Inhibition of MiR-20a by Pterostilbene Facilitates Prostate Cancer Cells Killed by NK Cells Via up-Regulation of NKG2D Ligands and Down-Regulation of TGF-β1

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Research Article

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Abstract

Purpose-Natural killer (NK) cells play a potent role in antitumor immunity via spontaneously eliminating tumor directly. However, some tumors such as prostate cancer constantly escape this immune response by down-regulating cell surface molecule recognition and/or secreting immune impressive cytokines. Pterostilbene, a dietary compound primarily found in blueberries, exhibits a potential anti-tumor activities and immunoregulation function. However, whether it could affect NK cells immune response against prostate cancer is not clear.

Methods-NK cell cytotoxicity against prostate cancer cells was analyzed using the CytoTox 96 cytotoxicity assay kit. Flow cytometry was used to detected NK cell degranulation, NK cell-surface and intracellular protein expression. The effects of pterostilbene on TGF-β1 secretion were determined by Enzyme-linked immunosorbent assay. The candidate target gene of miR-20a was determined by luciferase reporter assay and the expression of major histocompatibility complex class I chain-related proteins A and B (MICA/B) was detected by quantitative real-time polymerase chain reaction.

Results-We found pterostilbene could enhance expression of MICA/B on prostate cancer cells surface, which are ligands of the natural killer group 2 member D (NKG2D) expressed by NK cells, and inhibit TGF-β1 secretion by prostate cancer cells. Further, we discovered that these effects were caused by inhibition of miR-20a in prostate cancer cells by pterostilbene. MiR-20a could target the 3’ untranslated region (UTR) of MICA/B, resulting in their expression down-regulation. Inhibition of TGF-β1 function by its specific antibody attenuated its impairment to NKG2D on NK cells. Finally, we observed that pterostilbene-treated prostate cancer cells were more easily to be killed by NK cells.

Conclusions-Our findings demonstrated inhibition of miR-20a by pterostilbene in prostate cancer cells could increase MICA/B expression and decrease TGF-β1 secretion, which enhanced NK cell-mediated cytotoxicity againt prostate cancer cells, suggesting a potential approach for increasing anti-prostate cancer immune.

Introduction

Prostate cancer is the most commonly diagnosed malignancy in men in the US, and rank the second leading cause of cancer-related death in men [1]. It is a life-threatening disorder that is undruggable to date owing to stumbling blocks in the standardization of therapy. In response to its immunogenicity, anti-tumor immunotherapy has shown promising effects in prostate cancer patients [2]. As a first line of defence of the innate immune system, natural killer (NK) cells exert anti-tumor effects via their cytotoxic and immune-regulatory capacities, which are based on the interaction of their surface receptors recognizing ligands specifically expressed by malignant cells or stressed cells [3]. NKG2D and its ligands including major histocompatibility complex (MHC) class I chain-related proteins A and B (MICA/B) and unique long 16-binding proteins 1 through 6 (ULBP1-6) are such a pair of the most important costimulatory molecules modulating the cytotoxicity of NK cells against tumor cells [4, 5].

Downregulation or complete knockout of NKG2D in mice caused an impaired immune response against tumor cells, including prostate cancer [6, 7], yet increasing expression of NKG2D ligands in cancer cells enhanced NKG2D-mediated anti-tumor response [8, 9]. Overexpression of NKG2D ligands predicts favorable prognosis in some cancer types [10, 11]. Some constant important strategies by which tumor cells adapt to evade the immune response are to downregulate or lose expression of certain ligands on cells surface, and/or to secret immune suppressive cytokines like IL-10, TGF-β impairing activating receptors on immune cells [12, 13]. Therefore, exploring the approach improving or potential mechanisms regulating NKG2D ligands expression in prostate cancer cells or NKG2D expression on NK cells might be important for NKG2D-mediated NK cells immune response against this disease.

It is well known that microRNAs (miRNAs), single-strained noncoding RNAs of approximately 19–23 nucleotides, are confirmed to play a crucial role in biological process of cancer development by regulating expression of special targeted genes [14]. In prostate cancer, changes in expression of miRNAs are associated with clinically pathological parameters such as Gleason score and recurrence [15, 16]. In addition, many miRNAs regulate the effects of lymphocytes on immune-mediated diseases [17]. It has been shown that several miRNAs such as miR-17, miR-20a, miR-93 and miR-106b are involved in the regulation of NKG2D ligands expression in colon and glioma cells as well as breast cancer [18–20], and some miRNAs like miR-30c, miR-1245 can up-regulate NKG2D expression on NK cells [21, 22], suggesting the post-transcriptional regulation of NKG2D or its ligands by microRNAs. In previous publications, miR-20a was found to be highly expressed in both prostate cancer tissues and prostate cancer cell lines, playing an important role in biological behavior of prostate cancer [23–25].

Pterostilbene (Pte), a dietary compound that is primarily found in blueberries, exhibits a potential anti-tumor activities on a variety of cancer cells [26]. It has been reported that Pte suppresses the growth and promotes apoptosis of prostate cancer LNCap cells [27]. It also possesses properties like anti-inflammation [28] and immunoregulation [29]. However, whether it could affect NK cells immune response against prostate cancer is not clear. Here, we detected that treatment with pterostilbene inhibited miR-20a, resulting in up-regulation of NKG2D ligands MICA/B and decreased secretion of TGF-β1 in prostate cancer cells, which enhanced the susceptibility of tumour cells to natural killer cell-mediated cytotoxicity.

Materials And Methods

Cell lines and cell culture

Prostate cancer cell lines (DU145, PC3) and human NK cell line NK92 cell were obtained from the Chinese Academy of Sciences (Wuhan, China). Prostate cancer cell lineswere cultured in RPMI 1640 medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 100 mg/mL penicillin/streptomycin. NK92 cells were maintained in RPMI 1640 supplemented with 10% human FBS and 100 U/ml recombinant human IL-2 (Peprotech, Rocky Hill, NJ). All cells lines were maintained at 37ºC in a humidified atmosphere containing 5% CO2.

Cell transfection and pterostilbene treatment

For miRNA transfection, prostate cancer cells were plated in six well plates and after growingup to 70–90% confluence, the cells were transfected with the synthesized miR-20a mimics or scramble control (Guangzhou Ribobio Co, Guangzhou, CA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. For pterostilbene treatment, cells were cultured with adding of pterostilbene (Cayman Chemical, Michigan, USA) dissolved in DMSO (Sigma, Saint Louis, USA) at a final concentration of 50μmol/L in medium. Cell samples were collected at 24 h after transfection or treatment with pterostilbene for further analysis.

qRT-PCR

Cell samples were lysed in RNAiso (TaKaRa, Dalian, China) for miRNA extraction, and miRNAs was reverse-transcribed into complementary DNAs (cDNAs) using PrimeScript Reverse Transcriptase (TaKaRa, Dalian, China) to produce a template suitable for qRT-PCR. U6 was used as an internal control. qRT-PCR was performed using SYBR Green master mix kit (Exiqon, Vedbaek, Denmark). This experiment was repeated 3 times and data were analyzed with the $2^{-\Delta\Delta Ct}$.

Luciferase reporter assay

TargetScanHuman 7.1 (www.targetscan.org) predicted that MICA/B was a potential target ofmiR-20a, but not the TGF-β1. To generate the luciferase reporter vectors, MICA/B 3'UTR segments (MICA/B 3'UTR WT and MICA/B 3'UTR MUT) were amplified by PCR from the human cDNA and inserted into the pmiR-RB-Report™ vector (Guangzhou RiboBio Co., Ltd., China). Then MICA/B 3'UTR luciferase construct and miR-20a mimics were co-transfected into DU145 and PC3 cells using Lipofectamine2000 transfection reagent (Invitrogen, Shanghai, China) according to manufacturer's instructions. After 48 h transfection, relative luciferase activity of MICA/B 3'UTR was measured by the Dual-Luciferase Reporter Assay (Promega, Madison, USA).

Analysis of NK cell degranulation

NK92 cells were co-cultured with PC3 or DU145 cells at a 3 : 1 ratio for 4 h. PE-anti-107a or isotype control monoclonal antibody (BD Biosciences, NJ, USA)and Golgi-Plug and Golgi-Stop eagents (BD Biosciences, NJ, USA) at 1:1500 dilution were added to the co-culture system at the beginning of the assay. Afterward, the cells were collected for being analyzed by flow cytometry.

Flow cytometry

For cell-surface protein expression analysis, cells were harvested, and incubated with antibodies for 30 min at 4°C in the dark. These antibodies are: APC-conjugated CD3, APC-cy5-conjugated CD56, PE-conjugated anti-NKG2D, FITC-conjugated anti-MICA, PE-conjugated anti-MICB (BD Biosciencesn, NJ, USA). For intracellular staining, cells were fixated and permeabilizated using BD Perm/Fix kits following surface markers staining and then stained withPE/Cy7-anti-IFN-γ (Biolegend, CA, USA). Before being

collected, cells were cultured in the presence of Golgi-Plug and Golgi-Stop eagents at 1:1500 during the last 4 h. Isotype-matched monoclonal antibodies were used as controls.Cells were analyzed by flow cytometer (BD, NJ, USA). Data were analyzed using the FlowJo software (BD, NJ, USA).

Enzyme-linked immunosorbent assay

Culture supernatants were harvested and analyzed forTGF-β1production by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, CA, USA), following the manufacturer's instructions.

NK cell cytotoxicity

NK cells cytotoxic to prostate cancer cells was analyzed using the CytoTox 96 cytotoxicity assay kit (Promega, Madison, USA) according to the manufacturer's instructions. In brief, 1×10^4 DU145 or PC3 cells were seeded in a round-bottom 96-well plate as target cells. NK92 cells were then added as effector cells at E:T ratios of 10:1, 3:1 and 1:1. After 4 h of co-incubation, the supernatant was removed for analysis. The cytotoxicity of the effector cells against the target cells was assessed as: cytotoxicity = (experimental release - effector spontaneous release - target spontaneous release)/(target maximum release - target spontaneous release) × 100%.

Statistical analysis

Statistical analyses were performed using SPSS version 13.0. Values were expressed as the mean ± standard deviation. Statistically significant differences between groups in each assay were determined by ANOVA, or student's t-test. $P < 0.05$ was considered statistically significant.

Results

Pterostilbenetreatment enhanced the susceptibility of prostate cancer cells to NK cell-mediated cytotoxicity

To explore whether Pte could facilitate prostate cancer cells to be killed by NK cells, PC3 and DU145 cells were first treated with Pte at 50μmol/L for 24 h, and then co-cultured with NK92 cells. The treatment of Pte enhanced cytotoxicity of NK92 cells against both PC3 and DU145 cells (Fig. 1A, 1F). Meanwhile, the intracellular expression of IFN-γ and 107a, which represented the activity of NK cells, was also measured. It was showed that Pte treatment remarkably increased the IFN-γ and 107a expression in NK92 cells (Fig. 1B-1E, 1G-1J). These data demonstrated Pte-treated prostate cancer cells could enhance their susceptibility to the cytotoxicity of NK cells.

Prostate cancer cells treated with pterostilbene up-regulated MICA/B expression and decreased TGF-β1 secretion

To detect the potential mechanisms by which Pte increased the susceptibility of PC3 and DU145 cells to NK92 cells, due to the very important role of NKG2D ligands in NK cell-mediated anti-tumor immune

response according to the published studies [4,5], we analyzed the expression of MICA/B on tumor cells. Flow cytometry showed that MICA/B on both PC3 and DU 145 cells surface were highly up-regulated by Pte treatment (Fig. 2A-2H). Besides, we also found the level TGF-β1, which is one of the most critical and common cytokines contributing prostate cancer cells to escape immune responses [13], in the supernatants of PC3 and DU145 cells culture was dramatically decreased by Pte (Fig. 2I-2J). Further, it was discovered that co-culture of cancer cells and NK92 cells for 24 h decreased the expression of the NKG2D on NK cells surface, while when TGF-β1was neutralized by adding TGF-β1 antibody to the co-culture, the NKG2D decrease was obviously attenuated (Fig. 2K-2M). These data indicated that Pte-treated prostate cancer cells increased MICA/B expression on cell surface and decreased TGF-β1 secretion, which could attenuate down-regulation of NKG2D on NK cells by tumor cells.

Inhibition of miR-20a was involved in MICA/B up-regulation and TGF-β1 decrease in prostate cancer cells by pterostilbene treatment

To further make clear how Pte affected the MICA/B expression and TGF-β1 secretion in prostate cancer cells, we analyzed miR-20a, which was found to regulate MICA/B expression in several types of tumors [30,19,20]. Our findings showed Pte treatment dramatically decreased miR-20a level in both PC3 and DU145 cells (Fig.3A-3B). Further, we found the up-regulation of MICA/B expression and down-regulation of TGF-β1 secretion in cancer cells by Ptewere attenuated by treating with miR-20a mimics(Fig. 3C-3D, 3E). Next, to further elucidate how miR-20a regulates MICA/B and TGF-β1**,** the MICA/B and TGF-β1 3’ UTR was prepared in pmiR-RB-Report™ vector respectively, andluciferase activity was measured. After cells were transfected with synthetic miR-20a mimics, the relative luciferase activity in the wild-type group was obviously decreased compared with the mut-type group in targeting MICA/B, but not TGF-β1 (Fig. 3F-3G). The results suggested that inhibition of miR-20a by Pte up-regulated MICA/B expression by directly binding to the 3’ UTR of MICA/B in prostate cancer cells, but how reduced TGF-β1secretion was still unclear.

Discussion

The activating receptor NKG2D is a powerful stimulatory immunoreceptor expressed on NK cells, NKT cells, γδ + T cells and CD8 + T cells [31]. Engagement of NKG2D by its ligands causes anti-tumor immune response via either the direct activation of killing by NK cells or adaptive cytotoxicity by cytotoxic T-lymphocytes (CTLs). However, tumor cells constantly develop some strategies to downregulate their NKG2D ligands expression or inhibit NKG2D on immune cells, allowing them to avoid immune recognition and removal. Therefore, the NKG2D/NKG2D ligands system has brought much interest as a potential treatment target to improve antitumor immune responses [4].

In this study, we found pterostilbene enhanced the susceptibility of prostate cancer cells to NK cell-mediated cytotoxicity in vitro by up-regulating MICA/B expression and decreasing TGF-β1 secretion. Pterostilbene is a dietary compound that is primarily found in blueberries, showing various functions in anti-tumor application, including induction of apoptosis, regulation of autophagy and mediation of

immune response [26]. Here, we first discovered its another role in promoting prostate cancer cells to be killed by NK cells. Previous study showed MICA/B are ectopic expressed in prostate cancer tissue, and they are correlated to patient prognosis [32]. Up-regulation of NKG2D ligands in prostate cancer cell line DU145 enhanced NK cell-mediated cytotoxicity against the tumor [18]. TGF-β1 is a common immune suppressive cytokine, which can impair cytotoxicity of cytotoxic T lymphocytes against tumor cells [33], promote the generation of immunosuppressive regulatory T (Treg) cells [34], increase the production of immunosuppressive cytokine IL-10 by macrophages [35] and inhibit IFN-γ production in NK cells through Smad2, Smad3, and Smad4 and at least in part through inhibition of T-bet [36]. Here, our findings showed both PC3 and DU145 cells released TGF-β1, which resulted in inhibition of NKG2D in NK cells. However, this inhibition effect could be attenuated when TGF-β1 was neutralized by its antibody. Thus, inhibition of TGF-β1 secretion by prostate cancer cells via pterostilbene treatment would recover NKG2D on NK cells, enhancing NKG2D-mediated NK cells anti-tumor response. In addition to that, the most recent publication demonstrated the highly secreted TGF-β1 by prostate cancer also induced expression of inhibitory receptor (ILT2/LILRB1) and down-regulation of activating receptors NKp46 and CD16 by NK cells [37]. Therefore, up-regulating the MICA/B expression in prostate cancer cells and recovering NKG2D on NK cells by pterostilbene would be a potential promising approach to convert prostate cancer evasion from NK cells immune response.

The regulation of NKG2D and MICA/B expression is a complex process that involves various transcriptional and post-transcriptional mechanisms, including uumerous signals and pathways such as ATM (ataxia-telangiectasia, mutated) and ATR pathway, c-Myc/miR-17 pathway [38–40]. The previous studies showed the anti-cancer role of dietary compounds via their effect on microRNAs aberrantly expressed in malignant cells [41, 40]. Dhar et al. found significantly increased expression of miRs-17, -20a, -106a and − 106b and the ability of dietary resveratrol to downregulate these oncomiRs in miRNA profiling studies of prostate cancer cells [42]. It has been demonstrated that miR-20a is not only related to tumor development [43], but also associated with invasive activity in prostate cancer cells [23]. Therefore, we detected the changes of miR-20a in PC3 and DU145 under pterostilbene treatment. We found pterostilbene-treated tumor cells down-regulated miR-20a levels dramatically and up-regulation of MICA/B expression and down-regulation of TGF-β1 secretion in cancer cells by pterostilbene were attenuated by treating with miR-20a mimics. These data suggested the effects of MICA/B and TGF-β1 in tumors cells by pterostilbene were involved in miR-20a. Then we further confirmed that miR-20a targeted 3’UTR of MICA/B, and overexpression of miR-20a apparently inhibited the MICA/B expression. miR-20a overexpression also significantly inhibited the TGF-β1 expression, but not in mechanism ob by targeting to 3’UTR of TGF-β1, and potential mechanisms is still needed to further investigation.

Taken together, given exploiting NK cells as a therapeutic strategy for prostate cancer, which has shown promising benefits in preclinical studies [44], our findings demonstrated pterostilbene-treated prostate cancer cells enhanced their susceptibility to NK cell-mediated cytotoxicity with promoted IFN-γ and 107a, providing additional evidence for improving NK cell-based immunotherapeutic interventions against prostate cancer.

Declarations

Conflict of interest statement: There are no potential conflicts of interest in this paper.

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Figures

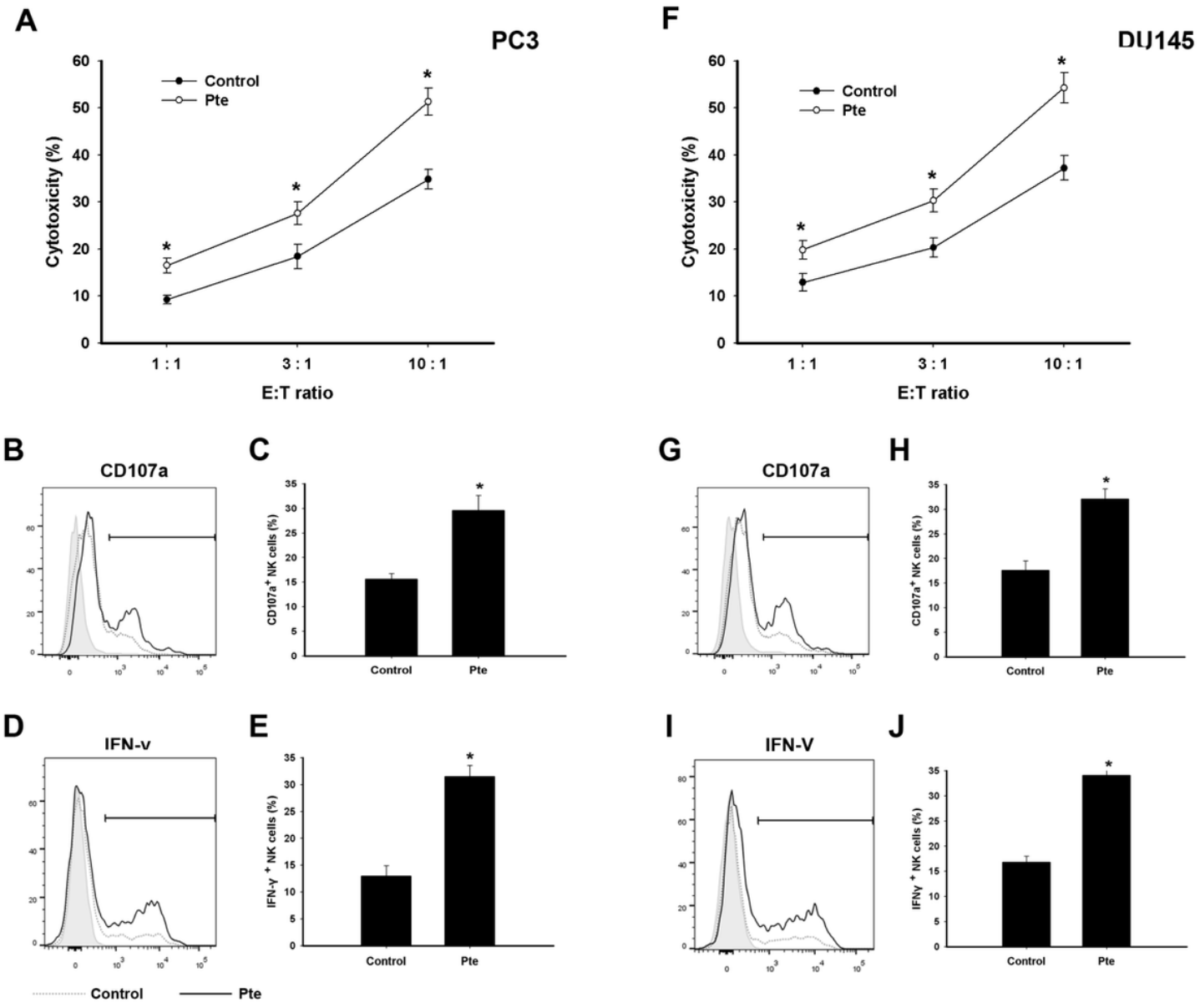

Figure 1

Pterostilbene enhanced the cytotoxicity of NK cells to PC3 and DU145 cells. (A, F) PC3 and DU145 cells treated with Pte at 50μmol/L for 24 h, were co-incubated with NK92 cells at an indicated E:T ratio for another 4 h. The cytotoxic activity of NK cells were evaluated using the CytoTox 96 Non-Radioactive Cytotoxicity Assay. (B-E, G-J) PC3 and DU145 cells treated with Pte were co-incubated with NK92 cells at an E:T ratio of 10:1 for another 4 h. The cells were then collected for flow cytometry to measure the expression of IFN-γ and 107a in NK92 cells. Data were presented as the mean ± SD of 3 independent experiments, each performed in triplicate (*P<0.05).

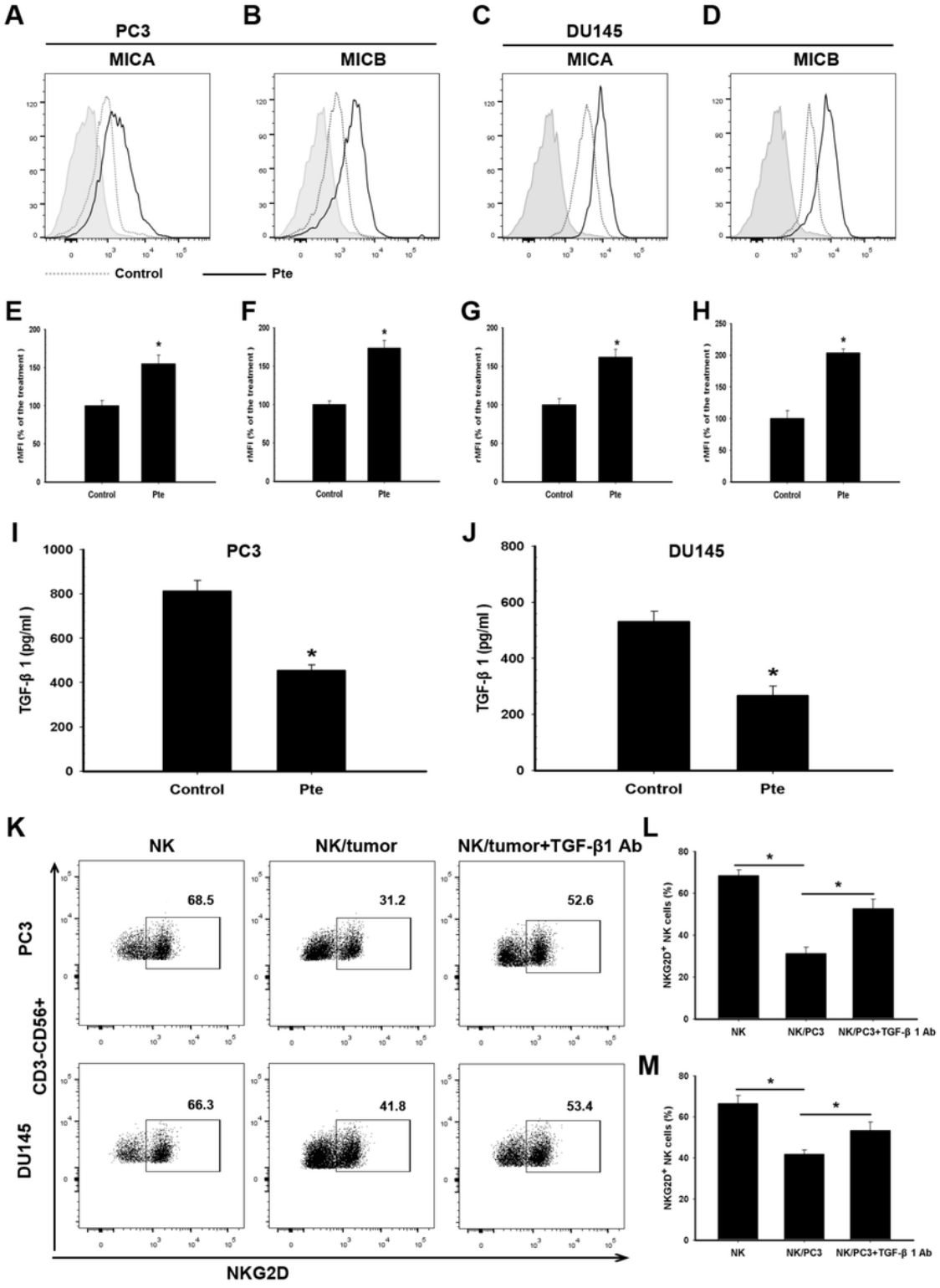

Figure 2

Pterostilbene up-regulated MICA/B expression and decreased TGF-β1 secretion in PC3 and DU145 cells. (A-H) PC3 and DU145 cells were treated with Pte at 50μmol/L for 24 h, and then collected for flow cytomery detecting the expression of MICA/B. (I-J) PC3 and DU145 cells were treated with Pte at 50μmol/L for 24 h. The supernatant was collected and subjected to the measurement of TGF-β1 by ELISA. (K-M) PC3 and DU145 cells were treated with neutralizing TGF-β1 antibody (10μg/ml) for 30 min

before co-cultured with NK92 cells for another 24 h. The expression of NKG2D was analyzed by flow cytometry. Data were presented as the mean ± SD of 3 independent experiments, each performed in triplicate (*P<0.05).

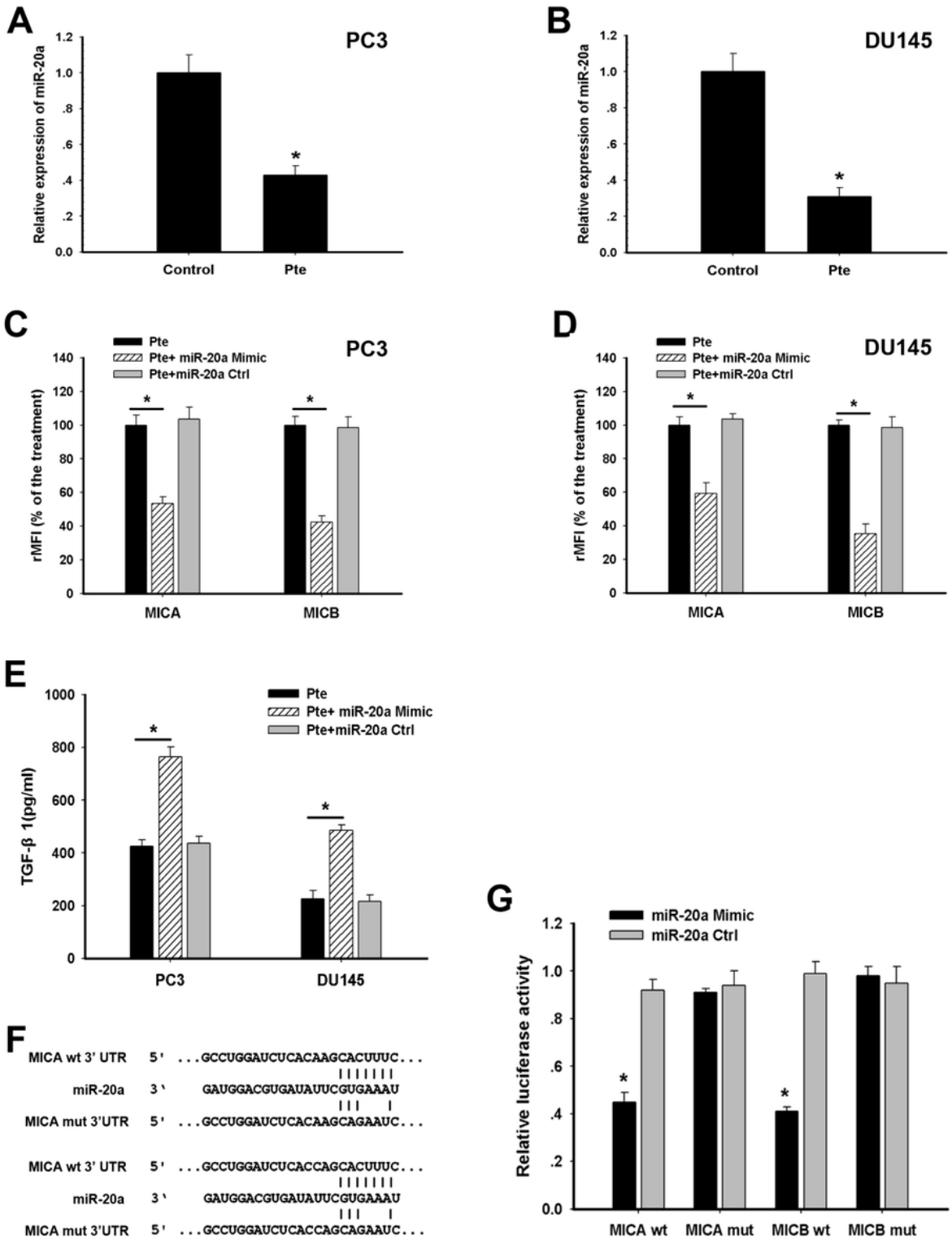

Figure 3

Inhibition of miR-20a induced MICA/B up-regulation and TGF-β1 decrease in PC3 and DU145 cells by pterostilbene. (A-B) PC3 and DU145 cells were treated with Pte at 50μmol/L for 24 h, and then the

expression of MICA/B was detected by qRT-PCR. (C-D) PC3 and DU145 cells were treated with Pte (50μmol/L) in the absence of miR-20a mimics (50nm) for 24 h, and then collected to measure the expression of MICA/B using flow cytometry. (E) PC3 and DU145 cells were treated with Pte (50μmol/L) in the absence of miR-20a mimics for 24 h. The concentration of TGF-β1 in the supernatant was measured by ELISA. (F-G) The predicted binding sites of miR-20a in the 3'-UTR of MICA/B was identified using TargetScanHuman 7.1 and the relative luciferase activity was measured after transfection of the indicated reporter plasmids (MICA/B 3'-UTR or the mutant MICA/B 3'-UTR) into DU145 cells. Data were presented as the mean ± SD of 3 independent experiments, each performed in triplicate (*P<0.05).