Original Research

The killing effect of Tanshinol on breast cancer cells: insight into the reversion of TGF-β1-mediated suppression of NK cell functions

Chunmei Yang[1,2,†], Cheng Qian[1,†], Tingting Zhang[1], Weiwei Zheng[1], Shan Zhang[1], Shijun Wang[3], Aiyun Wang[1,4,*], Yang Zhao[1,2,*], Yin Lu[1,4,*]

[1]*Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica, School of Pharmacy, Nanjing University of Chinese Medicine, 210023 Nanjing, Jiangsu, China,* [2]*School of Medicine & Holistic Integrative Medicine, Nanjing University of Chinese Medicine, 210023 Nanjing, Jiangsu, China,* [3]*Shandong co-innovation center of TCM formula, College of Traditional Chinese Medicine, Shandong University of Traditional Chinese Medicine, 250399 Jinan, Shandong, China,* [4]*Jiangsu Collaborative Innovation Center of Traditional Chinese Medicine (TCM) Prevention and Treatment of Tumor, Nanjing University of Chinese Medicine, 210023 Nanjing, Jiangsu, China*

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
3.1 Mouse tumor model
3.2 Cell lines and drug preparation
3.3 Histology
3.4 Cell proliferation assay
3.5 LDH cytotoxicity assay
3.6 Western blot analysis
3.7 Immunofluorescence
3.8 Co-immunoprecipitation (Co-IP)
3.9 Flow cytometry
3.10 Enzyme-linked immunosorbent assay
3.11 RT-PCR
3.12 Statistical analysis
4. Results
4.1 Tanshinol prevents the breast cancer metastasis in vivo
4.2 Tanshinol reverses TGF-β1-meidated inhibition of NK cell functions in the breast cancer cells
4.3 Tanshinol rescues the inhibitory effect of TGF-β1 on degranulation of NK cells
4.4 Tanshinol restores the inhibitory effect of TGF-β1 on NKG2DL-NKG2D signaling axis
4.5 Tanshinol abolishes TGF-β1-mediated upregulation of p-smad2/3 level and its translocation into the nucleus, increasing the expression of NKG2D
4.6 Tanshinol antagonizes the intervention effect of TGF-β1 on the formation of NKG2D-DAP10 complex
5. Discussion
6. Conclusions
7. Author contributions
8. Ethics approval and consent to participate
9. Acknowledgment
10. Funding
11. Conflict of interest
12. References

Submitted: 14 July 2021 Revised: 30 September 2021 Accepted: 19 October 2021 Published: 30 November 2021

1. Abstract

Background: Natural killer (NK) cells play an indispensable role in anti-tumor immunity. TGF-β1 is the main accomplice of tumor immune escape, inhibiting tumor immunity mediated by NK cells. It is reported that Salvia miltiorrhiza can promote the immune killing effect of NK cells. In this study, Tanshinol, a water-soluble active component of Salvia miltiorrhiza, was used to investigate its effect on the inhibition of NK cell functions mediated by TGF-β1 in breast cancer. **Methods**: We constructed a mouse model of breast cancer by tail vein injection, H&E staining and ELISA were used to verify the role of TGF-β1 and the effects of Tanshinol on breast cancer and NK cells. *In vitro*, we used CCK8 and cytotoxicity assays to preliminarily evaluate the effect of Tanshinol on the anti-tumor effect of NK cells intervention by TGF-β1. We explored the killing activity of NK cells and related signal pathways by immunofluorescence imaging technology, RT-PCR, ELISA and flow cytometry. Also, Western blot, RT-PCR and immunofluorescence experiments were applied to investigate the expression level of the natural killer group 2 member D (NKG2D)-NKG2D ligands (NKG2DL) signal axis, and combined with immunoprecipitation, to detect the formation of NKG2D-DNAX-activating protein of 10 kD (DAP10) complex. **Results**: TGF-β1 played a role in promoting lung metastasis of breast cancer and inhibiting the secretion of cytotoxic mediators from NK cells, but Tanshinol could reverse it. High-dose Tanshinol also significantly optimized the survival rate of tumor-bearing mice. TGF-β1 could destroy the NKG2D-NKG2DL axis, down-regulate the expression and nuclear accumulation of p-smad2/3. Moreover, TGF-β1 inhibited the activation of PI3K-ERK1/2-PLCγ2 signaling pathway that is related to the degranulation of NK cells, and diminished the expression of degranulation marker CD107a and the release of anti-tumor cytotoxic killing medium of NK cells. However, Tanshinol was able to interfere with the negative regulation of TGF-β1 on the functions of NK cells, mainly through promoting the expression of NKG2D and its molecular chaperone DAP10, thereby propelling the formation of NKG2D-DAP10 complex. **Conclusions**: Collectively, Tanshinol enables NK cells to activate and release multiple killing mediators to carry out immune attacks on tumor cells.

2. Introduction

In the immune system, NK cells are a subset of lymphocytes derived from the development and differentiation of hematopoietic stem cells. As a “natural killer”, NK cells do not need to be pre-sensitized, so they are the forerunners of the body’s anti-tumor immunity, which is essential to directly identify and kill tumor cells. However, most tumors can evolve immune escape mechanisms. On one hand, tumor cells change their antigenicity, immunogenicity and related molecular proteins to avoid recognition and killing by the immune system. On the other hand, tumor cells can also promote the production of anoxic acid immunosuppressive microenvironment and inhibit the cancer immunosurveillance [1].

TGF-β1 is one of the main accomplices of tumor immune escape, which is able to reduce the release of killing mediators and inhibit tumor immunity mediated by NK cells through various routes [2, 3]. TGF-β1 can drive NK cells to upregulate the expression of fructose-1,6-bisphosphatase (FBP1), inhibiting the glycolysis metabolism and weakening the killing function of NK cells [4]. It has been well recognized that tumor microenvironment (TME) rich in TGF-β promotes immune escape by differentiating NK cells into intrinsic lymphoid cell type 1 (ILC1) lacking cytotoxicity [5]. In addition, high concentration of TGF-β1 in tumor patients’ plasma tend to upregulate the expression of CD96 and dynamically change the balance of CD96, TIGIT and CD226 in NK cells, thereby suppressing the immune function of NK cells [6]. Also, blocking TGF-β signaling pathway is beneficial to enhance the killing effects of NK cells on breast cancer cells *in vitro* [7].

Our previous studies showed that the release of TGF-β1 was significantly increased in the activated platelet-tumor cell co-culture system, and repression of TGF-β1 could dramatically retard the malignant biological progression of breast cancer cells, suggesting that TGF-β1 may act as a key molecule mediating the interactions between tumor cells and platelets [8]. It has been documented that Salvia miltiorrhiza exerts an important effect on enhancing immunity, which was evidenced by the fact that it boosted the killing activities of cytotoxic T lymphocytes (CTL) and NK cells and stimulated the phagocytosis of macrophages. Meanwhile, it could potentiate the synthesis and release of perforin and granzyme B of NK cells and improve the anti-tumor immune function in the rats with gastric cancer [9]. In addition, it was reported that Cryptotanshinone and Tanshinone IIA, two predominant fat-soluble components, could reinforce the differentiation and maturation of NK cells induced by IL-15, and then promote their killing effect on target cells [10, 11].

In this study, our results showed that immunosuppressive factor TGF-β1 could destroy the NKG2DL-NKG2D signaling axis and restrict the release of anti-tumor cytotoxic killing mediators in the NK cells, whereas Tanshinol was able to reverse the immunosuppression activity of NK cells triggered by TGF-β1, restore the degranulation function of NK cells, enhance tumor immunity and inhibit the occurrence and development of tumor. Our study complements the anti-tumor mechanisms of Tanshinol and explores the role of Tanshinol in promoting tumor immunity and inhibiting tumor immune escape.

3. Materials and methods

3.1 Mouse tumor model

Four-week-old female Balb/c nude mice were purchased from Shanghai Slac Laboratory Co., Ltd. All animal procedures were conducted under the guidance of the Animal Ethics Committee and approved by the Institutional Animal Committee of Nanjing University of Chinese Medicine (Ethical Review Number: 202005A018).

Each of the Balb/c nude mouse was inoculated with 10^6 ZR-75-1 cells. Three treatment groups were given 15 mg/kg, 30 mg/kg and 60 mg/kg Tanshinol powder prepared with double distilled water by intragastric administration. Both control group and model group were given the same volume of double distilled water. The weight of mice was measured every week. The mice were sacrificed three weeks following cell injection, after which the tissues and serum were collected for further studies.

To demonstrate the effect of Tanshinol on the survival duration of tumor-bearing mice, we took the week of all mice in the model group deaths as the experimental end point, and made statistics on the survival rate of each group of mice.

3.2 Cell lines and drug preparation

The NK92MI cells from patients with human malignant non-Hodgkin lymphoma were purchased from Guangzhou Saiku Biotechnology Company. The NK92MI cells were cultured in Alpha MEM supplemented with 12.5% horse serum, 12.5% fetal bovine serum, 0.2 mM inositol, 0.1 mM mercaptoethanol and 0.02 mM folate. ZR-75-1 human metastatic breast cancer cells were kindly provided by Prof. Qiang Xu from Nanjing University, ZR-75-1 cells were grown in 1640 medium containing 10% FBS. All cells were cultured in a cell incubator at 37 °C, 5% CO_2, and 95% air.

Tanshinol (HPLC $\geq$98%, molecular weight: 198.17, Cat. NO.: A4544) was purchased from Shanghai Yuanye Biotechnology has a molecular weight of 198.17. Tanshinol was dissolved in PBS, made up into 16 mM stock solution, and stored at –20 °C in the dark. The working solutions were prepared from the stock solution. TGF-β1 (Cat. No.; 100-21) was obtained from the American PeproTech company, diluted with 10 mM citric acid solution (pH = 3) and 0.1% BSA solution to 1 μg/mL.

3.3 Histology

Liver, spleen, lung, and kidney tissues were harvested, fixed in 4% paraformaldehyde for 24 h, embedded with paraffin, and cut into 5 μm sections. The sections were stained with H&E, and the images were acquired on Mantra Pathology Workstation (PerkinElmer. Waltham, MA, USA).

3.4 Cell proliferation assay

The ZR-75-1 tumor cells were plated into the 96-well plate at the density of 1.2 $\times$ 10^4 cells/well. The adjustment group, the control group, and the treated groups (5 μM, 10 μM, 20 μM, 40 μM and 80 μM) were set with 6 replicates in each group. Cell proliferation assay was performed based on the instructions of the CCK8 kit, a BioTek microplate reader (model: 270133) was used to detect the absorbance at 450 nm and then the relative cell proliferation rate was calculated. The CCK8 kit (Cat. No.: C0038) was purchased from Shanghai Beyotime Biotechnology Company.

3.5 LDH cytotoxicity assay

Lactate dehydrogenase (LDH) cytotoxicity was performed according to the manufacturer's instructions. NK cells were seeded into 12-well plates at a density of 1 $\times$ 10^6 cells/well. TGF-β1 (10 ng/mL) and different concentrations of Tanshinol (5 μM, 10 μM, 20 μM, 40 μM and 80 μM) were added and cultured for 24 h. ZR-75-1 cells were seeded into the 24-well plates at a density of 1 $\times$ 10^4 cells/well. NK cells were pre-treated according to different target ratios (10:1, 20:1, and 40:1) to construct a co-incubation system. After co-cultivation for 3 h, the prescribed ratios of LDH release reagent were added to the sample's maximum enzyme activity control well. LDH content detection was performed according to the kit instructions. LDH kit (Cat. No.: C0017) was purchased from Shanghai Beyotime Biotechnology Company.

3.6 Western blot analysis

ZR-75-1 cells were seeded in a 6-well plate at a density of 2 $\times$ 10^5 cells/well. After the cells adhered, TGF-β1 (10 ng/mL), Tanshinol (5 μM 10 μM and 20 μM) were added and incubated with cells for 24 h. The protein extract (RIPA lysate:phosphatase inhibitor:protease inhibitor = 100:1:1) was used to lyse the cells, and then the BCA kit (Thermo, Cat. No.: 23227) was employed to detect the protein concentration. The denatured proteins were separated and transferred to PVDF membranes using SDS-PAGE electrophoresis and wet transfer, respectively. The PVDF membranes were blocked with blocking solution (5% skimmed milk powder/TBST) at room temperature for 2 h, and incubated with indicated primary antibodies at 4 °C overnight. TBST was eluted 4 times (5 min/time), followed by incubation with corresponding secondary antibodies at room temperature for 1.5 h. The proteins were developed by ECL kit (Millipore, Cat. No.: P36599). The gel imaging system (ChemiDoc™ XRS +) was used to acquire images, and protein bands were quantified using Image J software (version 1.8.0, National Institutes of Health, Bethesda, MD, USA).

MICA (A12622), MICB (A9802), ULBP1 (A10483), ULBP2 (A15194), KLRK1/NKG2D (A6123), P-PLCγ2 (AP0785) antibodies were from ABclonal

Technology. DAP10 (sc-133173) antibody was obtained from Santa Cruz Biotechnology. GAPDH (AP0063) was from Bioworld Technology. PI3K (4249), P-PI3K (4228), ERK1/2 (4695), P-ERK1/2 (4370), PLCγ2 (3872), smad2/3 (8685), p-smad2/3 (8828) antibodies were purchased from Cell Signaling Technology.

3.7 Immunofluorescence

ZR-75-1 cells were seeded in a 6-well plate at a density of 2×10^5 cells/well with a round cover glass pre-positioned at the bottom, TGF-β1 and different concentrations of Tanshinol were incubated with the cells for 24 h. The cells were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.2% Triton X-100/PBS, blocked with 1% BSA (BSA/PBS), and the indicated primary antibodies were incubated at 4 °C overnight. The corresponding fluorescent secondary antibodies were incubated in the dark for 2 h. Hoechst 33324 was used to stain the nuclei. Images were taken with ZEISS fluorescence microscope (Oberkochen, Germany, model: Vert.A1).

3.8 Co-immunoprecipitation (Co-IP)

The protein samples of NK92MI cells were extracted with IP lysate (Jiangsu Keygen Biotechnology. Cat. No.: KGP701. Nanjing, China), and the protein concentration was quantified by BCA method. 2 μg IP primary antibody was added and incubated at 4 °C overnight. 20 μL of fully resuspended Protein A/G PLUS-Agarose was added (Santa Cruz, Cat. No.: sc-2003. Dallas, TX, USA) and mixed slowly at 4 °C for 3 h. The supernatant was removed and the pellet was washed with PBS. The immunoblot detection was performed and the protein bands were developed with ECL reagents.

3.9 Flow cytometry

NK cells treated with TGF-β1 and Tanshinol were collected, seeded into a 6-well plate, and then ZR-75-1 cells were added at a 10:1 target ratio to construct a co-incubation system. After 4 h of co-culture, the cell suspension was collected, centrifuged at 1500 rpm for 5 min, washed twice with PBS, and fixed with 4% paraformaldehyde at room temperature for 10 min. 500 μL PBS was added to each tube to resuspend the sample. 5 μL PerCP-Cy5.5 CD56 and 20 μL PE-CD107a flow cytometry antibodies were added to each sample according to the instructions, and incubated on ice for 30 min. BD C6 flow cytometry was used to detect the expression of CD107a on the surface of NK cells.

In order to detect the levels of the intracellular factors Perforin, Granzyme B and IFN-γ, a protein transport blocker BFA or monensin was added to the co-incubation system. After cell fixation, 0.2% Triton X-100/PBS was permeabilized for 10 min at room temperature. Flow cytometry antibodies PerCP-Cy5.5 CD56 (Cat. No.: 560842) and PE-CD107a (Cat. No.: 555801) were from BD PharmingenTM. Perforin (Cat. No.: 12-9994-41), Granzyme B (Cat. No.: 12-8899-41), and IFN-γ (Cat. No.: 12-7319-42) antibodies were purchased from Invitrogen.

3.10 Enzyme-linked immunosorbent assay

The serum samples of mice were agglutinated at room temperature for 30 min, and left overnight at 4 °C to completely release TGF-β1. After Centrifugation at 1000 g for 10 min, the serum samples were detected according to the instructions. The cell suspension of the co-incubation system was collected and centrifuged at 1500 g for 10 min to obtain a supernatant sample. The results with 3 replicates in each group were detected at 450 nm with a microplate reader.

Human perforin ELISA kit (Cat. No.: RK00135) and human granzyme B ELISA Kit (Cat. No.: RK00089) were from ABclonal Technology. Human IFN-γ ELISA Kit (Cat. No.: 70-EK180-96) was purchased from Link Bio. Mouse TGF-β1 ELISA kit (Cat. No.: EK981-48), mouse perforin ELISA Kit (Cat. No.: JEB-13034), mouse granzyme B ELISA Kit (Cat. No.: JEB-12517) and mouse IFN-γ ELISA kit (Cat. No.: JEB-12796) were obtained from Nanjing Jin Yibai Biological Technology Co. Ltd.

3.11 RT-PCR

TRIzol extraction kit (Invitrogen. Carlsbad, CA, USA) was used to extract the total RNA in NK cells, and the purity was verified by the mRNA detection plate provided with BioTek microplate reader. cDNA was synthesized by the reverse transcription kit (Vazyme Biotech Co., Ltd, Nanjing, China) using gradient PCR instrument (Thermo Fisher Scientific, Applied Biosystems life Veriti96. Waltham, MA, USA). The Ct values of each cDNA were detected with a fluorescence quantitative PCR instrument (BIO-RAD, model: iQ5. Hercules, CA, USA), and the relative change of mRNA was calculated by $2^{(-\Delta\Delta Ct)}$. The primer sequences are shown in Table 1.

3.12 Statistical analysis

GraphPad Prism 5.0 software (San Diego, CA, USA) was used for statistical difference analysis. All data were expressed by mean $\pm$ standard deviation (SD). Comparison between groups was analyzed by one-way analysis of variance. $p < 0.05$ was considered statistically significant.

4. Results

4.1 Tanshinol prevents the breast cancer metastasis *in vivo*

In order to preliminarily verify the efficacy of Tanshinol, each of the Balb/c nude mouse was inoculated with ZR-75-1 cells to constructed an animal model of breast cancer including treatment groups and model group. Three treatment groups were given 15 mg/kg, 30 mg/kg and 60 mg/kg Tanshinol, and control group and model group were given the same volume of double distilled water. Compared with the model group, the body weights of treatment groups were significantly increased (Fig. 1A). Meanwhile,

Table 1. The primer sequences.

Name	Forward	Reverse
GAPDH	5′-GGTTGTCTCCTGCGACTTCA-3′	5′-TGGTCCAGGGTTTCTTACTCC-3′
NKG2D	5′-TCTCGACACAGCTGGGAGATG-3′	5′-GACATCTTTGCTTTTGCCATCGTG-3′
DAP10	5′-TCCATCTGGGTCACATCCTCTTCC-3′	5′-GAGTGATGATCTCTCTCCTGGAGTCGTCTGAGCTG-3′
Perforin	5′-ACCAGCAATGTGCATGTGTCTGTG-3′	5′-GAAGGAGGCCGTCATCTTGTGCTT-3′
Granzyme B	5′-TGCAGGAAGATCGAAAGTGCG-3′	5′-GAGGCATGCCATTGTTTCGTC-3′
IFN-γ	5′-TCCAACGCAAAGCAATACAT-3′	5′-GCAGGCAGGACAACCATTAC-3′

the spleen organ index of the medium or high dose group was significantly lower than that of model group, whereas the liver, lung and kidney indexes showed no significant changes (Fig. 1B). Notably, H&E staining results revealed that the mice in the model group exhibited obvious lung metastasis, and Tanshinol could alleviate the lung metastasis in a dose-dependent manner (Fig. 1C). In order to examine the expression of TGF-β1 in tumor-bearing mice and the effect of Tanshinol on TGF-β1 in the serum of mice, ELISA assay was thus performed. The results demonstrated that the level of TGF-β1 in the model group was significantly increased compared with that in the control group, and high-dose of Tanshinol administration significantly reduced TGF-β1 production in the tumor-bearing mice (Fig. 1D). Given the fact that NK cells can exert anti-tumor and anti-metastasis activities by secreting various effector molecules such as IFN-γ, perforin and granzyme B [12], we further detected the levels of IFN-γ, perforin and granzyme B in the tumor-bearing mice. It was found that high-dose of Tanshinol could effectively promote the release of above-mentioned killing mediators in the serum, which may be related to the activation and enhanced function of NK cells mediated by Tanshinol (Fig. 1E). In addition, we demonstrate the effect of Tanshinol on the survival duration of tumor-bearing mice, the results showed that all mice in the model group died in the seventh week, and the high-dose Tanshinol group significantly optimized the survival rate of tumor-bearing mice.

4.2 Tanshinol reverses TGF-β1-meidated inhibition of NK cell functions in the breast cancer cells

To verify the role of Tanshinol *in vitro*, we thus evaluated its effects on the proliferation of ZR-75-1 and NK92MI cells. As shown in Fig. 2A, there were no significant changes in cell proliferation between control and treated groups. To further explore the effects of Tanshinol on modulating NK cells and tumor cells, we used the LDH experiment to detect the killing effect of NK cells on ZR-75-1 cells. The results showed that TGF-β1 could significantly inhibit the LDH level in the supernatant of the co-culture system under different target-effect ratios, which indicates that TGF-β1 remarkably inhibits the immune killing effect of NK92MI cells on the ZR-75-1 cells (Fig. 2B). Together, the above results suggest that Tanshinol may exert anti-tumor effect by improving the NK cell-mediated immune killing function.

4.3 Tanshinol rescues the inhibitory effect of TGF-β1 on degranulation of NK cells

Since degranulation of NK cells plays a pivotal role in regulating NK cell functions, we therefore evaluated the effects of TGF-β1 and Tanshinol on influencing the degranulation function of NK cells by detecting the expression of CD107a on the membrane surface of NK cells using flow cytometry. Our data revealed that Tanshinol was able to significantly reverse the inhibition of CD107a expression mediated by TGF-β1 and promote the degranulation function of NK cells (Fig. 3A). Furthermore, we also investigated the effects of TGF-β1 and Tanshinol on modulating PI3K-ERK1/2-PLCγ2 signaling pathway, which emerges as a key cascade of NK cell degranulation. The western blot results showed that TGF-β1 markedly inhibited the phosphorylation of PI3K, ERK1/2 and PLCγ2, which could be reversed in the presence of Tanshinol. The activation of PI3K-ERK1/2-PLCγ2 by Tanshinol contributed to the degranulation of NK cells and reduced immune escape of tumor cells (Fig. 3B).

In addition, in order to further explore the effects of TGF-β1 and Tanshinol on the synthesis and release of killing mediators during NK cells degranulation, we thus detected the expression of Perforin, Granzyme B and IFN-γ. At the transcriptional level, Tanshinol could significantly increase the mRNA levels of *Perforin and IFN-*γ in a concentration-dependent manner, but had no significant effect on the expression of *Granzyme B* (Fig. 3C). This was also substantiated by the results of ELISA (Fig. 3D), all of which imply that Tanshinol can prominently reverse the inhibitory effect of TGF-β1 on the synthesis and release of cytotoxic mediators in the NK cells.

4.4 Tanshinol restores the inhibitory effect of TGF-β1 on NKG2DL-NKG2D signaling axis

It has been well accepted that the activity of NK cells depends on the interaction of NK cell surface receptors and their associated ligands on target cells. NKG2DLs expressed on the surface of tumor cells can bind to activated receptor NKG2D on the surface of NK cells, thereby activating NK cells to exert anti-tumor killing effects [13–15]. As shown in Fig. 4A, TGF-β1 declined the expression of NKG2DLs, but Tanshinol alleviated the inhibitory effects of TGF-β1 on the expression levels of MICA, MICB and ULBP2. Besides, ULBP1 was not significantly affected by TGF-β1 and Tanshinol (Fig. 4A). All of these could be vali-

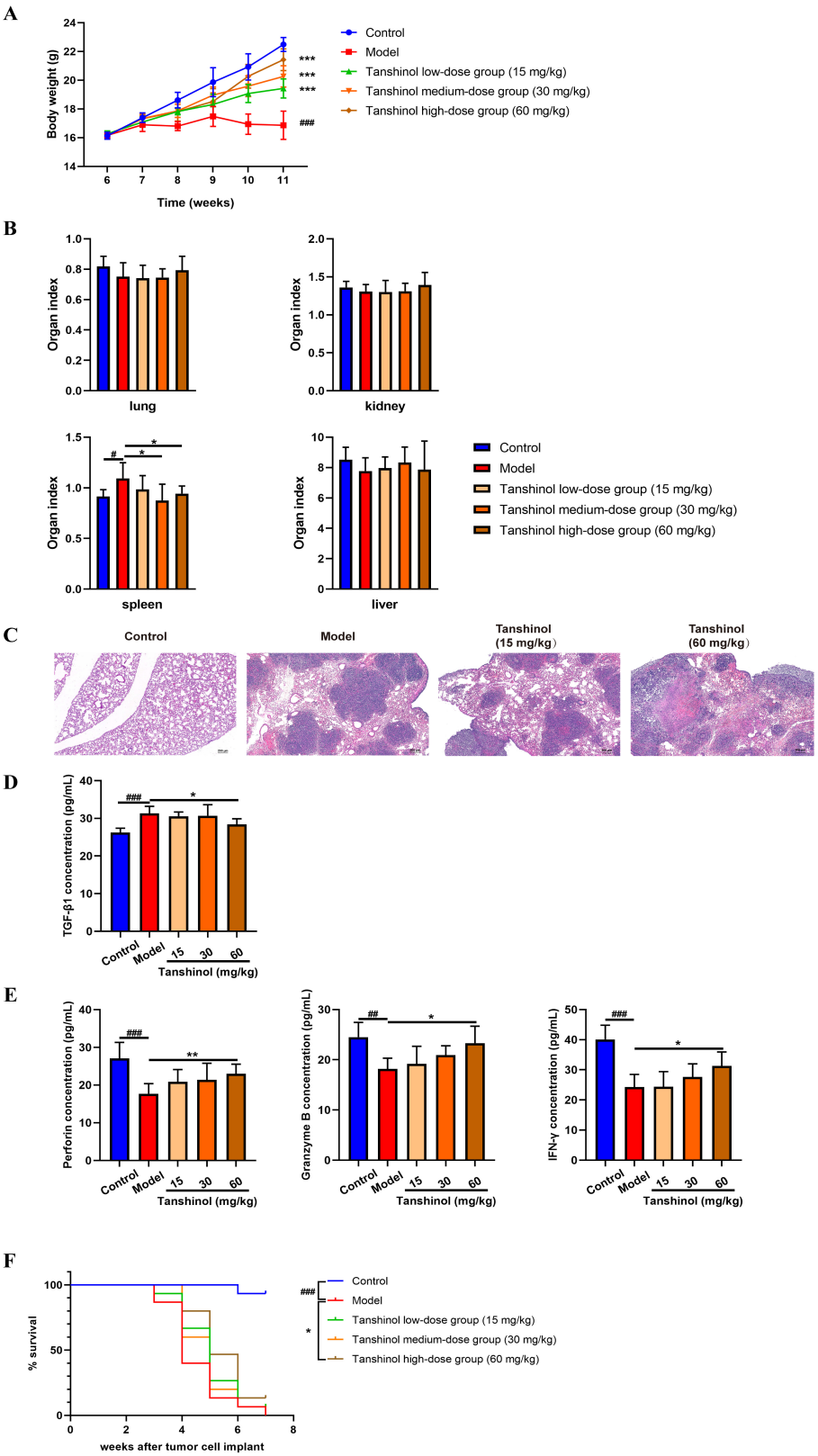

Fig. 1. The effects of Tanshinol on breast cancer metastasis. (A) Body weight of mice. (B) Organ index. (C) H&E staining of lung tissue sections (×50). (D) Serum TGF-β1 content in mice. (E) The levels of NK cell effector molecules. All data are presented as the means ± SD, n = 8. (F) The survival rate of tumor-bearing mice, n = 15. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ (vs. control group). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (vs. model group).

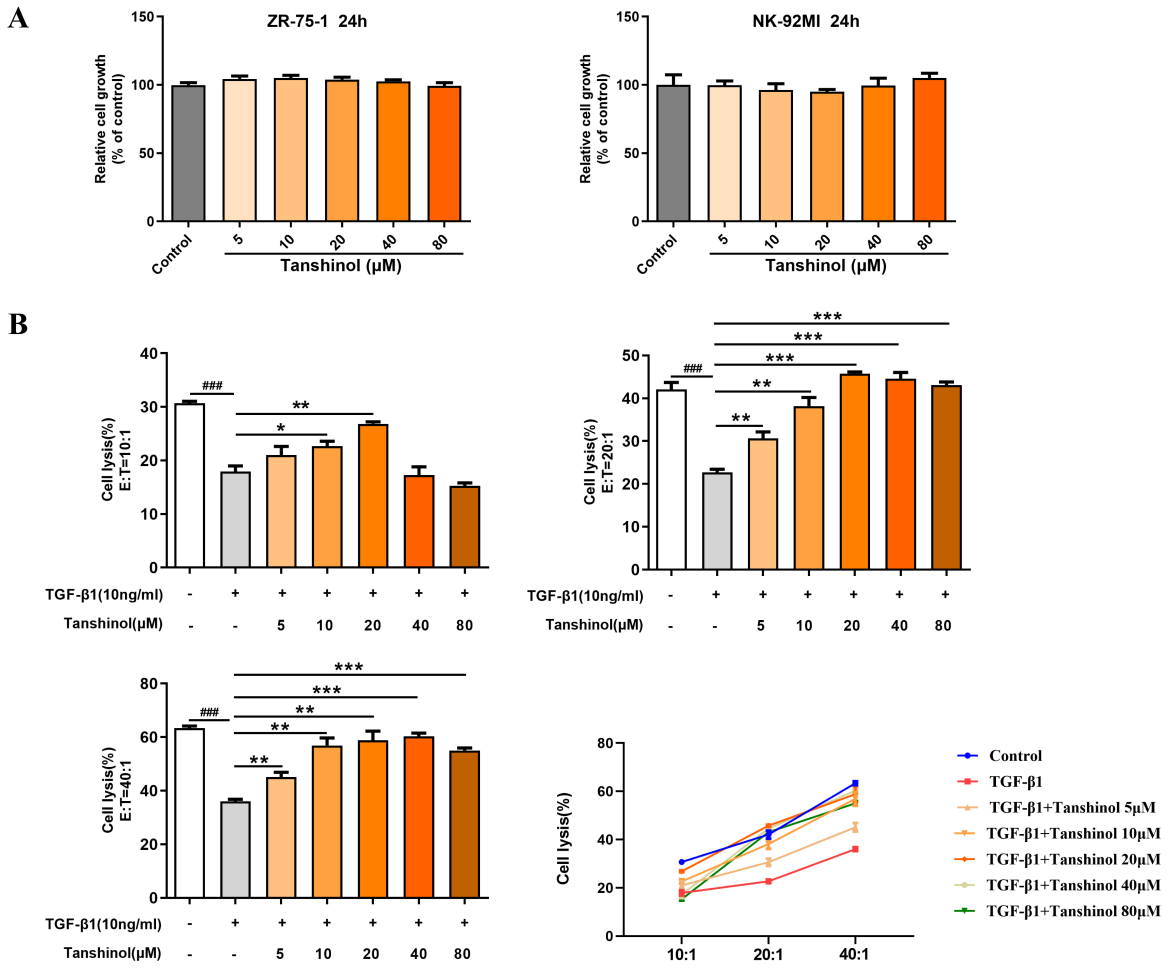

Fig. 2. Effects of Tanshinol on ZR-75-1 and NK92MI cells. (A) The relative growth rate of ZR-75-1 and NK92MI treated with Tanshinol (n = 6). (B) The effect of Tanshinol on the percentage of killing tumor cells by NK cells following the intervention of TGF-β1 (n = 3). The data are presented as the mean $\pm$ SD. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ (vs. control group). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (vs. TGF-β1 treated group).

dated by the immunofluorescence results (Fig. 4B), indicating that Tanshinol can reverse the repression of NKG2DL expression mediated by TGF-β1 on the surface of tumor cells.

Interestingly, after exogenous administration of human MICA recombinant protein (1 μg/mL), the killing effects of NK92MI cells under different effect target ratios were significantly enhanced, in comparison to the control group (Fig. 4C). However, the killing effect of MICA-mediated NK92MI cells was significantly inhibited following the stimulation of TGF-β1, which could be reversed following the treatment of Tanshinol (Fig. 4C). Further, the restoration of NK cell killing effect disappeared following the utilization of NKG2D neutralizing antibody to inhibit the expression of NKG2D on the surface of NK cells, indicating that the alleviation of TGF-β1-mediated immunosuppression of NK cells by Tanshinol is closely associated with the activation of functional receptor NKG2D (Fig. 4D). Based on the above results, it can be concluded that Tanshinol may improve the immune killing function of NK cells by rescuing the inhibitory effect of TGF-β1 on the NKG2D-NKG2DL signaling axis.

4.5 Tanshinol abolishes TGF-β1-mediated upregulation of p-smad2/3 level and its translocation into the nucleus, increasing the expression of NKG2D

Numerous studies have reported that TGF-β1 can promote the phosphorylation of the key transcription factor smad2/3, which results in the dramatic elevation of intracellular p-smad2/3 level, and in turn negatively regulates the expression of functional receptors in NK cells [16, 17]. As shown in Fig. 5A, the expression of p-smad2/3 was upregulated in a time-dependent manner following the stimulation of TGF-β1 (5 min, 15 min, 30 min and 60 min) in the NK92MI cells and reached the peak at 30 min compared with those without TGF-β1 treatment. However, after the intervention of Tanshinol, the elevation of p-smad2 mediated by TGF-β1 was rescued at 15 min, 30 min and 60 min, whereas the expression levels of p-smad3 and smad2/3 remained unchanged. Furthermore, it was observed that the fluorescence intensity of p-smad2/3 in the nucleus of

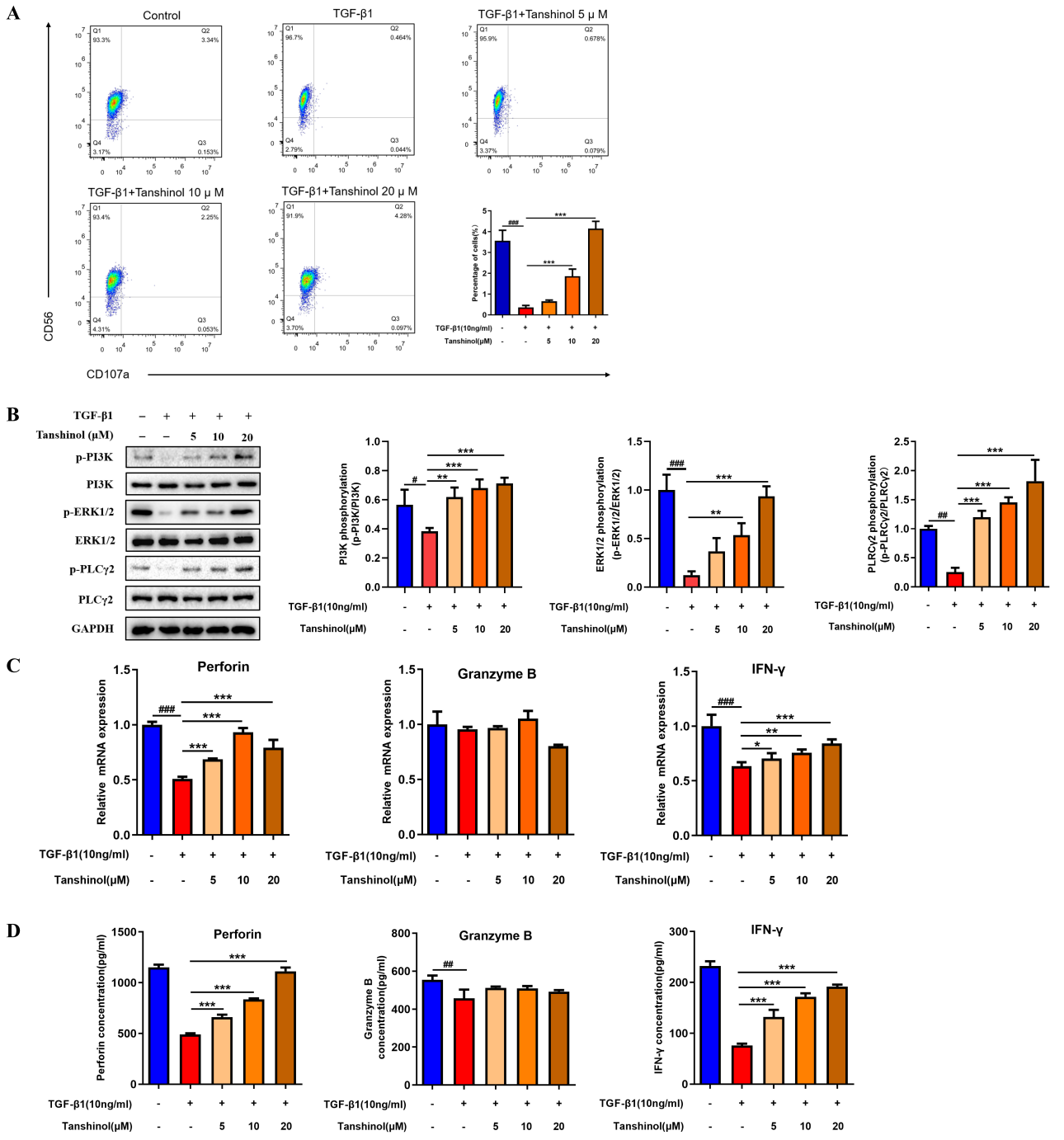

Fig. 3. Tanshinol restores the inhibited activity of NK cells mediated by TGF-β1. (A) The expression of CD107a, a degranulation marker on the surface of NK92MI cells. (B) The effect of Tanshinol on the PI3K-ERK1/2-PLCγ2 signaling pathway that is related to degranulation following the intervention of TGF-β1. (C) The effects of Tanshinol on *Perforin, Granzyme B and IFN-*γ mRNA levels in NK92MI cells following TGF-β1 intervention. (D) The effects of Tanshinol on the release of Perforin, Granzyme B and IFN-γ in NK92MI cells following the intervention of TGF-β1. The data are presented as the mean $\pm$ SD, n = 3. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ (vs. control group). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (vs. TGF-β1 treated group).

NK92MI cells treated with TGF-β1 for 30 min was significantly enhanced, indicating TGF-β1 plays an essential role in promoting the nucleus translocation of p-smad2/3. Nevertheless, it was shown that Tanshinol could inhibit the entry of p-smad2/3 into the nucleus (Fig. 5B). These results suggest that Tanshinol can reverse the upregulation of p-smad2/3 level and inhibit its translocation into the nucleus mediated by TGF-β1, which may be one of the underlying mechanisms that Tanshinol modulates the expression of key functional receptors on the surface of NK cells.

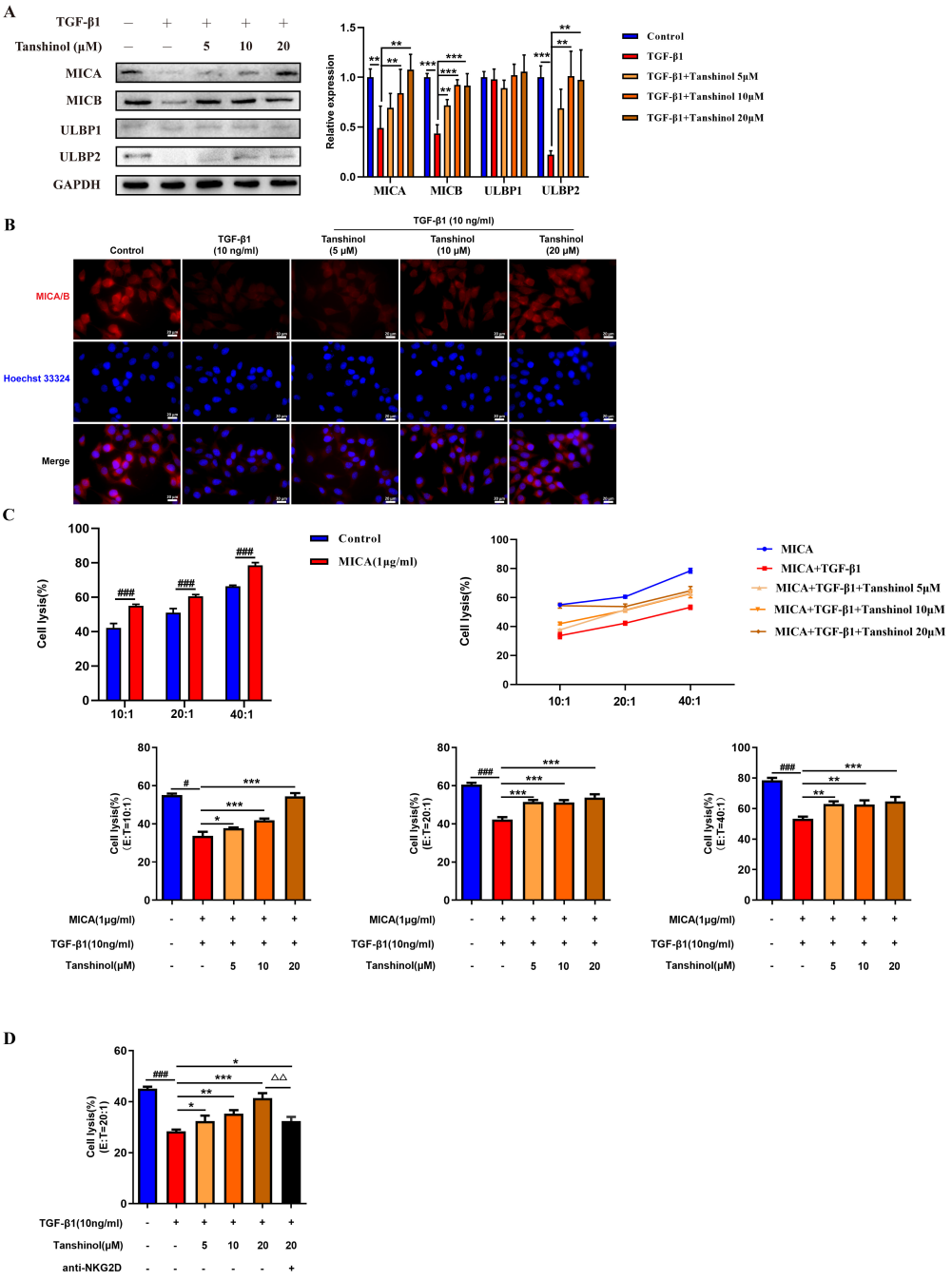

Fig. 4. Tanshinol activates the NKG2D-NKG2DLs signaling axis and reverses the effect of TGF-β1. (A) The effect of Tanshinol on the inhibition of NKG2DL protein expression by TGF-β1. (B) The effect of Tanshinol on the inhibition of MICA/B protein expression induced by TGF-β1 ($\times$400). (C) The activation of NK92MI by exogenous MICA and the intervention of Tanshinol on TGF-β1 inhibiting the activation of NK92MI cells by exogenous MICA. (D) Tanshinol reverses the TGF-β1-induced immunosuppression after neutralizing NKG2D. The data are presented as the mean $\pm$ SD, n = 3. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ (vs. control group). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (vs. TGF-β1 treated group). $\triangle\triangle p < 0.01$ (NKG2D neutralizing antibody vs. without antibody).

4.6 Tanshinol antagonizes the intervention effect of TGF-β1 on the formation of NKG2D-DAP10 complex

It has been known that the binding of NKG2DL to NKG2D on the tumor surface can trigger the coupling of NKG2D with the transporter DNAX activating protein (DAP10) to form an immune recognition receptor complex NKG2D-DAP10, which is the central link in mediating the release of immune killing mediators from NK cells [18–20]. Hence, we detected the expression of NKG2D and DAP10 by western blot and immunofluorescence assays, and found that TGF-β1 mitigated the expression levels of NKG2D and DAP10, while Tanshinol could interfere with this coupling process and restore the expression

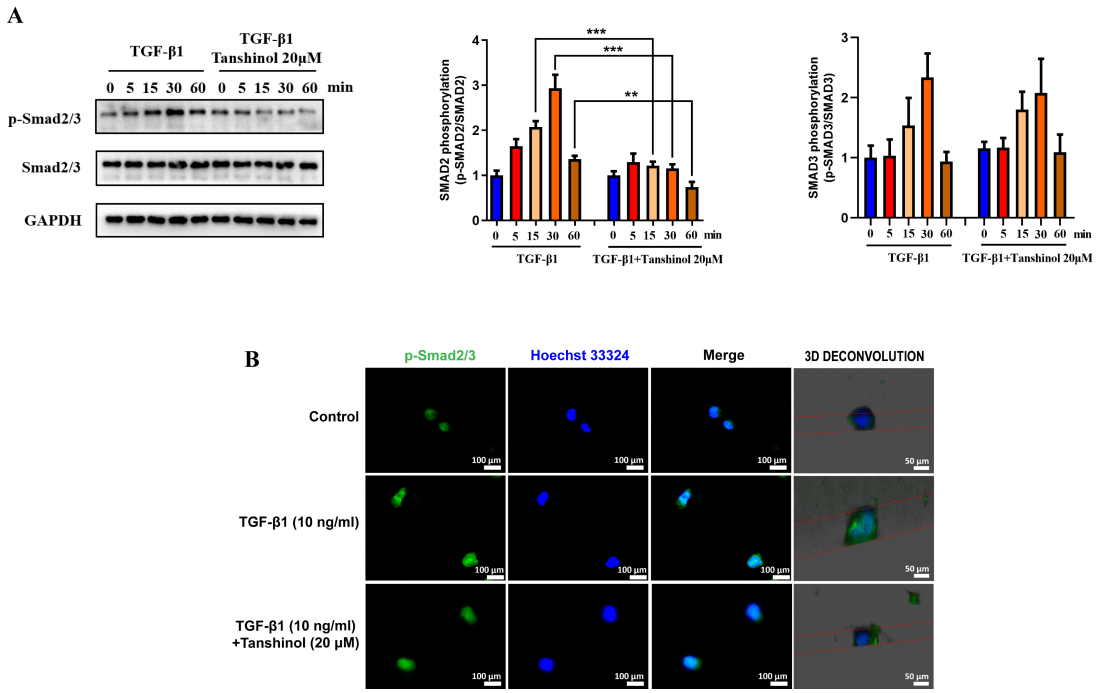

Fig. 5. Tanshinol reverses TGF-β1-mediated increased p-smad2/3 expression and translocation into the nucleus. (A) The effect of Tanshinol on the increased p-smad2/3 and smad2/3 protein expression mediated by TGF-β1 in the NK92MI cells. (B) Immunofluorescence images ($\times$400) of Tanshinol affecting TGF-β1-mediated p-smad2/3 nuclear translocation. The data are presented as the mean $\pm$ SD, n = 3. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

of NKG2D and DAP10 (Fig. 6A–D). The formation of NKG2D-DAP10 complex on the surface of NK cells is the key step to initiate the activation of NK cells. Therefore, we further investigated the binding of NKG2D to DAP10 in NK cells by co-immunoprecipitation. It was observed that TGF-β1 could significantly promote the dissociation of NKG2D and DAP10, but Tanshinol counteracted this process in a concentration-dependent manner and strengthen the binding of NKG2D and DAP10 (Fig. 6E). In order to further verify the key role of Tanshinol in the formation of NKG2D-DAP10 complex, we visualized the co-localization of NKG2D and DAP10 by immunofluorescence, the results of which were consistent with those of co-immunoprecipitation (Fig. 6F). Together, these data suggest that Tanshinol can promote the interaction and binding capability of NKG2D and DAP10, thus reconciling the inhibitory effect of TGF-β1 on the formation of NKG2D-DAP10 complex, which may be the potential driving force of Tanshinol promoting the cytotoxicity of NK cells.

5. Discussion

The International Agency for Research on Cancer (IARC) released the latest global data on cancer burden in 2020, which indicated that breast cancer overtook lung cancer as the leading cause of cancer-associated death worldwide. Traditional Chinese medicine has a long history of effectively treating breast cancer, in which Salvia miltiorrhiza plays an important role [21, 22]. Tanshinol is one of the main active components of Salvia miltiorrhiza, but its anti-tumor mechanism is not comprehensive. In this study, we robustly verified the role of Tanshinol in tumor immune escape from the perspective of immunity and outlined the potential mechanisms, which can be used as a basis supplement for Salvia miltiorrhiza in treating breast cancer.

As the main activating receptor of NK cells, NKG2D can trigger the coupling with the adaptor protein DAP10 after binding to various ligands on the surface of tumor cells, thus forming an immune recognition receptor complex NKG2D-DAP10. The complex then initiates the internalization mediated by DAP10 ubiquitin, which propels it to translocate from the cell membrane to the cytoplasm. This suggests that NKG2D-DAP10 endocytosis is a main route to hamper its abundance on the cell surface and regulate the signaling transduction of NK cells and other cytotoxic lymphocytes to kill tumor cells [23]. The recognition and binding of NKG2D and NKG2DLs acts as a vital step in activating NK cells. In the present study, we demonstrated that TGF-β1 could interfere with NKG2D-NKG2DL signaling axis and inhibit the expression of NKG2D and its related ligands such as MICA/B and ULBP2, while Tanshinol was prone to overcome the immunosuppressive effect induced by TGF-β1. The formation and endocytosis of NKG2D-DAP10 active complexes are critical in activating the downstream signaling pathway for NK cell degranulation to exert anti-tumor activity. During the hematogenous metastasis of tumor cells, activated platelets release a large amount of immunosuppressive fac-

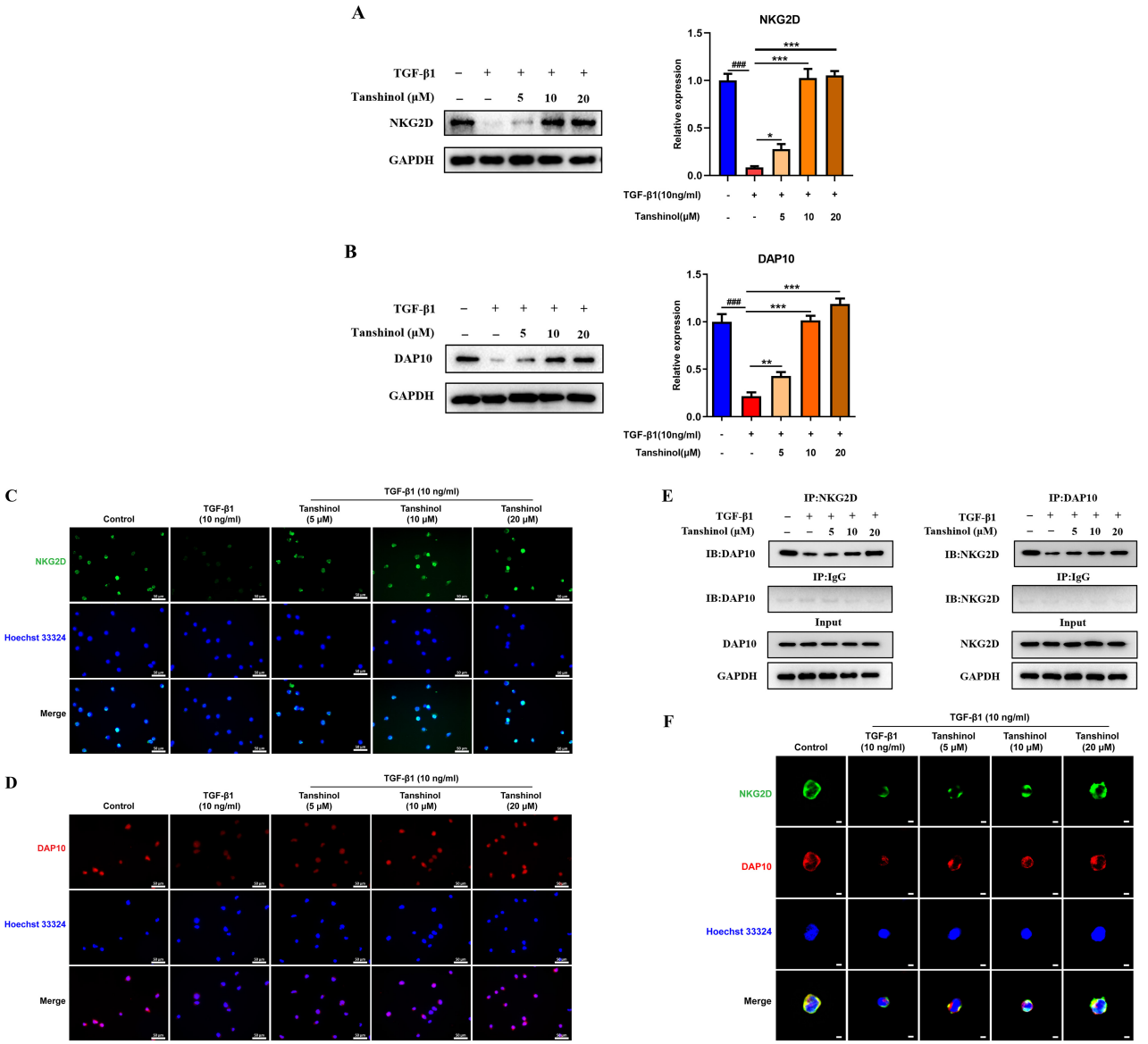

Fig. 6. Effects of TGF-β1 and Tanshinol on the formation of NKG2D-DAP10 complex. (A) Tanshinol promoted the expression of NKG2D protein following the intervention of TGF-β1. (B) The effect of Tanshinol on the expression level of DAP10 protein interfered by TGF-β1. (C) Fluorescence images ($\times$400) of NKG2D protein expression level following the treatment of Tanshinol in the presence of TGF-β1. (D) Fluorescence images of Tanshinol reversing the inhibition of DAP10 protein expression by TGF-β1 ($\times$400). (E) The effect of Tanshinol on the binding ability of NKG2D and DAP10 following the intervention of TGF-β1. (F) Fluorescence images ($\times$400) of Tanshinol on the co-localization of NKG2D-DAP10 following the intervention of TGF-β1. The data are presented as the mean $\pm$ SD, n = 3. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ (vs. control group). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (vs. TGF-β1 treated group).

tor TGF-β1. It has been illustrated that TGF-β1 can significantly downregulate the expression levels of NKG2D and DAP10 on NK cell membranes, and inhibit the synthesis and secretion of tumor-killing mediators from NK cells [24–26]. Our results revealed that Tanshinol attenuated the immunosuppressive effect mediated by TGF-β1 in the NK cells, which was responsible for restoring the expression of NKG2D and DAP10 and promoting the formation of NKG2D-DAP10 complex.

It has been widely held that TGF-β/SMAD signaling pathway plays an important role in the activation of NK cells. TGF-β1 can boost the phosphorylation of SMAD2/3, inhibit the expression of NKG2D, and restore the cytolytic ability of NK cells [27]. Our data demonstrated that the decreased expression of NKG2D by TGF-β1 was ascribed to the augmented phosphorylation and nuclear translocation of SMAD2/3, whereas Tanshinol could interfere with the activation of SMAD2/3 triggered by TGF-β1, thus restoring the expression level of NKG2D. It has been accepted that NK cells tend to promote the degran-

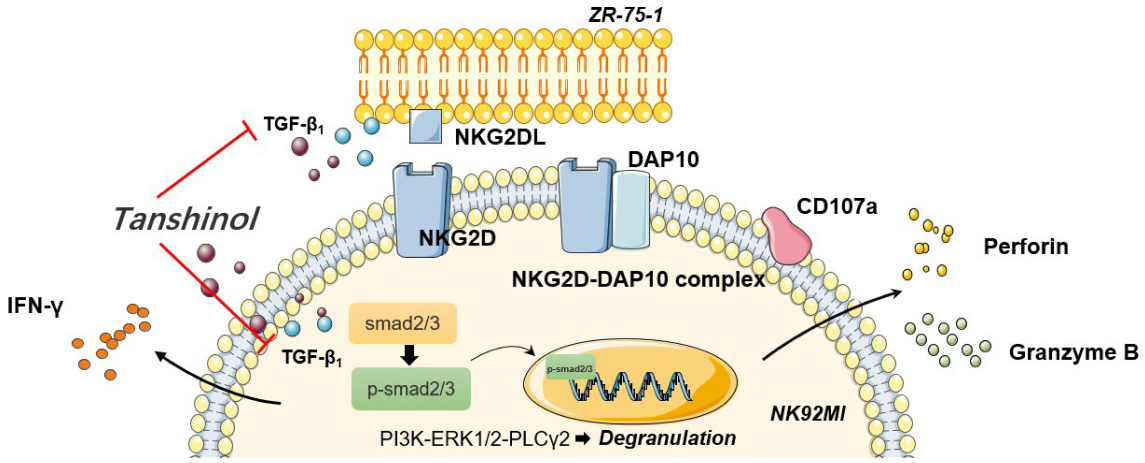

Fig. 7. Schematic diagram of Tanshinol preventing the malignant biological progression of ZR-75-1 breast cancer cells by reviving the inhibited tumor-killing effect of NK92MI NK cells mediated by TGF-β1.

ulation of NK cells when they interact with target cells. Perforin derived from NK cells can cleave the target cells, and granzyme can activate the apoptosis pathway of target cells [28, 29]. Additionally, IFN-γ released by NK cells can also activate macrophages, promote the synchronous activation of other immune cells, and promote the expression of major histocompatibility complex (MHC) and antigen presentation [30]. Our *in vivo* experimental results showed that Tanshinol prominently inhibited the lung metastasis of tumor-bearing mice, diminished the level of TGF-β1 and increased the content of effector molecules in the serum. Moreover, the *in vitro* results revealed that Tanshinol could activate PI3K-ERK1/2-PLCγ2 signaling cascade that is involved in NK cell degranulation. The synthesis and secretion of Perforin and IFN-γ interfered by TGF-β1 could be restored though those of granzyme B remained unchanged in the presence of Tanshinol.

6. Conclusions

In summary, our research focused on the NKG2D-NKG2DL signaling axis and NKG2D-DAP10 complex, and confirmed the TGF-β1-induced immunosuppression of NK cells. It was demonstrated that Tanshinol exerted the anti-tumor effects on breast cancer via reviving the tumor-killing activity of NK cells, featuring the enhanced activity of the NKG2D-NKG2DL signaling axis and increased expressions of NKG2D-DAP10 complex (Fig. 7). However, the underlying mechanisms that how Tanshinol offset TGF-β1-mediated immunosuppression of NK cells via the signaling axis and the complex needs to be further explored.

7. Author contributions

CY and CQ—methodology, writing-original draft, writing review and editing. TZ—conceptualization and formal analysis. WZ and SZ—investigation and validation. SW—project administration. AW, YZ and YL—writing revision, funding acquisition and supervision.

8. Ethics approval and consent to participate

The animal experiments follow the regulations set by the Experimental Animal Ethics Committee of Nanjing University of Chinese Medicine and international animal welfare and health standards. Ethical Review Number: 202005A018.

9. Acknowledgment

Not applicable.

10. Funding

This project was supported in part by National Natural Science Foundation of China (81973587), National TCM Innovation Backbone Training Program (Chinese medicine practitioners letter [2019] No.128), Natural Science Foundation of Higher School of Jiangsu Province (18KJA360007), the Open Project Program of Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica (No. JKLPSE201812).

11. Conflict of interest

The authors declare no conflict of interest.

12. References

[1] Du N, Bai R, Cui J. Mechanism of tumor immune escape and treatment strategy. Chinese Journal of Cancer Biotherapy. 2019; 454–462. (In Chinese)

[2] Viel S, Marçais A, Guimaraes FS, Loftus R, Rabilloud J, Grau M, *et al*. TGF-β inhibits the activation and functions of NK cells by repressing the mTOR pathway. Science signaling. 2016; 9: ra19.

[3] Wang Y, Chu J, Yi P, Dong W, Saultz J, Wang Y, *et al*. SMAD4 promotes TGF-β-independent NK cell homeostasis and maturation and antitumor immunity. The Journal of Clinical Investigation. 2018; 128: 5123–5136.

[4] Cong J, Wang X, Zheng X, Wang D, Fu B, Sun R, *et al*. Dysfunction of Natural Killer Cells by FBP1-Induced Inhibition of

Glycolysis during Lung Cancer Progression. Cell Metabolism. 2018; 28: 243–255.e5.
[5] Cortez VS., Ulland TK, Cervantes-Barragan L, Bando JK, Robinette ML, Wang Q, *et al*. SMAD4 impedes the conversion of NK cells into ILC1-like cells by curtailing non-canonical TGF-β signaling. Nature Immunology. 2017; 18: 995–1003.
[6] Sun H, Huang Q, Huang M, Wen H, Lin R, Zheng M, *et al*. Human CD96 Correlates to Natural Killer Cell Exhaustion and Predicts the Prognosis of Human Hepatocellular Carcinoma. Hepatology. 2019; 70: 168–183.
[7] Zhao Y, Hu J, Li R, Song J, Kang Y, Liu S, *et al*. Enhanced NK cell adoptive antitumor effects against breast cancer in vitro via blockade of the transforming growth factor-β signaling pathway. OncoTargets and Therapy. 2015; 8: 1553–1559.
[8] Liu Y, Zhao Y, Wang Y, Zhu P, Wei Z, Wang S, *et al*. Suppressive role of diallyl trisulfide in the activated platelet-mediated hematogenous metastasis of MDA-MB-231 human breast cancer cells. International Journal of Molecular Medicine. 2017; 39: 1516–1524.
[9] Wei W, Zhou P, Lin C, Wang W, Li Y, Gao K. Diterpenoids from Salvia miltiorrhiza and their Immune-Modulating Activity. Journal of Agricultural and Food Chemistry. 2017; 65: 5985–5993.
[10] Quan FS, Compans RW, Cho Y, Kang S. Ginseng and Salviae herbs play a role as immune activators and modulate immune responses during influenza virus infection. Vaccine. 2007; 25: 272–282.
[11] Dong Z, Ma D, Gong Y, Yu T, Yao G. Salvianolic acid B ameliorates CNS autoimmunity by suppressing Th1 responses. Neuroscience Letters. 2016; 619: 92–99.
[12] Chen X, Chen X, Gao J, Yang H, Duan Y, Feng Y, *et al*. Astragaloside III Enhances Anti-Tumor Response of NK Cells by Elevating NKG2D and IFN-γ. Frontiers in Pharmacology. 2019; 10: 898.
[13] Guillerey C, Huntington ND, Smyth MJ. Targeting natural killer cells in cancer immunotherapy. Nature Immunology. 2016; 17: 1025–1036.
[14] Stojanovic A, Correia MP, Cerwenka A. The NKG2D/NKG2DL Axis in the Crosstalk Between Lymphoid and Myeloid Cells in Health and Disease. Frontiers in Immunology. 2018; 9: 827.
[15] Ding H, Yang X, Wei Y. Fusion Proteins of NKG2D/NKG2DL in Cancer Immunotherapy. International Journal of Molecular Sciences. 2018; 19: 177.
[16] Rouce RH, Shaim H, Sekine T, Weber G, Ballard B, Ku S, *et al*. The TGF-β/SMAD pathway is an important mechanism for NK cell immune evasion in childhood B-acute lymphoblastic leukemia. Leukemia. 2016; 30: 800–811.
[17] Schilling B, Halstead ES, Schuler P, Harasymczuk M, Egan JE, Whiteside TL. IRX-2, a novel immunotherapeutic, enhances and protects NK-cell functions in cancer patients. Cancer Immunology, Immunotherapy. 2012; 61: 1395–1405.
[18] Roda-Navarro P, Reyburn HT. The traffic of the NKG2D/Dap10 receptor complex during natural killer (NK) cell activation. The Journal of Biological Chemistry. 2009; 284: 16463–16472.
[19] Upshaw JL, Arneson LN, Schoon RA, Dick CJ, Billadeau DD, Leibson PJ. NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. Nature Immunology. 2006; 7: 524–532.
[20] Zompi S, Hamerman JA, Ogasawara K, Schweighoffer E, Tybulewicz VLJ, Di Santo JP, *et al*. NKG2D triggers cytotoxicity in mouse NK cells lacking DAP12 or Syk family kinases. Nature Immunology. 2003; 4: 565–572.
[21] Ateba SB, Mvondo MA, Ngeu ST, Tchoumtchoua J, Awounfack CF, Njamen D, *et al*. Natural Terpenoids against Female Breast Cancer: a 5-year Recent Research. Current Medicinal Chemistry. 2018; 25: 3162–3213.
[22] Dong YZ, Morris-Natschke SL, Lee KH. Biosynthesis, total syntheses, and antitumor activity of tanshinones and their analogs as potential therapeutic agents. Natural Product Reports. 2011; 28: 529–542.
[23] Quatrini L, Molfetta R, Zitti B, Peruzzi G, Fionda C, Capuano C, *et al*. Ubiquitin-dependent endocytosis of NKG2D-DAP10 receptor complexes activates signaling and functions in human NK cells. Science Signaling. 2015; 8: ra108.
[24] Crane CA, Han SJ, Barry JJ, Ahn BJ, Lanier LL, Parsa AT. TGF-beta downregulates the activating receptor NKG2D on NK cells and CD8+ T cells in glioma patients. Neuro-oncology. 2010; 12: 7–13.
[25] Fujii R, Jochems C, Tritsch SR, Wong HC, Schlom J, Hodge JW. An IL-15 superagonist/IL-15Rα fusion complex protects and rescues NK cell-cytotoxic function from TGF-β1-mediated immunosuppression. Cancer Immunology, Immunotherapy. 2018; 67: 675–689.
[26] Sun C, Fu B, Gao Y, Liao X, Sun R, Tian Z, *et al*. TGF-β1 downregulation of NKG2D/DAP10 and 2B4/SAP expression on human NK cells contributes to HBV persistence. PLoS pathogens. 2012; 8: e1002594.
[27] Yin S, Chen X, Zhang J, Xu F, Fang H, Hou J, *et al*. The effect of Echinococcus granulosus on spleen cells and TGF-β expression in the peripheral blood of BALB/c mice. Parasite immunology. 2017; 39.
[28] Dotiwala F, Mulik S, Polidoro RB, Ansara JA, Burleigh BA, Walch M, *et al*. Killer lymphocytes use granulysin, perforin and granzymes to kill intracellular parasites. Nature Medicine. 2016; 22: 210–216.
[29] Ge MQ, Ho AW, Tang Y, Wong KH, Chua BY, Gasser S, *et al*. NK cells regulate CD8+ T cell priming and dendritic cell migration during influenza A infection by IFN-γ and perforin-dependent mechanisms. Journal of Immunology. 2012; 189: 2099–2109.
[30] Guo Y, Luan L, Rabacal W, Bohannon JK, Fensterheim BA, Hernandez A, *et al*. IL-15 Superagonist–Mediated Immunotoxicity: Role of NK Cells and IFN-γ. The Journal of Immunology. 2015; 195: 2353–2364.

Keywords: Tanshinol; Tumor; TGF-β1; NKG2D; DAP10; NK cell functions

Send correspondence to:

Aiyun Wang, Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica, School of Pharmacy, Nanjing University of Chinese Medicine, 210023 Nanjing, Jiangsu, China, Jiangsu Collaborative Innovation Center of Traditional Chinese Medicine (TCM) Prevention and Treatment of Tumor, Nanjing University of Chinese Medicine, 210023 Nanjing, Jiangsu, China, E-mail: wangaiyun@njucm.edu.cn

Yang Zhao, Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica, School of Pharmacy, Nanjing University of Chinese Medicine, 210023 Nanjing, Jiangsu, China, School of Medicine & Holistic Integrative Medicine, Nanjing University of Chinese Medicine, 210023 Nanjing, Jiangsu, China, E-mail: y.zhao@njucm.edu.cn

Yin Lu, Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica, School of Pharmacy, Nanjing University of Chinese Medicine, 210023 Nanjing, Jiangsu, China, Jiangsu Collaborative Innovation Center of Traditional Chinese Medicine (TCM) Prevention and Treatment of Tumor, Nanjing University of Chinese Medicine, 210023 Nanjing, Jiangsu, China, E-mail: luyingreen@126.com

† These authors contributed equally.