Original Article

Inhibitory Role of Pentraxin-3 in Esophageal Squamous Cell Carcinoma

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Abstract

Background: Esophageal cancer is the sixth leading cause of cancer-related death worldwide. Pentraxin-3 (PTX3) is a member of the PTX superfamily. Here, we investigated the role of PTX3 in esophageal squamous cell carcinoma (ESCC).
Methods: The effect of PTX3 on ESCC cell proliferation, colony formation, apoptosis, migration, and invasion was investigated using cell viability assays, colony formation assays, flow cytometry, and migration and invasion assays. The effect of PTX3 on the tumorigenicity of ESCC *in vivo* was investigated with xenograft studies in nude mice.
Results: PTX3 overexpression in ESCC cells reduced cellular proliferation and colony formation ($P < 0.05$) and increased the rate of apoptosis ($P < 0.05$). PTX3 expression had no significant effect on the migratory or invasive potential of ESCC cells. In our mouse model of human ESCC, we achieved 100% successful tumor establishment. Compared with the control and empty vector-expressing groups, the PTX3-expressing group formed significantly smaller tumors ($P < 0.05$).
Conclusions: This study indicates that PTX3 might play an inhibitory role in ESCC.

Key words: Apoptosis; Esophageal Squamous Cell Carcinoma; Pentraxin-3; Proliferation

Introduction

Esophageal cancer is the eighth most common cancer and the sixth most common cause of cancer-related death worldwide; in 2012, there were an estimated 456,000 new cases and 400,000 deaths. About 80% of the esophageal cancer cases occur in developing countries, and China accounts for approximately half of the total cases and deaths worldwide.[1] Based on histology, more than 90% of the esophageal cancers are categorized as either squamous cell carcinoma or adenocarcinoma. Esophageal squamous cell carcinoma (ESCC) is the most common esophageal cancer worldwide.[2] The mechanisms underlying ESCC carcinogenesis are currently unclear.

Pentraxins (PTXs) are a superfamily of evolutionarily conserved proteins that are characterized by their pentameric structure.[3-5] PTXs play a pivotal role in vascular biology and are the essential components of innate humoral immunity.[6,7]

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Quick Response Code:

Website: www.cmj.org

DOI: 10.4103/0366-6999.189921

PTXs are divided into two subfamilies: short-PTXs and long-PTXs. Long-PTXs differ from short-PTXs by the presence of an unrelated N-terminal domain that is coupled to the C-terminal domain.[8]

PTX3 is the prototypical long-PTX.[9-11] Unlike short-PTXs, PTX3 is produced at the extrahepatic sites of inflammation by a number of different cell types;[6,12] at these sites, PTX3 appears to regulate complement activation.[6,9,13] PTX3 is produced by dendritic cells, macrophages, fibroblasts, and activated endothelial cells

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Received: 28-03-2016 **Edited by:** Peng Lyu
How to cite this article: Ma D, Zong Y, Zhu ST, Wang YJ, Li P, Zhang ST. Inhibitory Role of Pentraxin-3 in Esophageal Squamous Cell Carcinoma. Chin Med J 2016;129:2233-40.

in response to inflammation,[6,12] as well in other tissues, such as the heart and kidney.[7,13,14]

Recent findings suggest an insidious relationship between the complement cascade and cancer. PTX3 facilitates the dysregulation of mitogenic signaling pathways, sustains cellular proliferation, angiogenesis, insensitivity to apoptosis, cancer cell invasion and migration, and tumor immunosurveillance escape.[9,13]

Our previous study showed that PTX3 expression is reduced in ESCC tumor tissues compared with adjacent nontumor tissues.[15] In the present study, we aimed to determine the effects of PTX3 in ESCC *in vitro* and *in vivo*.

Methods

Cell lines and cell culture

EC109 and KYSE-450 cells were provided by the Cancer Institute and Hospital, Chinese Academy of Medical Sciences. EC109 and KYSE-450 cells were routinely cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and antibiotics (100 U/ml penicillin G and 100 mg/ml streptomycin) at 37°C in a humidified atmosphere with 5% CO_2.

Cloning of pentraxin-3 cDNA and expression vector construction

An expression construct containing the full-length human *PTX3* cDNA sequence was generated by polymerase chain reaction (PCR) amplification (primers: Sense 5′-TATGGATCCATGCATCTCCTTGCGATTCTG-3′; antisense 5'-CTAGTCTAGATTATGAAACATACTGAGCTCCTCC-3'). PCR products were cloned into the pEasy-T1 vector (Transgene, Beijing, China), and the *PTX3* fragment was confirmed by sequencing (Invitrogen, Carlsbad, CA, USA). The *PTX3* fragment was excised using BamHI and XbaI restriction enzymes (NEB, USA) and cloned into the BamHI/XbaI sites of pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA).

Establishment of stable clones

EC109 and KYSE-450 cells were transfected with the pcDNA3.1(+)-PTX3 expression construct using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Briefly, exponentially growing EC109 and KYSE-450 cells seeded in 60 mm dishes were washed with serum-free medium and treated with DNA-Lipofectamine 2000 complexes containing 4 μg of PTX3 plasmid and 10 μl of Lipofectamine 2000. After 6 h incubation, complete medium with serum was added. Once confluence was reached, the cells were split in a ratio of 1:10 into the selection medium (complete medium supplemented with 600 or 500 mg/L G418) to isolate the transformed clones. EC109 and KYSE-450 cells were also transfected with the empty pcDNA3.1(+) expression vector as a control. Positive clones were routinely maintained in the selection medium.

Western blotting

Equal amounts of total protein cell lysates were separated by 12% sodium dodecyl sulfate-polyacrylamide electrophoresis and electrophoretically transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated with an anti-PTX3 antibody (1:1000; Epitomics, Burlingame, USA) at 4°C overnight, washed in tris-buffered saline-Tween 20, and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. β-actin, used as the loading control, was detected using a mouse anti-β-actin monoclonal antibody (Santa Cruz, TX, USA).

Cell viability assay

ESCC cell lines were cultured in 200 μl culture medium at a density of 5×10^3 cells/well in 96-well plates with 20 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) added to each well. After 24 h, 48 h, and 72 h of culture, cell viability was assessed by replacing the culture medium with serum-free RPMI-1640 containing 0.2 mg/ml MTT and the cells were incubated for 4 h at 37°C. The supernatant was then removed, and 150 μl dimethyl sulfoxide was added to each well to dissolve the MTT formazan. After lightly vortexing the plate for 10 min, the optical density of each well at 490 nm was recorded using a microplate reader (Model 450, Bio-Rad, CA, USA).

Colony formation assay

A total of 250 cells were seeded into 6-well plates and cultured in 3 ml of medium at 37°C with 5% CO_2 for approximately 14 days until colonies are formed. To determine the number of colonies, the supernatant was removed, and the cells were fixed with methanol and stained with Giemsa (Sigma-Aldrich, USA).

Migration and invasion assays

Migration and invasion assays were performed *in vitro*. Approximately, 0.7×10^5 EC109 cells or 1×10^5 KYSE-450 cells, suspended in 0.5 ml of serum-free medium, were seeded in the upper chamber of BD chambers for migration assays or BD BioCoat™ Matrigel™ chambers for invasion assays (Becton Dickinson, USA). In the lower chambers, 0.75 ml medium containing 10% FBS was added. After incubation for 24–36 h, nonmigratory or noninvasive cells, on the upper surface of the Boyden chambers, were wiped off, and the cells on the lower surface of the Boyden chamber, considered to be the migratory or invasive cells, were fixed with methanol and stained with Giemsa.

Annexin V/7-amino-actinomycin D apoptosis assay

Apoptosis was measured using the Annexin V-phycoerythrin (PE)/7-amino-actinomycin D (7-AAD) Kit (Becton Dickinson, Franklin Lakes, NJ, USA), according to the manufacturer's instructions. Briefly, collected cells were washed with cold phosphate-buffered saline and resuspended in binding buffer at a density of 1×10^6 cells/ml. Cells (1×10^5) were then incubated with 5 ml of Annexin V-PE

and 7-AAD at 25°C for 15 min in dark before adding 400 ml of binding buffer to each tube. Apoptosis was analyzed using an ABI flow cytometer (ABI, USA) within 1 h.

Tumor xenograft study

Five-week-old male athymic BALB/c nude (nu/nu) mice were purchased from the Academy of Military Medical Sciences (Beijing, China, No. SCXK [Military] 2012-0004) and maintained under specific pathogen-free conditions according to the Institutional Guidelines for the Care and Use of Laboratory Animals. They were divided into three groups (n = 6): PTX3, vector, and control. Wild-type, vector-expressing, or PTX3-expressing cells (4×10^6) were injected subcutaneously into the right flank mice, and tumors were allowed to develop. Tumors were measured with digital calipers, and the tumor volume was calculated using the following formula: Volume (mm^3) = $(width)^2 \times length/2$.

Statistical analysis

All data are representative of at least three independent experiments, which were conducted using cells from separate cultures. Results are expressed as the mean ± standard deviation (SD). Differences between groups were analyzed using the Student's t-test, and repeated measures analysis of variance (ANOVA), followed by Tukey's multiple comparison, was used for the comparison of more than three groups. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Pentraxin 3 suppresses the proliferation of esophageal squamous cell carcinoma cell lines

Cellular proliferation was measured using the MTT assay. The expression of PTX3 in EC109 and KYSE-450 cells [Figure 1] reduced proliferation [Figure 2], indicating that PTX3 negatively regulates ESCC cellular proliferation. The rates of growth inhibition in EC109 and KYSE-450 cells were 16.18% and 17.26%, respectively.

Colony formation assay

Representative images of colonies and the average number of colonies formed in wild-type, vector-expressing, and PTX3-expressing EC109 and KYSE-450 cells are shown in Figure 3a and 3b. Both the PTX3-expressing cell lines formed significantly fewer and smaller colonies than those formed by the wild-type and vector-expressing EC109/KYSE-450 cells. These results indicate that PTX3 expression reduces colony formation in ESCC cells.

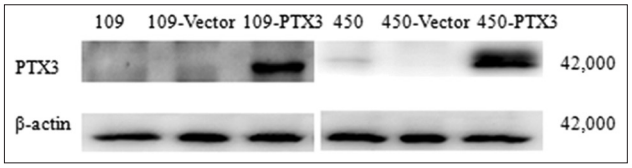

Figure 1: Western blotting analysis of PTX3 expression after transfection. Expression of PTX3 in wild-type, vector-expressing, and PTX3-expressing stably transfected EC109 and KYSE-450 cells. β-actin was used as an endogenous control. PTX3: Pentraxin-3.

Pentraxin 3 increases esophageal squamous cell carcinoma apoptosis *in vitro*

The percentage of apoptotic cells in EC109-PTX3, EC109-vector, and EC109-wild-type cell cultures was 10.35%, 2.24%, and 1.82%, respectively. In KYSE-450-PTX3, KYSE-450-vector, and KYSE-450-wild-type cells, the rates of apoptosis were 10.10%, 2.73%, and 3.01%, respectively. Expression of PTX3 significantly increased apoptosis in EC109 and KYSE-450 cells [Figure 4a and 4b].

Pentraxin 3 expression does not affect esophageal squamous cell carcinoma migration and invasion *in vitro*

We used two modified Boyden chamber assays to investigate the migration and invasion of ESCC cell lines. The expression of PTX3 did not affect the migration or invasion of the studied ESCC cell lines [Figure 5a and 5b].

Upregulation of pentraxin 3 inhibits the tumorigenicity of esophageal squamous cell carcinoma cells *in vivo*

Our *in vitro* studies indicated that PTX3 plays an important role in ESCC cell proliferation, colony formation, and apoptosis. To investigate the effect of PTX3 in ESCC cells *in vivo*, we performed a tumor xenograft study using EC109 and KYSE-450 cells. EC109-PTX3 and KYSE-450-PTX3 cells formed smaller tumors in nude mice than EC109-vector, EC109-wild-type, KYSE-450-vector, or KYSE-450-wild-type cells [Figure 6a]. The average tumor volume in mice bearing PTX3-expressing EC109 and KYSE-450 cells was 875.97 and 902.26 mm^3, respectively. In mice bearing EC109-vector or KYSE-450-vector tumors, the average tumor volume was 1485.00 and 1433.66 mm^3, respectively; this was similar to that observed with wild-type EC109 and KYSE-450 cells, which produced average tumor volumes of 1452.07 and 1425.19 mm^3, respectively [Figure 6b]. No significant weight differences were observed between the three groups [Figure 6c].

Discussion

Inflammatory cells and molecules are the essential components of the tumor microenvironment.[16-19] It is widely accepted that there is a causal relationship between inflammation, innate immunity, and cancer. PTX3 has been shown to facilitate the dysregulation of mitogenic signaling pathways and promote cellular proliferation, angiogenesis, insensitivity to apoptosis, cancer cell invasion and migration, and tumor escape from immunosurveillance.[9,13] As such, the relationship between PTX3 and cancer has been studied in many different tumor types.

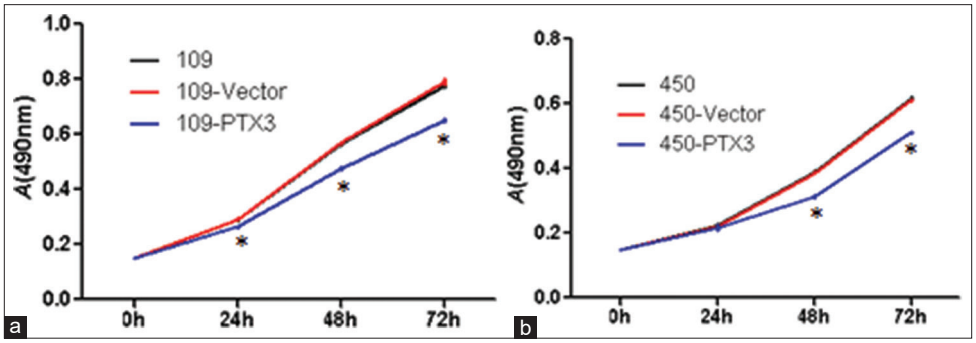

Figure 2: PTX3 expression suppresses esophageal squamous cell carcinoma cell proliferation. Proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide. Proliferation was significantly reduced in PTX3-transfected EC109 and KYSE-450 cells relative to control cells ($P < 0.05$). The rates of inhibition for PTX3-expressing EC109 and KYSE-450 cells were 16.18% (a) and 17.26% (b), respectively. *A*: Absorbance; PTX3: Pentraxin-3.

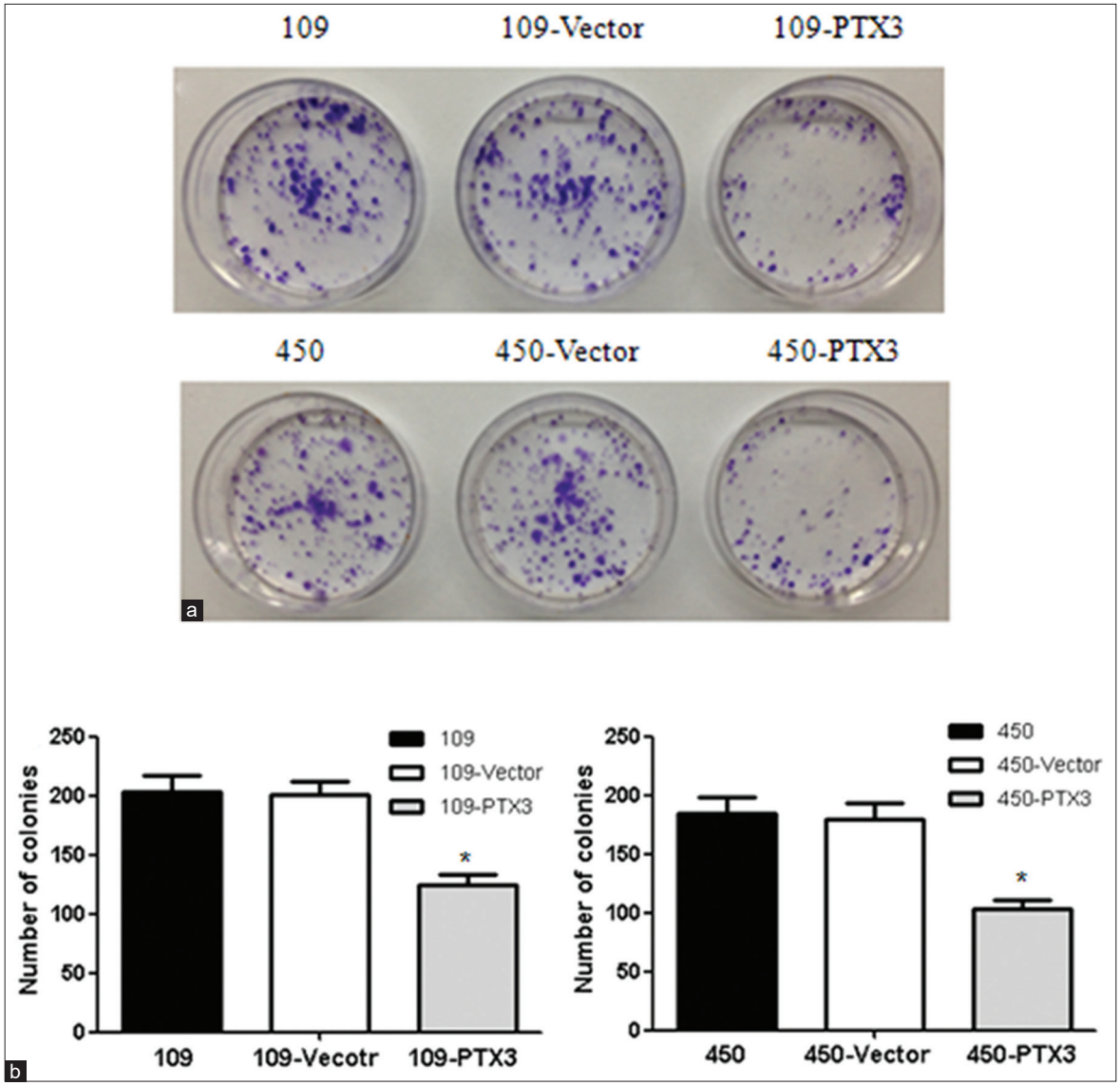

Figure 3: PTX3 expression inhibits esophageal squamous cell carcinoma cell colony formation. (a) Results from the EC109 and KYSE-450 cell colony formation assays. (b) PTX3-expressing cells formed significantly fewer colonies than wild-type and vector-expressing cells (*$P < 0.05$). PTX3: Pentraxin-3.

Studies on human ESCC have shown that the *PTX3* promoter is hypermethylated in ESCC, and that this results in *PTX3* gene silencing.[15] Bonavita *et al.*[20] found that methylation of the *PTX3* promoter region, CpG islands, were progressively increased from normal colon epithelium to adenoma and colorectal cancer, independent of tumor stage. They also

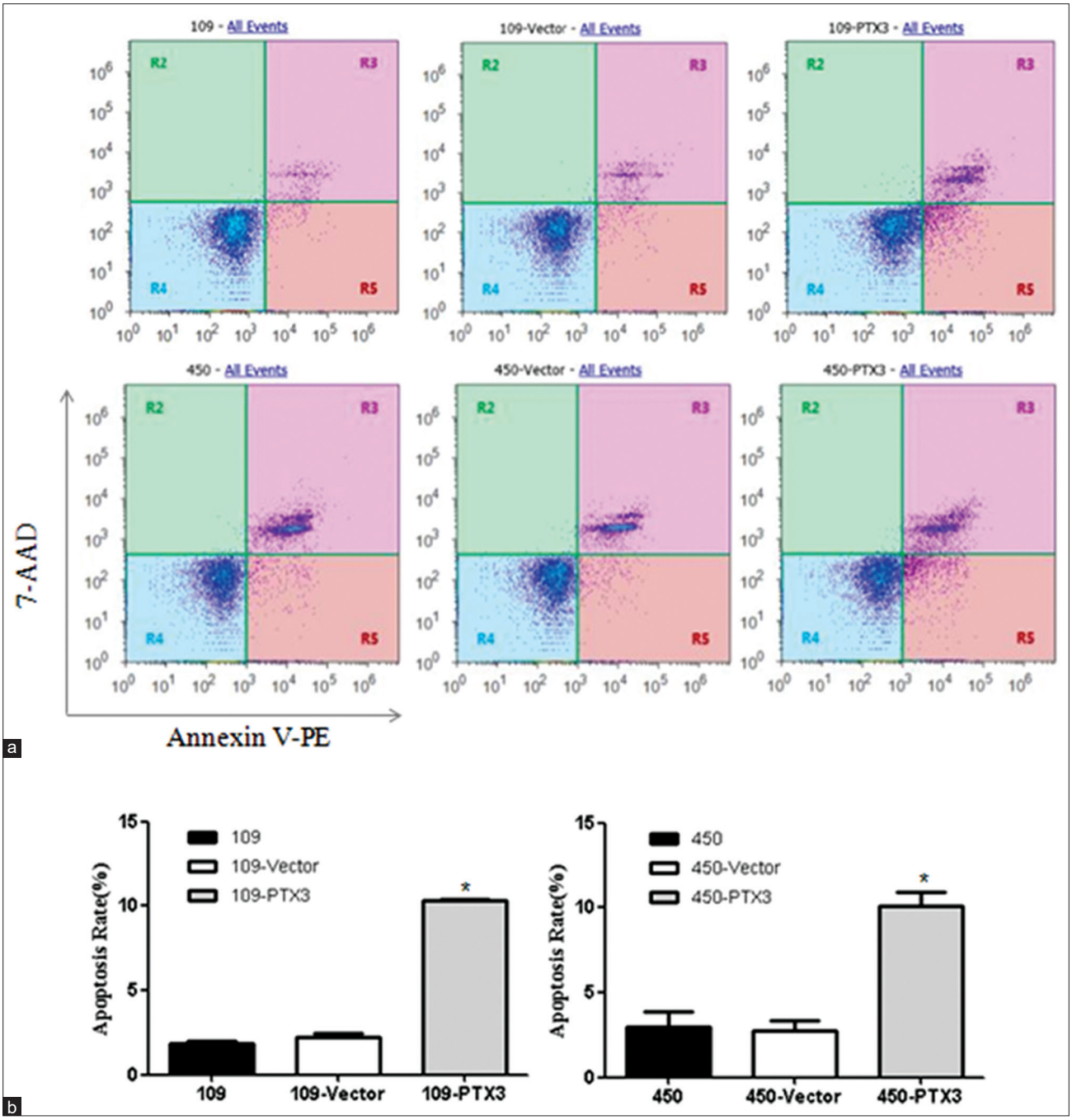

Figure 4: PTX3 expression increases esophageal squamous cell carcinoma cell apoptosis. (a) Rates of cell apoptosis were assessed by flow cytometry, and cells were stained with Annexin V-PE/7-AAD. (b) The total apoptosis rate is the sum of the early apoptosis rate and the late apoptosis rate. PTX3 expression significantly increased the rate of EC109 and KYSE-450 apoptosis (*$P < 0.05$). PE: Phycoerythrin; 7-AAD: 7-amino-actinomycin D; PTX3: Pentraxin-3.

showed that PTX3 plays a pivotal role in controlling tumor growth, and that the tumor suppressor role of PTX3 is related to its ability to modulate the inflammatory response. These studies suggest that *PTX3* expression is epigenetically repressed in selected human tumors; this potential evidence of an innate humoral immunity effector molecule acting as a candidate cancer gene could provide a vital missing link in the relationship between inflammation and cancer.

A number of studies also indicate a potential therapeutic role for PTX3 in selected tumors: Ronca *et al.*[21] identified PTX3 as a potent fibroblast growth factor (FGF) antagonist endowed with anti-angiogenic and anti-neoplastic activity in prostate cancer; Margheri *et al.*[22] stably transfected two breast cancer cell lines with *PTX3* and found that it reduced tumor growth *in vivo*; and a long PTX3-derived pentapeptide has been shown to inhibit the proliferation and angiogenic potential of FGF8b-dependent tumor cells.[23]

There is currently some controversy regarding the exact role of PTX3 in carcinogenesis, and some studies indicate an oncogenic role for PTX3. Choi *et al.*[24]

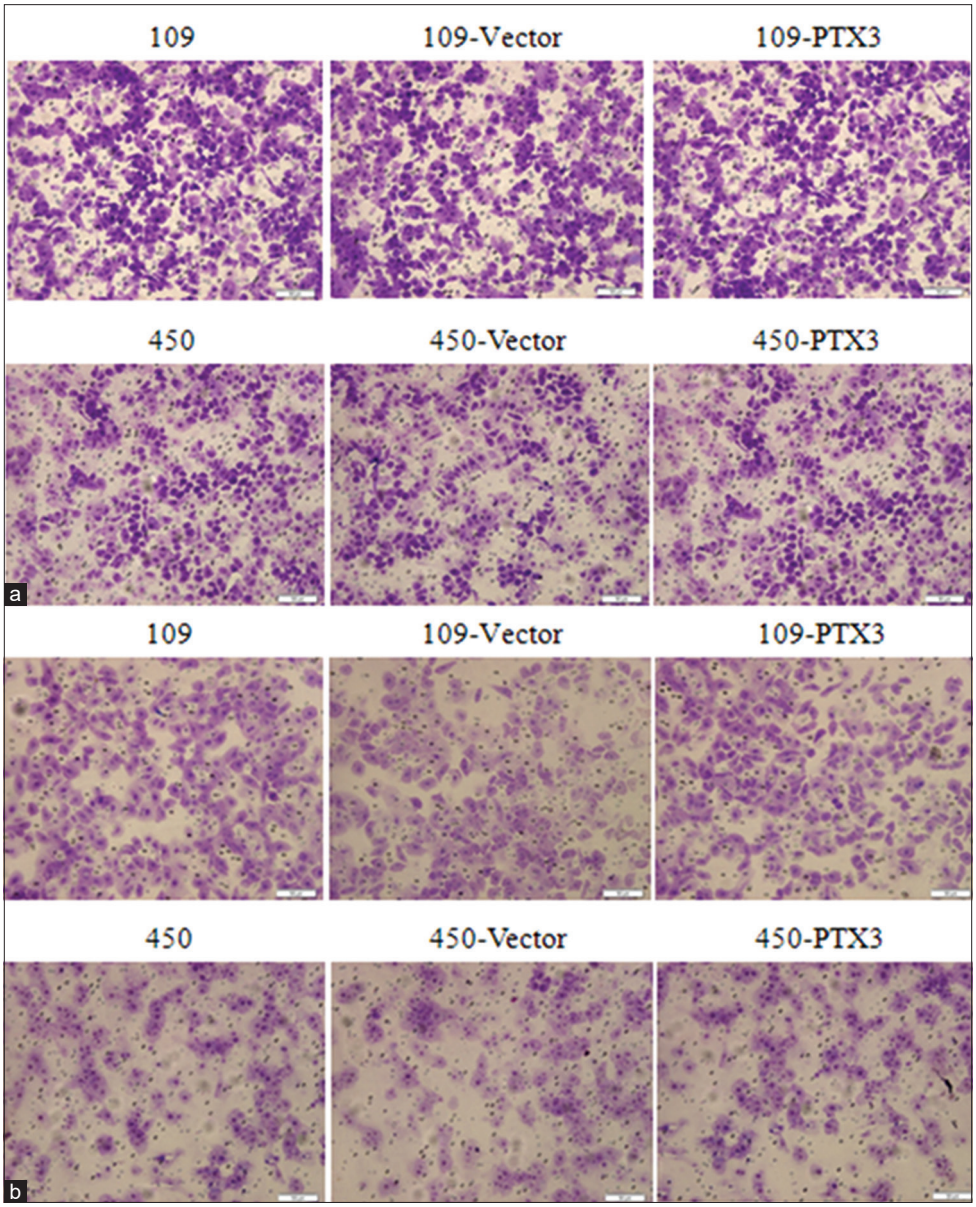

Figure 5: PTX3 expression does not affect the migration or invasion of esophageal squamous cell carcinoma cells *in vitro*. Wild-type, vector-expressing, or PTX3-expressing EC109 and KYSE-450 cells were seeded in Boyden chambers for migration and invasion assays. Migratory or invasive cells were fixed, stained with Giemsa, and counted using a microscope with ×200. No differences in migration (a) or invasion (b) were observed between the groups. PTX3: Pentraxin-3.

showed that elevated expression of PTX3 was correlated with a poor survival in patients with breast cancer, and showed that *PTX3*-silencing, using a *PTX3*-specific siRNA, prevented breast cancer cell migration. These conflicting results could be due to PTX3 serving a multifunctional role, or by PTX3 exerting specific effects in different tumor types.

The study had several limitations. The level of PTX3 is neither tested in the serum nor the tissue of the healthy population and the esophageal cancer patients in this study, although our previous study showed that PTX3 expression is reduced in ESCC tumor tissues compared with the adjacent nontumor tissues.[15] The functional form of the constructed PTX3 expression vector and the mechanism of the inhibitory role of PTX3 in ESCC are not studied in this article. All these issues will be studied further.

We have investigated the role of PTX3 in ESCC carcinogenesis. We have shown that in ESCC cell lines, PTX3 reduces proliferation and colony formation, increases apoptosis, and exerts no effect on the migratory or invasive capacity of cells. PTX3 expression in ESCC cell lines also inhibited tumor growth *in vivo*. Our results suggest that PTX3 plays an oncosuppressor role in ESCC. Further studies are required to elucidate the exact mechanism underlying this and to determine the potential therapeutic role of PTX3 in ESCC.

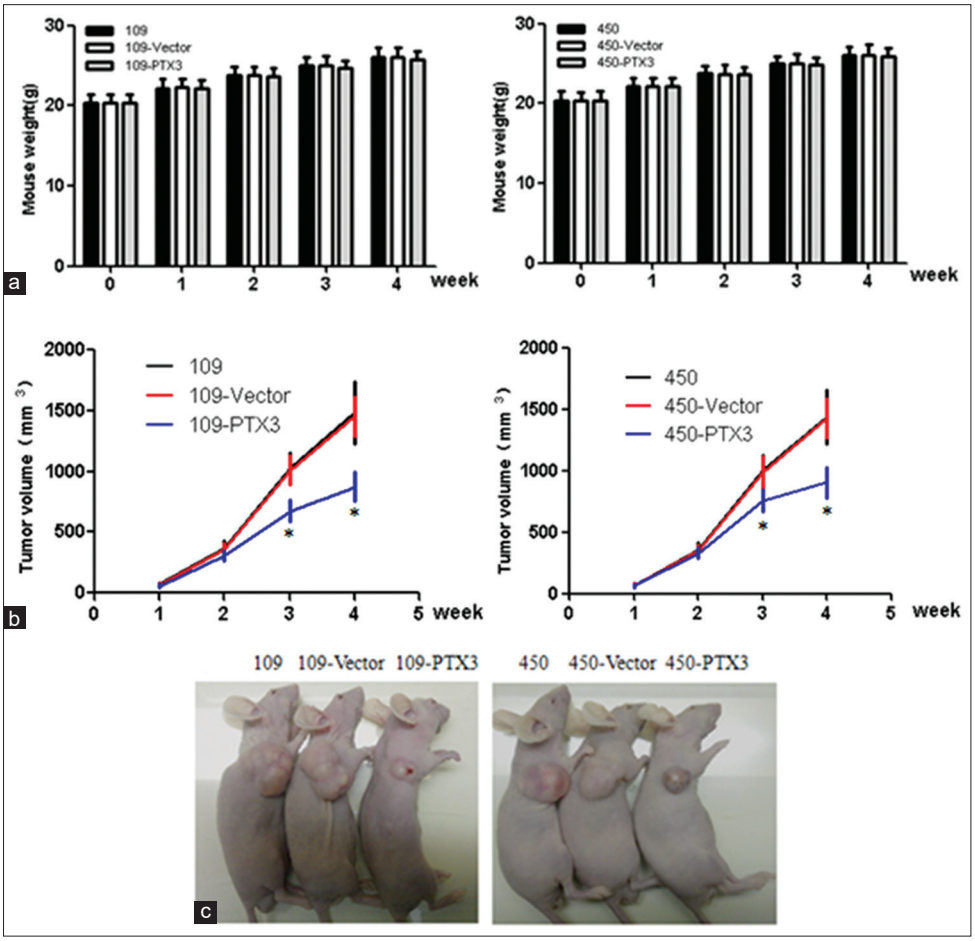

Figure 6: PTX3 expression suppresses esophageal squamous cell carcinoma cell growth *in vivo*. Wild-type, vector-expressing, or PTX3-expressing EC109 or KYSE-450 xenografts were established in nude mice ($n = 6$). (a) Average mouse weights ± standard deviation. There were no significant weight differences in mice among the three groups. (b) Tumor growth curves showed PTX3 expression suppressed xenografts growth (*$P < 0.05$). (c) Representative images of the tumors formed. PTX3: Pentraxin-3.

Financial support and sponsorship

This research was supported by grants from National Natural Science Foundation of China (No. 81101842, No. 81272447, No. 81570507, and No. 81302160), Beijing Training Project for the Leading Talents in S and T (No. ljrc201518), Beijing Municipal Administration of Hospitals Clinical Medicine Development of Special Funding Support (No. ZY201308), Beijing Natural Science Foundation Program and Scientific Research Key Program of Beijing Municipal Commission of Education (No. KZ201410025024), and Capital Medical Development Research Foundation (No. 2014-1-2021).

Conflicts of interest

There are no conflicts of interest.

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