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The effects of Tim-3 activation on T-cells in gastric cancer progression

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Abstract. The incidence of gastric cancer is high, especially in China. The present study aims to provide a novel therapeutic target for gastric cancer. Peripheral blood, cancerous and paracancerous tissues were collected from patients with gastric cancer. T-cell immunoglobulin mucin domain-3 (Tim-3) expression in T-cells was measured and the correlation between Tim-3 expression and the T staging of gastric cancer was analyzed. The levels of T-cell secreted interferon (IFN)-γ and tumor necrosis factor (TNF)-α were assessed following Tim-3 signaling pathway activation. A nude mouse model of gastric cancer was established and Tim-3-stimulated T-cells were injected into the mice to evaluate tumor growth. The results of the present study demonstrated that Tim-3 expression levels from the paracancerous and cancerous gastric tissues were significantly increased compared with the peripheral blood, while its expression was significantly increased in cancerous compared with paracancerous gastric tissues. With the T staging of gastric cancer increasing, the expression of Tim-3 gradually increased. The activation of the Tim-3 signaling pathway in T-cells may inhibit IFN-γ and TNF-α secretion, and the results from the nude mice tumor model demonstrated that the inhibitory effect on tumor growth by T-cells was reduced by Tim-3 signaling pathway activation. The expression level of Tim-3 on the surface of tumor infiltrating T-cells in gastric cancer tissue increases significantly and the increased Tim-3 signaling may inhibit the function of T-cells. The results suggest that the increased expression of Tim-3 on T-cells may be involved the development of gastric cancer.

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Key words: gastric cancer, T-cells, flow cytometry, T-cell immunoglobulin and mucin domain-3, nude mice

Introduction

Differences exist between the incidence of gastric cancer in a number of countries due to several factors including race and dietary habits. Gastric cancer incidence in eastern Asia, especially in China, where there were ~400,000 new cases in 2010, accounting for 42% of the global total (1). At present, gastric cancer is one of the most common diseases resulting in the mortality of patients with cancer (2,3). Gastric cancer is difficult to be detected early and the majority of patients with gastric cancer are diagnosed at Stage II or III (4). Studies demonstrate that the five-year survival rates of Chinese patients with gastric cancer at Stage II and III are ~70 and 40%, respectively (4,5). Clinical data demonstrated that there are a number of difficulties in treating patients with gastric cancer due to high recurrence rates and metastasis risks (6). T-cells may serve an important role in suppressing tumor progression. A previous study demonstrated that the immune system of gastric cancer patients and the antitumor function of T-cells are suppressed in gastric cancer, and therefore, resulting in tumor escape from the immune surveillance and disease progression (7).

Programmed death-1 (PD-1) and T-cell immunoglobulin mucin domain-3 (Tim-3) (8-11) are known to be associated with the inhibition of T-cell function. The expression and function of the T-cell surface PD-1 molecules in gastric cancer patients have been reported in several studies (8-10). The results demonstrate that the average expression levels of PD-1 on the surface of T-cells in the peripheral blood and cancer tissues of patients with gastric cancer increases significantly, and the ability of T-cells that express PD-1 to secrete interferon (IFN)-γ decreases significantly (8-10). However, to the best of our knowledge, no research regarding the expression of Tim-3 on the surface of the T-cells of gastric cancer patients, and the association with the development of gastric cancer has been reported. Therefore, a study of the expression of Tim-3 on the surface of T-cells in paracancerous and cancerous tissues of patients with gastric cancer and the effect on the function of T-cells may reveal the mechanism of immune inhibition in gastric cancer patients. Interfering with the immune inhibitory state of T-cells may be a novel therapeutic target for the treatment of gastric cancer.

In the present study, the expression of Tim-3 on the surface of T-cells in the peripheral blood, paracancerous and cancerous gastric tissues was analyzed using flow cytometry,

and the association between Tim-3 expression and T staging of gastric cancer was analyzed. Recombinant galectin-9 was used to activate the Tim-3 signaling pathway. Flow cytometry following intracellular staining was used to detect the ability of T-cells to secrete IFN-γ and tumor necrosis factor (TNF)-α. In addition, the effects of Tim-3 on T-cells inducing the inhibition of tumor growth were evaluated in a human gastric cancer xenograft model. The expression of Tim-3 in T-cells in paracancerous and cancerous gastric tissues, and the effect of Tim-3 activation on T-cell function were assessed to provide a scientific basis for novel therapeutic targets for gastric cancer.

Materials and methods

Reagents. The key instruments and reagents used in the present study included: The 37˚C constant temperature incubator with 5% CO_2 purchased from Thermo Fisher Scientific, Inc., (Waltham, MA, USA); the flow cytometer was purchased from BD Biosciences (Franklin Lakes, NJ, USA); cluster of differentiation (CD)3, Tim-3, IFN-γ/TNF-α antibody purchased from Biolegend Inc., San Diego, CA, USA; galectin-9 purchased from Cloud-Clone Corp., (Katy, TX, USA); the RPMI-1640 medium and fetal bovine serum were purchased from HyClone (GE Healthcare Life Sciences, Logan, UT, USA); Pan T Cell Isolation Kit was purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany); collagenase and DNase were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany); 70-μm cell strainer was purchased from BD Biosciences; and a total of 24 female BALB/c nude mice (6-7 weeks old; mean weight, 20 g) were purchased from Beijing Vital River Experimental Animals Co., Ltd., (Beijing, China). Mice were maintained in a regulated environment (23±1˚C, 50-60% humidity) under a 12 h light/dark cycle and provided food and water *ad libitum*.

Clinical data. A total of 22 patients (male:female, 12:10) with gastric cancer had been hospitalized in Weihai Municipal Hospital (Jinan, China) and Provincial Hospital Affiliated to Shandong University (Weihai, China), and underwent surgery between January 30, 2015 and December 30, 2015. None of the patients in the group received chemotherapy, radiotherapy or immunotherapy prior to the operation. Paracancerous and cancerous tissue specimens were obtained from patients with gastric cancer during the operation. The tissue specimens were immediately placed in physiological saline solution and tumor-infiltrating T-cells (TILs) were extracted within 30 min for the experiment. Histopathological diagnosis was performed using the specific diagnostic criteria described in the literature (4). The average age of the 22 patients was 55.06±16.83 years. The present study was approved by the Medical Ethics committee of Provincial Hospital Affiliated to Shandong University and Weihai Municipal Hospital. Written informed consent was received from all patients involved.

Peripheral blood mononuclear cells (PBMCs). Peripheral blood was obtained from patients with gastric cancer and then diluted using sterile RPMI-1640 to the ratio of 1:1. Following the dilution, the blood was added into a centrifuge tube, which contained the lymphocyte separation medium (the ratio of diluted blood to lymphocyte separation medium was 2:1) and then centrifuged for 20 min at room temperature, at a rate of 250 x g. White lymphocytes were extracted using a glass pipette and then the cells were added into a centrifuge tube containing 10 ml sterile RPMI-1640 and centrifuged at room temperature for 15 min at the rate of 250 x g. RPMI-1640 was then discarded and the cells were precipitated using 10 ml sterile RPMI-1640 and finally centrifuged at room temperature for 8 min at the rate of 200 x g.

Isolation of TILs. The gastric cancer tissue was placed into sterile PBS, then cut into pieces using surgical scissors and mechanical grind using the grinding rod. Subsequently, tissues were treated with 1 μg/ml collagenase, 25 μg/ml DNase, and 2% fetal bovine serum in PBS at 37˚C for 1-1.5 h. The tissue homogenates were filtered with a 70-μm cell strainer, and then centrifuged at room temperature for 15 min at the rate of 250 x g (12).

T-cell isolation. All types of T-cells (>90%) were purified by negative selection using a Pan T Cell Isolation kit (cat. no. 130-096-535; Miltenyi Biotec GmbH), according to manufacturer's protocol. In brief, non-T-cells were indirectly magnetically labeled by using a cocktail of biotin-conjugated antibodies and anti-Biotin MicroBeads. Highly pure untouched T-cells are obtained by depletion of the magnetically labeled cells. The cells were confirmed to be $CD3^+$ T-cells by flow cytometry.

Flow cytometry and intracellular staining method. A total of $1x10^5$ negative selection $CD3^+$ T-cells were washed using 1 ml PBS containing 1% bovine serum albumin (BSA) (Sigma-Aldrich; Merck KGaA) twice, each time for 5 min. After the supernatant was discarded, 0.1 μl PBS containing 1% BSA was added. Then, 5 μl fluorescein isothiocyanate (FITC) anti-Tim-3 (cat. no. 345021; Biolegend, Inc.) antibody was added for the incubation for 30 min at 4˚C. The solution was washed using 1 ml PBS containing 1% BSA twice, each for 5 mins. After the supernatant was discarded, 0.1 ml PBS containing 1% BSA was added for flow cytometry detection. Cytometric data were acquired by using a BD Accuri C6 flow cytometer (BD Biosciences). For intracellular staining, Galectin-9 was added into $1x10^5$ purified T-cells to a final concentration 5 μg/ml to activate the Tim-3 signaling pathway (11). After 72 h, cells were stimulated with 2 μl of the cell activation cocktail (cat. no. 423303; Biolegend, Inc.) at 37˚C for 6 h. The cells were washed using 1 ml PBS containing 1% BSA twice, and fixed cells using 0.5 μl fixation buffer (cat. no. 420801; Biolegend, Inc.) per tube and incubated in the dark for 20 min at room temperature. Subsequently, cells were washed using permeabilization buffer (cat. no. 421002; Biolegend, Inc.) twice and then 5 μl antigen presenting cell anti-TNF-α antibody (cat. no. 502913; Biolegend, Inc.) and 5 μl FITC-anti-IFN-γ antibody (cat. no. 502505; Biolegend, Inc.) were added for 30 min in the dark at 4˚C. The cells were washed using permeabilization buffer twice. Cells were analyzed using a flow cytometer.

Cell culture. The human breast cancer cell line MGC803 was purchased from the Cell Center of Institute of Basic

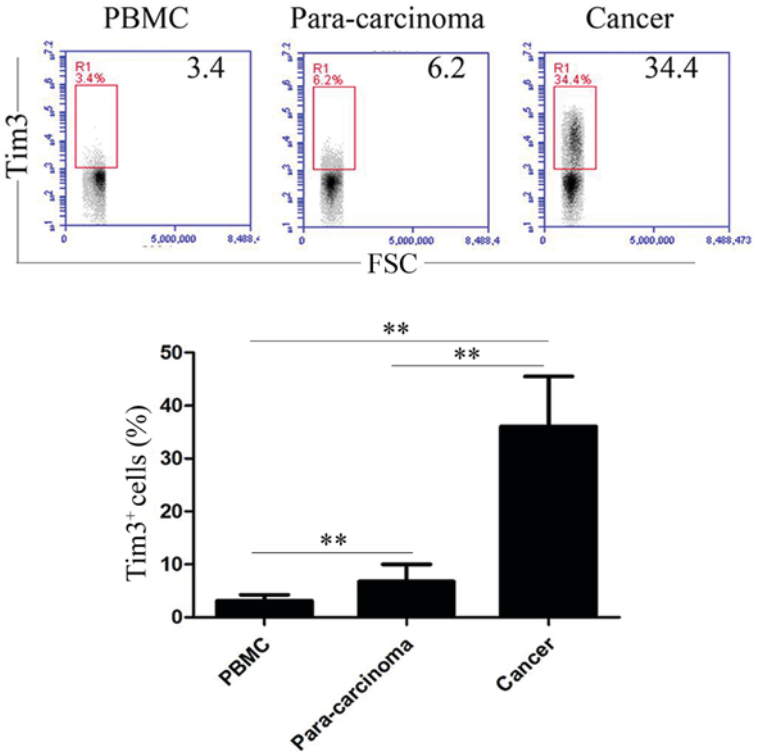

Figure 1. Expression of Tim-3 by T-cells in the peripheral blood, para-carcinoma tissue and gastric cancer tissue. T-cells using here were purified by negative selection using a Pan T Cell Isolation Kit, and purity was verified to be >90%. Tim-3, T-cell immunoglobulin mucin domain-3; PBMC, peripheral blood mononuclear cells; CD, cluster of differentiation. **P<0.01.

Medical Sciences Chinese Academy of Medical Sciences (Shanghai, China). The cells were placed into RPMI-1640 medium containing 10% fetal bovine serum and incubated in the 37˚C constant temperature incubator containing 5% CO_2. Subsequent experiments were then conducted when the cells were at the logarithmic growth stage.

Gastric cancer xenograft model and therapeutic method. MGC803 cells were digested with 0.25% trypsin (cat. no. 59427C; Sigma-Aldrich; Merck KGaA), then washed using sterile PBS for three times, and injected into nude mice subcutaneously in the right flank of nude mice ($1x10^6$/100 μl). Day 0 was marked when subcutaneous tumor nodules grew up to ~0.1 mm^3 (~10 days). The nude mice were randomly divided into three experimental groups, with each group containing 8 mice: The untreated T-cells treatment group (PBS-treated $CD3^+$ T-cells), the treatment group using galectin-9-stimulated T-cells ($CD3^+$ T-cells that were cultured for 72 h with 5 μg/ml final concentration of galectin-9) and the PBS control group. Then, the intratumor injection treatment was performed. All experiments were approved by the Institutional Animal Care and Utilization Committee of Medical Ethics committee of Provincial Hospital Affiliated to Shandong University and Weihai Municipal Hospital. The treatments were performed on day 5 after MGC803 cells injection, and there were 4 treatments, with the injection being repeated every 3 days. The untreated $CD3^+$ T-cells ($1x10^6$/50 μl per mice; total of 8 mice) were injected into mice in the conventional T-cells treatment group; Tim-3-stimulated $CD3^+$ T-cells ($1x10^6$/50 μl, 8 mice) were injected into the mice in the treatment group of the Tim-3-stimulated $CD3^+$ T-cells; the same volume of PBS was injected into the mice in the PBS control group (8 mice) instead of the cell suspension. At the same time, 5,000 U/kg interleukin-2 (Biolegend, Inc.) was injected into abdominal cavity or each mouse. The general state of the mice and the volume of the tumor nodules were observed. The duration of the experiments was 1 month, and following that all mice were anesthetized/euthanized using cervical dislocation. The computational formula for tumor volume used was: Tumor volume (mm^3)=$axb^2x0.5$, where 'a' denotes the long diameter of tumor (mm) and 'b' denotes the short diameter of tumor (mm).

Statistical method. The results are expressed as the mean ± standard deviation. The experiments were at repeated ≥3 times. Data analysis was conducted using Graph Pad software 5 (GraphPad Software, Inc., La Jolla, CA, USA). Data between groups were analyzed using a Student's t-test or one-way analysis of variance followed by a Bonferroni-Dunn multiple comparison post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of Tim-3 by T-cells. The expression of Tim-3 by T-cells in the peripheral blood, paracancerous and cancerous tissues was assessed by flow cytometry (Fig. 1). The percentages of $Tim\text{-}3^+$ cells within T-cells in the peripheral blood the paracancerous gastric tissues were 3.14±1.13 and 6.83±3.18%, respectively. The percentage of $Tim\text{-}3^+$ cells within T-cells in cancerous gastric tissues was 36.11±9.42% (Fig. 1). Compared with the expression levels of Tim-3 on the surface

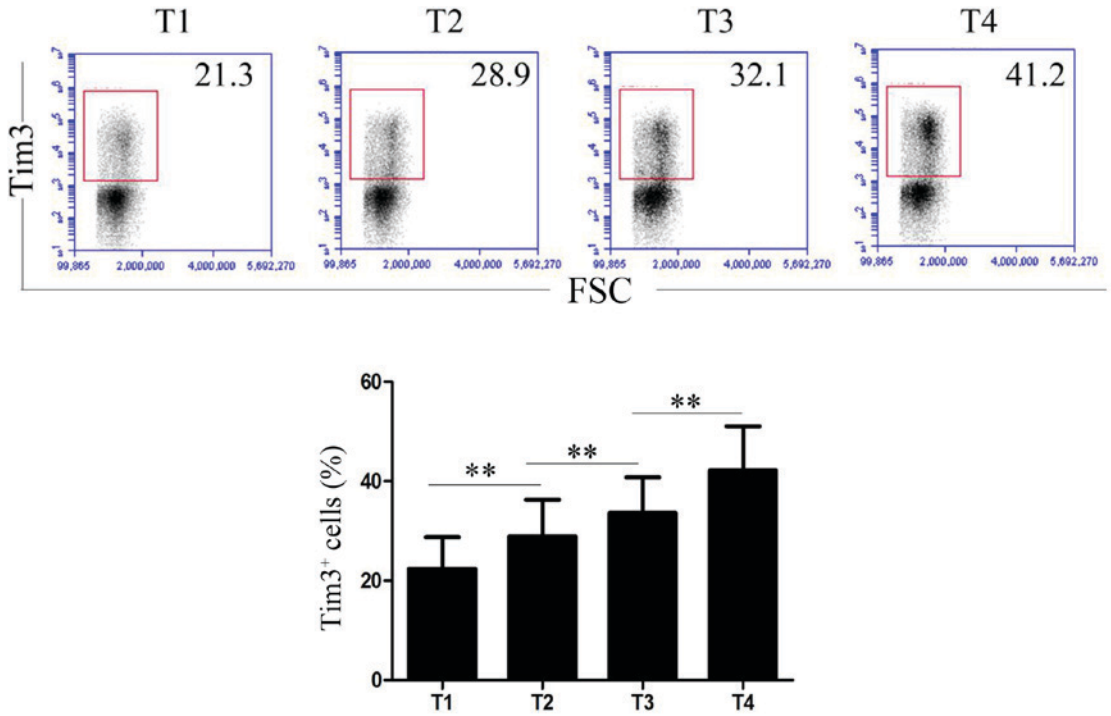

Figure 2. Tim-3 expression is increased at advanced stages of gastric cancer. T-cells using here were purified by negative selection using a Pan T-cell Isolation Kit, and purity was verified to be >90%. Tim-3, T-cell immunoglobulin mucin domain-3; CD, cluster of differentiation. **P<0.01.

of T-cells in the peripheral blood, the expression levels of Tim-3 on the surface of para-carcinoma tissue and cancer tissue of the patients with gastric cancer increased significantly (P<0.01; Fig. 1). Additionally, the expression of Tim-3 within T-cells was significantly increased in cancerous gastric tissues compared with that in paracancerous gastric tissues (P<0.01; Fig. 1).

Tim-3 expression is increased at advanced stages of gastric cancer. The association between Tim-3 expression level and T staging of gastric cancer is illustrated in Fig. 2. The percentage of Tim-3$^+$ T-cells in gastric cancer tissues at stages T1, T2, T3 and T4 were 22.41±6.35, 28.94±7.35, 33.62±7.16 and 42.16±8.93%, respectively. With the increase of gastric cancer stages, the expression level of Tim-3 on T-cell was significantly increased (P<0.01; Fig. 2).

Effects of galectin-9-mediated Tim-3 activation on cytokine secretion of T-cells. T-cells were treated with control or galectin-9 antibody and IFN-γ and TNF-α expression was assessed by flow cytometry The effects of Tim-3 activation on cytokine secretion of T-cells is demonstrated in Fig. 3. The percentages of IFN-γ$^+$ and TNF-α$^+$ T-cells in the control groups were 38.34±9.04 and 35.73±8.26%, respectively. Following the activation of Tim-3 signaling pathway, the percentages of IFN-γ$^+$ and TNF-α$^+$ T-cells were 19.45±6.36 and 20.35±7.42%, respectively. Therefore, galectin-9-mediated Tim-3 activation may significantly inhibit the capability of T-cells to secrete IFN-γ and TNF-α (P<0.01; Fig. 3).

Galectin-9-mediated Tim-3 activation affects tumor growth in vivo. A tumor xenograft model was used, and the mice were treated with 3 types of cells and tumor growth was assessed after 30 days Experimental results of nude mice tumor-bearing test are illustrated in Fig. 4. The tumor volumes of tumor-bearin g mice in control, untreated T-cells and Tim-3-stimulated T-cell groups were 1.27±0.38, 0.12±0.10 and 0.68±0.33 cm^3, respectively. Tim-3 stimulation significantly weakened the ability of T-cells to inhibit tumor growth (P<0.01; Fig. 4).

Discussion

The Tim family is a transmembrane glycoprotein encoded by the Tim gene. Its basic structure includes a signal peptide, immunoglobulin V area, mucin area, transmembrane region and intracellular tail region containing phosphate sites (13). Tim family members include Tim-1, Tim-3 and Tim-4 (14). Tim-3 is one of the newly-discovered family members (15-17). Previous studies have demonstrated that Tim-3 is an immune negative regulating molecule and expressed on the surface of differentiated and mature Th1 cells (11,18). Previous studies demonstrated that Tim-3, cytotoxic T lymphocyte associated antigen-4 (*CTLA*-4) and PD-1 are classified as the immune system's inhibitory receptors (11,19). A number of studies demonstrated that the expression of Tim-3 in a variety of tumor tissues is associated with tumor development (20,21).

In addition to the close association with the occurrence and development of tumors, Tim-3 is also associated with T-cell depletion (22). Previous studies demonstrated that the expression level of Tim-3 increases on the surface of T-cells infiltrating various tumor tissues (11,23). There are a number of studies regarding Tim-3 expression on the T-cell surface of gastric cancer patients (11,22,23). The present study assessed the expression of Tim-3 on the surface of T-cells in patients with gastric cancer. According to the results of the present study, the expression levels of Tim-3 on the surface

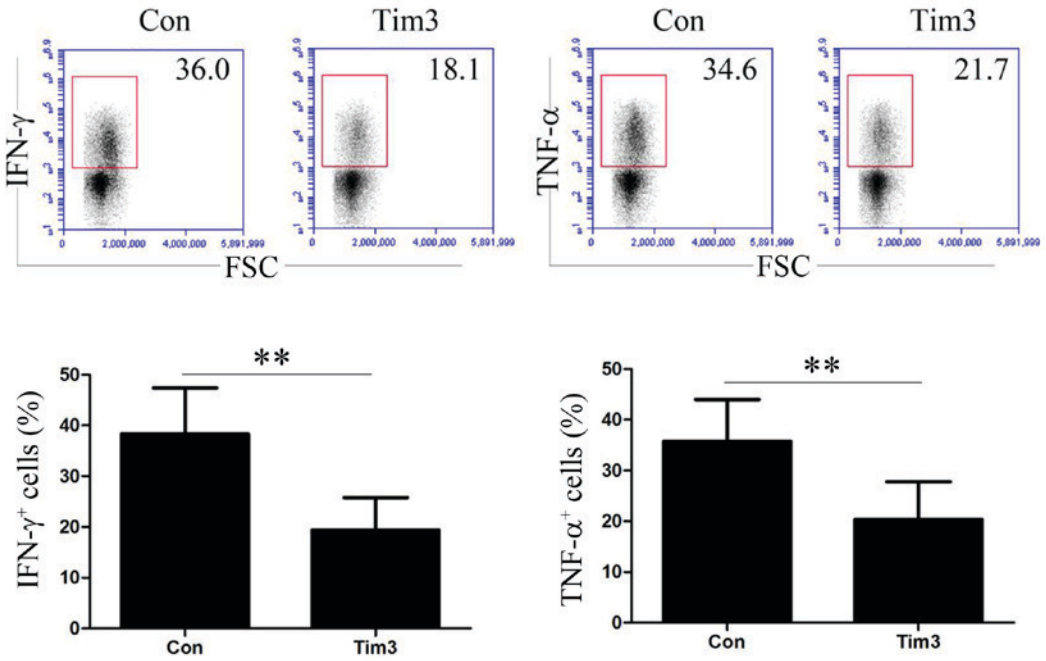

Figure 3. Effect of Tim-3 on the secretion of T-cells cytokines. The Con group includes T-cells with no treatment, and the Tim-3 group includes T-cells treated with a Galectin-9 antibody. T-cells using here were purified by negative selection using a Pan T-cell Isolation Kit, and purity was verified to be >90%. IFN, interferon; TNF, tumor necrosis factor; CD, cluster of differentiation; Con, control; Tim-3, T-cell immunoglobulin mucin domain-3. **P<0.01.

of T-cells was significantly increased in para-carcinoma and cancer gastric tissues compared with that in peripheral blood. Furthermore, the expression level of Tim-3 on the surface of T-cells is significantly increased in cancer tissues compared with that in para-carcinoma tissues. At the same time, with the increase in the T staging of gastric cancer, the expression level of Tim-3 on the surface of T-cells gradually increases. The results of the present study suggest that Tim-3 may participate in the decrease of T-cell function during tumor development due to it inhibiting the secretion of TNF-α and IFN-γ by T-cells, and therefore promote the occurrence and development of the tumor.

To further clarify the association between Tim-3 expression on the surface of T-cells in gastric cancer patients and T-cell function, the present study used recombinant ligand galectin-9 to activate Tim-3 on the surface of T-cells. Galectin-9 is the most commonly used ligand to activate the Tim-3 pathway in the study of all types of diseases (11,22-24). The present study used a Galectin-9 antibody, a ligand to Tim-3 to activate the Tim-3 signaling pathway in T-cells. Results demonstrated that, following the activation of Tim-3 signaling, the ability of T-cells to secrete IFN-γ and TNF-α decreased. Furthermore, a human gastric cancer xenograft model was employed. The mice were treated with T-cells that were activated or non-activated by Tim-3 stimulation and tumor growth was evaluated. The results demonstrated that the effect of Tim-3-stimulated T-cells on inhibiting tumor growth decreased. The results of the present study suggest that Tim-3 may inhibit T-cell function by inhibiting IFN-γ and TNF-α secretion, therefore participating in gastric cancer initiation and progression. In conclusion, the increase of expression level of Tim-3 on T-cell surface in gastric cancer tissues may be associated with the development gastric cancer. The present data provide a new understanding of the effect of Tim-3 on T-cells, which may have implications for T-based cancer immunotherapy by optimizing T-cell effector function, possibly by neutralization of the effect of Tim-3 on T-cells.

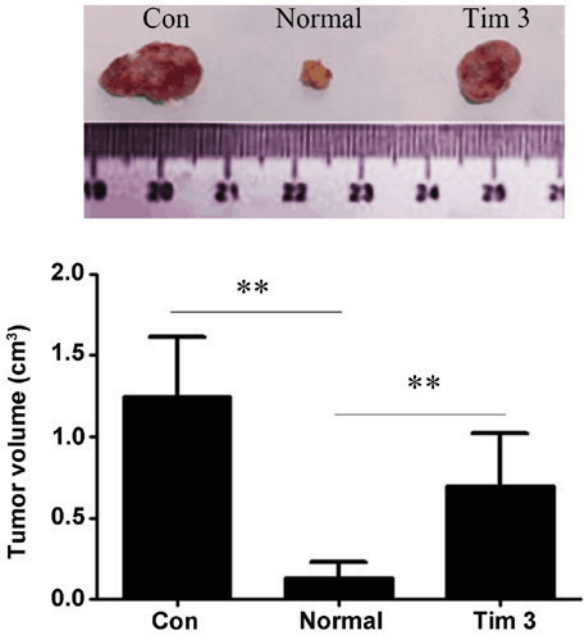

Figure 4. Effect of Tim-3 on the inhibition of the growth of the tumor. The normal group includes T-cells without treatment. Tim-3, T-cell immunoglobulin mucin domain-3; Con, control. **P<0.01.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LL, JY and HZ made substantial contributions to conception and design. HZ, SBS and SWS were responsible for the analysis and interpretation of data. LL, JY and SBS were involved in drafting the manuscript or revising it critically for important intellectual content. LL agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of Weihai Municipal Hospital (Weihai, China). Written informed consent was received from all patients involved.

Patient consent for publication

All of the study participants provided consent for the publication of data.

Competing interests

The authors declare they have no competing interests.

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