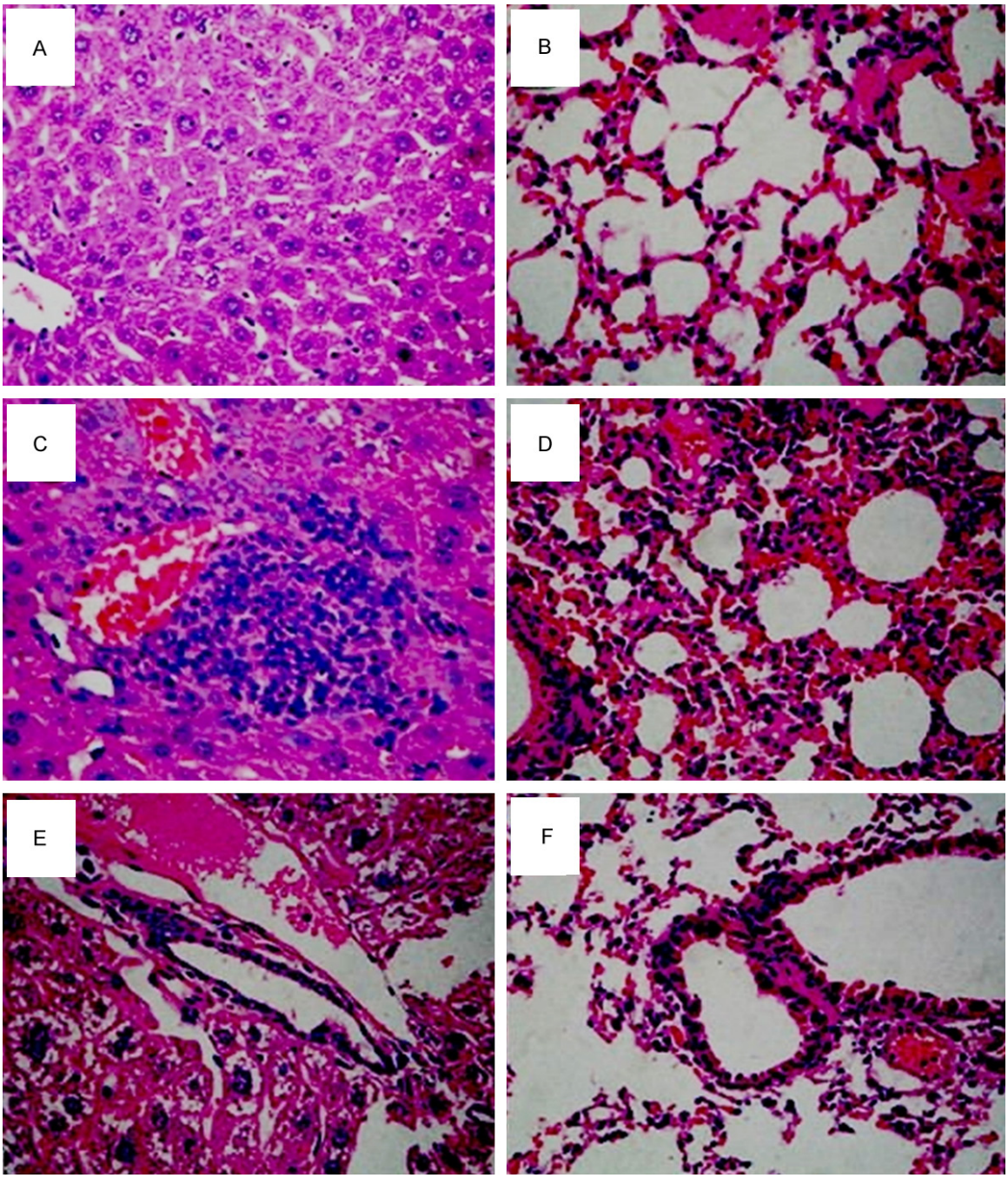

Figure 5. Microscopically observation of livers and lungs of nude mice in each group (HE*400). A: Liver of nude mice in transfected group under microscope. B: Lung of nude mice in transfected group under microscope. C: Liver of nude mice in mock group under microscope. D: Lung of nude mice in blank group under microscope. E: Liver of nude mice in blank group under microscope. F: Lung of nude mice in blank group under microscope.

time. Our data suggested that TSLC1 gene when stably expressed in human Esophageal Carcinoma Cell Line Eca109 inhibited the tumor growth of the nude mice xenografts, and the rate of tumor suppressor = (272.42 - 162.07) /272.42 × 100% = 40.51%.

Histopathology examination

Subcutaneous tumors: Compared with blank and mock, tumor size in the group of xenograft with TSLC1 transfectant Eca109 was smaller and the infiltrating range was low, the degree of

differentiation of tumor cells was slightly better, and tumor angiogenesis was more pronounced. There was no obvious difference between blank group and mock group (**Figure 2**).

Histopathologically, in control and blank groups, the cancerous lesions contained mostly malignant cells, with slurry nuclei and less cytoplasm. They contained less interstitial tissue. Cancer cells were of different forms and could be presented fusiform, round, oval or irregular shapes. The volumes of the cells were greatly varied. Pathological mitotic and multinucleated giant cell could be seen with the lesions, and the mucilage could be seen in part of the cells. Large amount of small blood vessels were seen in the tumor lesions, suggesting of active angiogenesis and rich blood supply to the tumor lesions. The cancer cells penetrated into blood vessels and reached myometrial (**Figures 3**, **4**). In blank group in which the nude mice were xenografted with parental Eca109 cells, angiogenesis could also be identified in the tumor lesions, and had rich blood supply to them, but not keratosis and intercellular junction. The cancer cells infiltrated into blood vessels and reached myometrial (**Figure 4**).

Visceral tumors: One mouse was selected randomly from three groups of nude mice at the fourth week and was then sacrificed by cervical dislocation. There were no metastases to lungs and liver tissue of these nude mice according to our observation. The inflammatory cells could be observed in the internal organs of the blank and control groups of nude mice and distributed densely along the vessel, but not in the experimental group nude (**Figure 5**).

Discussion

TSLC1 is expressed in normal tissues of the human body, especially in the tissues of skin, lungs and liver [14]. But in tissue and passaged cell lines of tumors of different origins, its expression is low or absent.The loss of its expression has been observed in laryngeal, cervical, breast, nasopharyngeal, lung and liver cancers, as well as in adult acute lymphoblastic cell leukemia (ALCL) and other malignant tumors. Although the role of TSLC1 in the genesis of esophageal cancer is not very clear yet, the promoter by DNA methylation of CpG islands and heterozygosity deletion (LOH) leading to inactivation of TSLC1 has been observed, and known to play an essential role in esophageal cancer occurrence, with impacts on tumor invasion, metastasis and apoptosis. Ito et al [11] have reported that TSLC1 expression was lost in 75% of esophageal squamous cell carcinoma (ESCC) cell lines and 50% of primary esophageal cancer as assayed with RT-PCR. Clinicopathologically, the loss of TSLC1 expression is correlated with the degree of infiltration and metastasis of esophageal cancer. Fukami [15] has found that absence of expression of TSLC1 in esophageal carcinoma was due to methylation on upstream promoter region of TSLC1. The expression of TSLC1 gene was also absent in lung cancer cell lines [16], malignant meningioma cell line IOMM-Lee [17] and prostate cancer cell line T3B [18]. Lu found that the expression of TSLC1 was significantly reduced in laryngeal squamous cell carcinoma (LSCC) tissues and cell lines [3].

It has been reported that its restored expression suppresses the cells of above-mentioned cancers to proliferate. Mao et al [19] compared the inhibition of tumor growth and invasion by A549 cells transfected with TSLC1 and the parental cells by assaying cell proliferation, colony-forming ability and the rate of in vivo tumor formation, and significant difference was demonstrated. He et al. have shown that fused protein SD55-TSLC1 inhibits growth of Huh7 liver cancer cells xenograft and might be a potent antitumoral agent [20]. Huh7 cells have lower expression of TSLC1. The re-expression of TSLC1 gene is believed to exert tumor suppression by inhibiting cell growth and proliferation; it induces apoptosis in tumor cell through activation of death proteases caspase-8 and caspase-3. Ectopic expression TSLC1 potentiates death receptor induced apoptosis by inducing caspase-8, which is one of the upstream caspases recruited to the death inducing signaling complex (DISC) formed in death receptor pathway of apoptosis and its activation triggers the cascade of downstream effectors caspases, leading to the induction of morphological apoptosis. Caspase-3 is among downstream caspases and directly executes the end effects in apoptotic pathway [12]. Liang et al. has shown that the network and pathway of TSLC1-mediated tumor suppression via its multi-regional structure, cell adhesion and intracellular signaling [12].

Clinicopathologically, the loss of TSLC1 expression is correlated with the degree of infiltration and metastasis of esophageal cancer. Liang et al [21] also found that the expression of TSLC1 could be used as biomarkers in clinical diagnosis and potential therapeutic targets. But the study of TSLC1 has been limited to in vitro system of cultured cells but not in vivo xenograft in nude mice. In the present study, the possible mechanism of TSLC1 in the genesis and development of esophageal cancer was investigated by inoculating Eca109 cells stably transfected with TSLC1, subcutaneously to nude mice and the phenotypic change in terms of tumor suppression exerted by the introduced gene was characterized in the xenograft based on the line Eca109.

TSLC1 gene inhibited the growth, proliferation, infiltration and tumor angiogenesis in Eca109 as assayed in nude mice xenograft tumor. Its expression level may serve as an effective indicator on progression and prognosis of esophageal cancer; and the current findings warrant future efforts with different methods to further clarify the pathogenesis of esophageal cancer and the work would provide a theoretical basis for using TSLC1 as the early diagnosis of tumors and an ideal target for gene therapy.

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Disclosure of conflict of interest

None.

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