miR-221-5p and miR-186-5p are the critical exosomal miRNAs in bladder cancer induced natural killer cells dysfunction

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Abstract

Bladder cancer (BC) is the 10th most commonly diagnosed cancer worldwide, its carcinogenesis mechanism has not been fully elucidated. BC is able to induce natural killer (NK) cells dysfunction and escape their immune surveillance. The present study found that exosome plays an important role in BC induced NK cell dysfunction by impairing viability, and inhibit the cytotoxicity of NK cell on target cells. Meanwhile, BC derived exosome inhibited the expression of important functional receptors NKG2D, NKp30, and CD226 on NK cells and the secretion of perforin and granzyme-B. Through high-throughput sequencing, the critical miRNAs with high expression in BC exosomes were identified. Furthermore, after Dual-Luciferase reporter assay and transfection experiment, miR-221-5p and miR-186-5p was confirmed can interfere the stability of mRNAs of DAP10, CD96, and perforin gene in NK cells and may be the potential targets using in the therapeutic application of BC.

Introduction

Bladder cancer (BC) is among the most prevalent cancers with a high incidence and fatality rate. In 2020, bladder cancer is ranked the 10th most commonly diagnosed cancer worldwide, with approximately 573,000 new cases and 213,000 deaths (1). Despite receiving years of attention and research, the mechanism of BC oncogenesis remained unexplained. Multiple structural and genetic abnormalities, as well as variations in cellular metabolism and intracellular signaling pathways, were postulated to contribute to the oncogenesis of BC (2). On the other hand, inadequate anti-tumor immunosurveillance and the ability of malignancies to evade immune clearance may partially explain the cause of oncogenesis. By gradually accumulating a set of mutations, tumors downregulate the MHC Class I expression on their surface to avoid being killed by $CD8^+$ T cells (3). Tumors could also induce dysfunction and exhaustion of a variety of immune cells, including $CD4^+$ T cells, $CD8^+$ T cells, natural killer (NK) cells, and dendritic cells (DC), by secreting tumor-related factors and extracellular vesicles (EVs) into the tumor microenvironment (4–7). Exhausted anti-tumor immune function have been reported in numerous of tumors, including breast cancer, lung cancer, colon cancer (8), kidney cancer, pancreatic cancer, ovarian cancer (9), glioma, and melanoma (10). To clarify the mechanism of anti-tumor immune fatigue is vital to improve the immunotherapeutic efficacy and prognosis of cancer patients.

NK cells ($CD56^+$ $CD3^-$) are a subset of large granular lymphocytes, independent of prior activation, they can directly kill transformed and viral-infected cells, and stimulate adaptive immunity by secreting pro-inflammatory cytokines and chemokines (11). NK cells serve a crucial role in anti-tumor immune surveillance; they are able to identify and eliminate tumor cells that have evaded T cell lysis, and enhancing the anti-tumor activity of T cells by releasing cytokines (12). NK cells are activated or maintained in a quiescent state based on the delicate balance between activating and inhibitory signals. According to clinical investigations, the decreased number and hindered activity of NK cells were detected in patients with BC (13, 14) suggesting that BC cells can evade anti-tumor immune surveillance by inducing NK cell dysfunction via an unknown mechanism.

Exosomes are nano lipid bilayer vesicles with a 30–120 nm (15) diameter that are secreted by virtually all live cells and have been discovered in various body fluids (16). Exosomes, and exosomal cargos such as DNA, RNA, lipids, and proteins, have garnered a great deal of attention due to their essential role in intercellular communication and regulation of target cells activity. Exosomes, which mirror the properties of their parent cells, have been exploited as biomarkers in illness diagnosis, prenatal sex determination, and the production of vaccines (17). Tumor cells secrete around 10 times more exosomes than normal cells (18–20). These tumor derived exosomes (TEXs) play a vital role in carcinogenesis, development, metastasis, angiogenesis, and drug resistance. The immunological regulatory function of TEXs has been gradually uncovered in recent years. The research revealed that through reducing the expression of CD3 and JAK3 in primary activated T-cells and mediating Fas/FasL (Fas ligand)-driven $CD8^+$ T-cells death, TEXs impaired T-cell activity in tumor microenvironment (21). As a result of exposure to TEXs, NK cells display a similar reduction of activity as T cells. By inducing SMAD phosphorylation and reducing NKG2D receptor expression, TEXs may inhibit the cytotoxicity of NK cells in myelogenous leukaemia patients (21). Another study showed that clear cell renal cell carcinoma (ccRCC) can release exosomes to induce NK cells dysfunction and evade innate immune surveillance by regulating the TGF-β/SMAD pathway (22). More evidence has confirmed the suppressed cytotoxicity capacity of NK cells in cancer patients, as described in the review of Wioletta *et al*. (23).

In the tumor microenvironment, there is a undeniable interaction between TEXs and NK cells, but how BC-derived TEXs influence NK cells has not been thoroughly investigated. The purpose of the present study is to examine the impact of BC-derived exosomes on NK cells. Exosomes derived from a BC cell line (T24) were used to treat human primary NK cells, after which, the cytotoxic activity, the expression of essential functional receptors as well as the cytotoxic cytokines secretion of NK cells were detected. Furthermore, the miRNA signature of T24 exosome was profiled by using high-throughput sequencing, and the essential miRNAs that play a crucial role in NK cell activity were screened and verified.

Materials And Methods

Ethics statement

This study was approved by the Ethic Committees of Northwestern Polytechnical University; Ethics approval number: 202102043. The ethics form is shown in the supplementary materials

Cell lines

The BC cell lines T24, human urothelium immortalized cells SV-HUC-1, K562 cell and HEK 293 cell were obtained from Cell Bank of Chinese Academy of Sciences (BioVector NTCC, Shanghai, China). The three cell lines of T24, SV-HUC-1 and HEK 293 were normal cultured in DMEM cell culture medium (Gibco, No. 11965-084, USA), supplemented with 100 units/mL penicillin, 0.1 mg/mL streptomycin and 10% (v/v) exosome-depleted FBS (SBI, Mountain View, CA, USA), K562 cells were maintained in RPIM-1640 culture medium with the same supplements. All cells were cultured in a humidified 5 % CO_2 atmosphere at 37 °C.

NK cells expansion

Human NK cells were expanded from peripheral blood mononuclear cell (PBMC) according to Li *et al*. (24). Briefly, peripheral venous blood was collected from healthy donors (10 mL, n = 12) and the PBMC were separated by using lymphocyte separation liquid (Haoyang TBD, Tianjin, China). Genetically modified stimulating cell for NK cell expansion was prepared according to our previous work (24) and cultured in RPIM-1640 cell culture medium supplemented with 100 units/mL penicillin, 0.1 mg/mL streptomycin and 10% (v/v) FBS (Gibco, No 10082139, USA). PBMC were co-cultured with an equal number of irradiation killed stimulating cells and maintained in RPIM-1640 cell culture medium, supplemented with 100 units/mL penicillin, 0.1 mg/mL streptomycin, 10% (v/v) FBS and 100 units/mL IL-2. After 14 days culture, the proportion of NK cells in PBMC was determined by flow cytometry (BD FACS Calibur, San Jose, CA, USA) through labeled with CD56-PE (BD, Cat No. 561904, CA, USA) and CD3-FITC monoclonal antibodies (mAbs) (BD, Cat No. 561806, CA, USA).

Exosomes isolation and identification

The culture supernatant of T24 and SV-HUC-1 cells was collected to isolate exosomes according to the instruction of ultracentrifugation method (25). Briefly, the cell culture supernatant was prepared by initial centrifugation (1×10 min, 300×g; 1×30 min, 2000×g) to remove cell debris. The exosomes contained supernatant was collected and filtered by 0.22 μm filter. The filtrate was ultracentrifuged (1×30 min, 10,0000×g) by using a fixed-angled rotor (Ti-70, Beckman Coulter, Inc., Brea, CA, USA) at 4°C. After washed by PBS, the filtrate was ultracentrifuged (1×70 min, 10,000×g) at 4 °C. The sediment was stored in -80 °C for the subsequent detection.

Transmission electron microscope (TEM) was employed to detect the morphology of exosome. The exosome sediment was resuspended in 2% paraformaldehyde aqueous solution and then 3-5 μL exosome suspension was dripping onto cleaned mica chips. After critical point drying, mica chips were imaged by (TEM) (FEI, Tecnai G2 Spirit BioTwin, USA) at 10 kV with a CCD camera (Gatan, Warrendale, PA, USA). Western blot method was used to examine the exosomal marker CD9, CD63 and CD81 on isolated exosome. Further, the Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) was used to detected the particle size and concentration of isolated exosomes. Each sample was measured 3 times at room temperature. The concentrations of exosomes were evaluated by BCA assay (BCA Protein Assay Kit, Beyotime, No. P0012-1).

Exosomes uptake assay

The exosomes uptake assay was following the protocol of Li *et al.* (24). Briefly, NK cells were added into 12 well plate and co-cultured with PKH67 labeled exosome for 24h. After then, the NK cells were washed by PBS and labeled with DAPI (Beyotime Institute of Biotechnolog, No. C1005, China). Then NK cells were observed under an inverted fluorescence microscope (Leica, Inverted Fluorescence Microscope, DM IL LED, Germany) and tested by flow cytometry. The positive rate of the NK cells (only the NK cells which take up labeled exosome showed the green fluorescence signal) was calculated.

NK cell viability and cytotoxicity

NK cells (2×10^6 in each group) were treated by T24 exosome for 24 h to 48 h. After centrifuged at 1000×g for 5 min and washed by PBS, NK cells in each group were resuspended in 1 mL RPMI-1640 media (IL-2 free). NK cell suspension (200 μL) were added into a well of 96-well plate and repeated for 5 well. Twenty microliter of CCK-8 (Cell Counting Kit-8, Dojindo, Japan) was added to each well of NK cell and then the plate was incubated in a 5 % CO_2 incubator at 37 ℃ for 2 h. The optical density (OD) value of each well was recorded at 450 nm in a microplate reader (BioTek Synergy-4, Germany). The viability of the NK cells was examined.

The cytotoxicity of NK cells was tested according to our previous publication (26). Briefly, NK cells in each group were centrifuged and washed by PBS. After then, NK cells (2×10^6) were re-suspended in RPMI-1640 (IL-2 free) and added into the well of a 96-well plate at 100 μL. Target cell (K562) (4×10^5) which re-suspended in 100 μL medium were added to the same well of NK cell (mix well) to make the effector-to-target ratio (E:T) at 5:1 and co-cultured for 4 h. The wells contain only NK cell (effector (e) well) and K562 cell (target (t) well) respectively were set and cultured in same condition. After 4 h incubation, 20 μL of CCK-8 was added to each well, and the plate was incubated for another 2 h at normal culture condition. The OD values were recorded at 450 nm. The cytotoxicity was determined by evaluating the rate of NK cells killed the target cells; the killing rate was calculated by following equation:

Killing rate (%) = [1- (OD(mix)-ODe)/ODt]×100%.

NK cell apoptosis and receptor expression

After T24 exosome treatment, the apoptosis of NK cell was detected by Annexin-V/PI double dye method. NK cells in each group were collected and washed by PBS. After labeled with Annexin V-FITC and PI (AnnexinV-FITC Apoptosis Detection Kit, Cat No. C1062M. Beyotime Institute of Biotechnolog, China), NK cells in each group were tested by flow cytometry (BD Calibur, Biosciences, California, USA).

After T24 exosome treatment, the NK cells in each group were washed and collected to divided into 4 groups (1×10^6). Different antibodies of NK cell receptors (NKG2A, NKG2D, NKp30, NKp44, NKp46, and CD226) were used to labeled each group of NK cells. All the cells were tested by flow cytometry separately and analyzed by Cellquest (BD) software. The information of all antibodies are showed in Table 1.

Exosomal miRNA profile of T24 cell

The miRNA profiles of T24 and SV-HUC-1 cell were identified by next-generation sequencing. By compared to SV-HUC-1 exosomal miRNA profile, the aberrant expressed miRNAs in T24 cell derived exosome were identified and verified.

According to the sequencing data, miR-146-3p is the highest expressed miRNA in T24 exosome with large number of reads and significant up-regulated expression compared to the exosome of SV-HUC-1 cell. The other high expressed miRNAs in T24 exosomes are miR-100-5p, miR-21-5p, miR-21-3p, miR-30a-5p, let-7i-5, miR-221-3p, miR-22-3p, miR-186-5p, miR-28-3p, and miR-378a-3p. The expression of this group of miRNAs in T24 exosome was verified by q-PCR method following the instruction of EasyPure miRNA Kit (Transgen Biotech, Beijing, China) and TransScript Green miRNA Two-Step qRT-PCR SuperMix (Transgen Biotech, Beijing, China). q-PCR circle was implement in CFX96 Touch qPCR System (Bio-Rad Laboratories, Hercules, CA, USA). Small RNA U6 was used as internal reference gene, and $2^{-\Delta\Delta Ct}$ method (27) was used to calculate the relative expression ratio of each miRNA in T24 exosome. The specific primers and the expression of miRNAs in BC exosome are showed in our previous study (28)

Target Gene Prediction

Four online programs TargetScan, miRDB, miRTarBase and miRWalk were employed to predict the potential target genes of these miRNAs in NK cells. The diagrammatic sketch of the interactions of exosomal miRNAs and potential target genes in NK cells was drew by the Cytoscape software (version 3.6.1) (Supplementary material figure1). miR-221-5p and miR-186-5p were selected for downstream analysis.

Luciferase Reporter Assay

TargetScan 7.2 database (http://www.targetscan.org/) were employed to predict the binding site of miRNAs matched to the target genes. Please see the algorithm predicted binding site of miRNA to the target genes in figure 5. Luciferase reported assay was used to confirm the combination of miRNA and target genes. Following the manufacturer's protocol, the position of sequence region, containing the putative binding sequence of each miRNA, was inserted into a luciferase reporter vector pGLO-basic (Promega, Madison WI, USA). Mutated sequence of the target gene was constructed into pGLO-basic vector. The sequences constructed in the reporter vector and mutant vector were confirmed by sequence analysis. pRL-TK Renilla Luciferase Reporter vector (Promega, Madison WI, USA) was used as an internal control vector. HEK 293 cells were seeded into 96-well plates with the confluent rate about 80 % and co-transfected with either reported vector (0.01 mg/well each), miRNA mimic/mutated vector (10 nM) and internal control vector. After 48 h co-transfection, Luciferase activities were measured by microplate reader (Infinite M1000, TECAN, China). Renin luciferase activity was defined as the standardization of firefly luciferase activity.

NK cell transfection via overexpression target miRNAs in SV-HUC-1 exosome

Because the very low efficiency of transfecting NK cell by conventional transfection methods, we attempt to use exosome as the vector to carry miRNA and transfect NK cells. The mimics of miR-186-5p (Cat no. miR10000456-1-5; RiboBio, Guangzhou, China), miR-221-5p (Cat no. miRB0004568-2-1; RiboBio, Guangzhou, China) and the NC mimic has-miR-NC (Cat no. miR1190315051351, RiboBio, Guangzhou, China) were purchased from Guangzhou RiboBio Co.,LTD. The miRNAs were loaded into exosomes via

electroporation according to the protocol by Lamichhane *et al* (29). Briefly, SV-HUC-1 derived exosomes (5 μg) were mixed each kind of with miRNA (5 μg) in 50 μL electroporation buffer (1.15 mM K_3PO_4, pH: 7.2, 25 mM KCl, 21% OptiPrep). Electroporation was carried out using Gene Pulser/Micropulser Cuvettes (Bio-Rad, Cat no.165-2089, USA) in a GenePulser Xcell electroporator (Bio-Rad, USA). Electroporation was carried out at 400 V and 125 μF with three pulses. Then the samples were transferred into tube (0.5 mL) and 1 mM EDTA was added. After incubated at RT for 15 min, the samples were centrifuged at 5000×g at 4°C for 5 min to remove buffer and unincorporated miRNA. Then, After 4 h treatment, the content of target miRNAs mimic and NC mimic in transfected NK cells were verified by q-PCR method.

The verification of target genes in NK cell

After miRNAs carrying exosomes treatment for 48 h, the expression of DAP10, CD96, Foxo1, perforin and NKG2Din NK cells were tested in protein level. The information of antibodies was showed in table 1.

Western blot method was used to test the protein expression. After washed with PBS, NK cells in each group (2×10^7 each) were lysed by RIPA lysis buffer (Cat No. P0013C, Beyotime Institute of Biotechnology) supplemented with protease inhibitor (Cat No. P1051, Beyotime Institute of Biotechnology) on ice for 10 min. The lysate of the cells was quantified by BCA assay (BCA Protein Assay Kit, Cat No. P0012S, Beyotime Institute of Biotechnology). Briefly, lysate was separated by 12 % Tris-glycine gels in equal amounts, and then transferred onto polyvinylidene fluoride (PVFD) membranes. Under gentle shaking, the blots were first blocked in 5 % nonfat milk for 1h. After washing in TBST three times, the blots were incubated with primary antibodies overnight at 4 °C. β-actin was used as internal control. After washing in TBST three times, the blots were incubated with the secondary antibodies at room temperature for 1h with gentle shaking. The blots were washed again with TBS, and then were immersed in the luminous liquid (EasySeeVR Western Blot Kit, Cat No. DW101-02, TransGen Biotech, Beijing, China) for 1 min. The signals were detected by exposure of the film in Chemiluminescence instrument (Tanon-5200Multi, China).

Data analysis

Statistical analyses were performed by using Graphpad prism8 software. The data were presented as the mean ± SD. The results were analyzed by using analysis of student' *t* test and Analysis of Variance (ANOVA). Multiple comparisons were performed using the LSD test to evaluate significant differences between the groups. Statistical significance was defined as $p<0.05$.

Results

Exosome identified

After the multistep centrifugation, the collected vesicles from T24 and SV-HUC-1 culture supernatant were morphological analyzed by TEM. It was showed that, these vesicles presented typical sphere and cup-like structure with the diameter between 30-120 nm under TEM (figure 1A), and mean diameter is 93.8 ± 37.5

nm which was detected by Zetasizer Nano ZS (figure 1B). The WB results indicated the expression of the bio-markers of CD63, CD81, and Hsp70 on these vesicles (figure 1C). This result indicated that the collected vesicle confirm the characteristics of exosomes.

NK cell identified

After 3 weeks *in vitro* expansion, by CD56 and CD3 antibodies double stained, the flow cytometry data indicated that the proportion of NK cells ($CD56^+CD3^-$) in cultured cells is 90.33 ± 1.65 % (data from 5 donor), with little NKT ($CD56^+CD3^+$) cells (7.74 ± 1.1 %) be present (figure 2).

NK cell can take up exosomes efficiently

The picture captured by inverted fluorescence microscope indicated that most of NK cells showed green signal which derived from labeled exosomes (figure 3 A, B, C), and the positive rate is about 91.6 ± 2.4% (figure 3D).

The effect of T24 exosome on NK cells

The T24 exosome (T24-EXO) was used to treat NK cell for 24 h-48 h with the SV-HUC-1 exosome (SV-EXO) treated group as control. The viability data showed that compare to the SV-EXO group, T24 EXO impaired NK cells viability as well as inhibited the cytotoxicity of NK cell on target cells (K562) after 48 h treatment. The OD_{450} value of NK cell in T24-EXO group is 0.44 ± 0.09 compared with 0.61± 0.07 in control (figure 4A). Meanwhile, the killing rates of 48 h treatment are 80.55 % ± 3.43 % (T24-EXO group) and 91.09 % ± 1.94% (control) respectively (figure 4B).

The apoptosis assay confirmed that the T24-EXO induced more NK cell apoptosis compared to the control after 48 h treatment. The early apoptosis and late apoptosis cell in T24-EXO treated group occupied 15.69 % ± 2.62 % and 4.13 % ± 1.94 % compared to 13.33 % ± 3.19 % and 4.23 % ± 2.08 % in the control group at 24 h, as well as 30.14 % ± 8.94 % and 9.83 % ± 7.35 % in T24-EXO group compared to 17.72 % ± 9.02 % and 5.06 % ± 4.13 % in the control group (48 h) (figure 4C, D). Western blot results showed that, decreased perforin and granzym-B secretion of NK cells may be the reason for their lost cytotoxicity after T24-EXO treatment. There is no significant change of IFN-γ secretion between T24 and SV-HUC-1 exosome treated (figure 4E).

To further explore the underlying reason of T24-EXO inhibiting NK cell cytotoxicity, the key functional receptors of NK cells were evaluated. The data of flow cytometry indicated that, the NKG2D, NKp30, and CD226 are significant effected by T24-EXO, the percentage of NKG2D, NKp30, and CD226 in T24-EXO group are 82.24 ± 5.61 %, 56.21 ± 10.99 %, and 81.47 ± 3.31 %, respectively compared to 95.93 ± 6.18 % (NKG2D), 88.56 ± 3.19 % (NKp30), and 90.20 ± 2.07 % (CD226) in control. Meanwhile, the MFI are 66.35 ± 6.04 (NKG2D), 21.96 ± 9.09 (NKp30) and 51.78 ± 6.65 (CD226) in T24-EXO group, compared to 84.19 ± 7.00 (NKG2D), 67.05 ± 8.65 (NKp30) and 70.25 ± 2.68 (CD226) in control. Nevertheless the receptors of

NKG2A, NKp44 and NKp46 showed no significant changes by T24-EXO treatment compared to the control (figure 5 A, B, C).

The effects of exosomal miRNAs on NK cells

Through calculating by the online programs, the miRNA and their predicted target genes in NK cells were showed in supplementary material figure 1. According to the results of calculation, miR-221-5p and miR-186-5p were chose to be further analyzed. The results of Dual-Luciferase reporter assay showed that, the gene of DAP10 (*HCST-001*) is practical target gene of miR-185-5p, and the gene of CD96 (*CD96*) and perforin (*PRF1*) are the target genes of miR-221-5p (figure 6).

As the low efficiency of transfecting NK cells directly by routine method, we employed SV-HUC-1 derived exosomes carrying miRNA mimic to transfect NK cells. After 48 h transfection, the content of miRNA in NK cell was evaluated by q-PCR method. It was showed that, the content of miR-221-5p and miR-186-5p were significant increase in NK cells, which indicated exosomes are idea carriers and can transport miRNA cargo to target cells efficiently (figure 7A).

After 48 h treatment, the NK cells were collected to test the target genes in protein level. The result showed that, the DAP10, CD96 and perforin in NK cells were significantly downregulated. The western blot data confirmed this result (figure 7B).

Discussion

The mechanism of BC carcinogenesis is not fully understood at this time. In addition to the accumulated variation in tumor cells, increasing experimental and clinical evidence suggested that tumor-induced immune dysfunction plays crucial roles in the development and occurrence of malignancies. NK cell dysfunction has been observed in many cancer patients, such as colorectal carcinoma (30), gastric cancer (31), pancreatic cancer (32), and chronic lymphocytic leukaemia (33), and NK cells play a crucial role in BC. Hermann *et al.*, found that there are decreased NK cells counts and impaired lymphokine-activated killer (LAK) cytotoxicity in PBMC from the patients with noninvasive and invasive transitional-cell bladder cancer (34). Similarly, Zhang *et al.*, reported that compared to the healthy volunteers, the circulating NK cells from bladder cancer patients presented significantly reduced cytotoxicity, which in part because there are overrepresented Tim-3^+ NK cells in bladder cancer patients (35). The study from Neelam *et al.*, showed that NK cells are the important intratumoral lymphocytes in BC, among them, $CD56^{dim}$ NK cells were associated with higher pathologic stage, and $CD56^{bright}$ NK cells are prognostically relevant to BC, which is a promising target in tumor treatment (36).

The accumulation of evidence suggests that in tumor microenvironment, tumor remodeling the function of immune cells via releasing TEXs (37). For example, tumor-derived exosomes contain soluble NKG2D ligands and TGF-β, which can inhibit the surface NKG2D expression and reduce NKG2D-dependent cytotoxicity on NK cells and $CD8^+$ T cells (38). In the present investigation, it was demonstrated that BC-derived exosomes can also affect the activity of NK cells, as evidenced by the decreased vitality and

impaired cytotoxicity of NK cell following T24 exosomes treatment. The diminished cytotoxicity of NK cells may be attributable to the down-regulated activated receptors NKG2D, NKp30, and CD226, as well as the decreased production of perforin and granzyme B.

miRNAs regulate the expression of protein-coding genes in a sequence-specific way by inhibiting their translation or cleaving RNA transcripts. Due of their amazing biological impacts, exosomal microRNAs have gained increasing interest. More than 200 miRNAs and miRNA families/clusters have been identified abnormally expressed in BC to date (37). Using high-throughput sequencing, we discovered the important exosomal microRNAs with significant abnormal expression in BC cells. Calculations revealed that miR-221-5p and miR-186-5p, are highly linked with the dysfunction of NK cells. The moderating impact of miR-186-5p on DAP10, and miR-221-5p on perforin and CD96 were validated using Dual-Luciferase reporter and western-blot assay.

miR-221-5p is the miRNA that is frequently dysregulated in cancer patients. The research of Liu *et al*. revealed that miR-221-5p is considerably elevated in clear cell Renal cell carcinoma (ccRCC) tissues and ccRCC cell lines, and acted as a crucial oncogene (40). Similarly, it was discovered that miR-221-5p increases cell proliferation and metastasis in human prostate cancer (41). However, other publications have demonstrated that miR-221-5p is downregulated in prostate cancer compared to normal prostate tissue (42), as well as in GC tissues and cell lines (43), suggesting that this microRNA may play negative roles in these tumors. miR-221-5p is significantly upregulated in T24 exosomes compared to SV-HUC-1 exosomes, as demonstrated by our previous results. It suggested that each miRNA has a distinct pattern of expression in different malignancies and various samples, such as tissue, body fluid, and extracellular vesicles. Other investigations demonstrated the possible effect of miR-221-5p in immunologic processes, such as regulation of inflammatory responses in acute gouty arthritis by targeting IL-1 (44) and anti-inflammatory function in human colonic epithelial cells by targeting interleukin 6 receptor (IL-6R) (45). Our findings demonstrated that miR-221-5p can target CD96, and perforin in NK cells, indicating that is a promising target worthy of consideration to be intervened in tumor immunotherapy.

miR-186-5p is also a well-studied miRNA that exhibits complex effects in tumor, such as play a positive role in lung adenocarcinoma by targeting PTEN (46) and inhibit tumor growth in osteosarcoma (47), non-small-cell lung cancer cells (NSCLCs) (48), colorectal cancer cell (49), and neuroblastoma (50). In NK cells, miR-186-5p can target DAP10, an essential adapter protein of NKG2D, and inhibiting NKG2D-DAP10 mediated activating signal in activating NK cell cytotoxicity. However, the cause of decreased expression of NKG2D, NKp30, CD226 and granzyme-B, as well as increased apoptosis in NK cells following T24-EXO treatment remains to be further studied.

Exosomes were deemed as an excellent nucleic acid carrier due to their inherent qualities, and have been utilized by numerous researchers to deliver therapeutic nucleic acid medications to target cells (51). For example, Kamerkar *et al*. confirmed that exosomes have a greater capacity to transfer siRNA and shRNA to inhibit tumor growth *in vivo* (52). In the present investigation, we attempted to use SV-HUC-1 exosomes as the vehicle for miRNA- mimetic delivery to NK cells. It was showed that miRNA mimics can be

conveyed to NK cells and effectively inhibit target gene expression, demonstrating the potential of exosomes in nucleic acid medication delivery.

Conclusion

The present study demonstrated that, T24 derived exosome can induce NK cell dysfunction by impairing viability, and inhibit the cytotoxicity of NK cell. The important functional receptors NKG2D, NKp30, and CD226 on NK cells and the secretion of perforin and granzyme-B were downregulated after T24-EXO treatment. miR-221-5p and miR-186-5p are the critical exosomal miRNAs in T24-EXO and can effect NK cells function by interfering the stability of mRNAs of DAP10, CD96, and perforin gene. Exosomes can be used as the nucleic acid carrier to delivery miRNA into NK cells efficiently.

Declarations

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Tables

Table 1
The information of all antibodies

Antibody	Brand	Catalog Number.
PE Mouse Anti-human NKG2A	R&D	FAB1059P
PE Mouse Anti-human NKG2D	BD Pharmingen	554680
PE Mouse Anti- human NKp30	BD Pharmingen	558407
PE Mouse Anti-human NKp44	BD Pharmingen	558563
PE Mouse Anti-human NKp46	BD Pharmingen	557991
PE Anti-human CD226 [DX11]	abcam	ab33337
Hsp70	abcam	ab2787
CD63	abcam	ab134045
CD81	abcam	ab79559
IFN-γ	abcam	ab267369
Granzyme-B	abcam	ab255598
Perforin	abcam	ab256453
DAP10	Santacruze	sc-374196
FOXO1	abcam	ab179450
CD96	abcam	ab264416
NKG2D	abcam	ab96606
β-actin	abcam	ab8226

Table 2
Primers sequences used for quantitative real time PCR of miRNAs

miRNA ID	Accession number	Forward Primer Sequence(5’ – 3’)	Tm	%GC
hsa-miR-221-5p	MIMAT0004568	CGCGACCTGGCATACAATGT	61.6	54.55%
hsa-miR-186-5p	MIMAT0000456	ATGCGCGCCAAAGAATTCTCC	62.78	52.38%

Figures

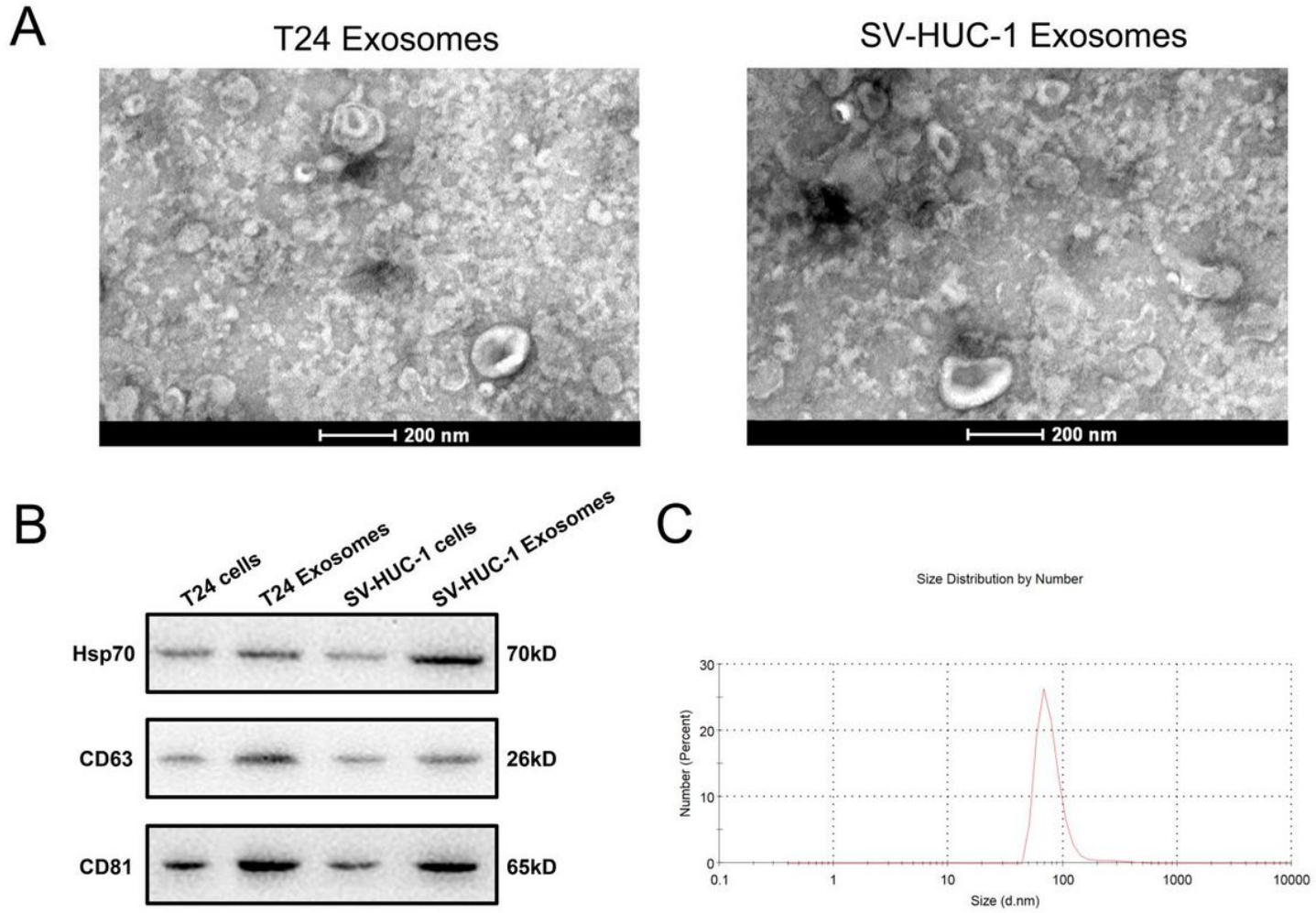

Figure 1

Identification of exosomes

A: Exosome morphology under TEM. Exosomes were re-suspended and fixed using 2% w/v paraformaldehyde aqueous and added onto cleaned mica chips, critical point drying and imaged by TEM. B: WB analysis of the exosome markers (Hsp70, CD63, CD81). C, Particle size distribution of exosome; exosomes were diluted by 1 × PBS, and measured by a Zetasizer Nano (n=3)

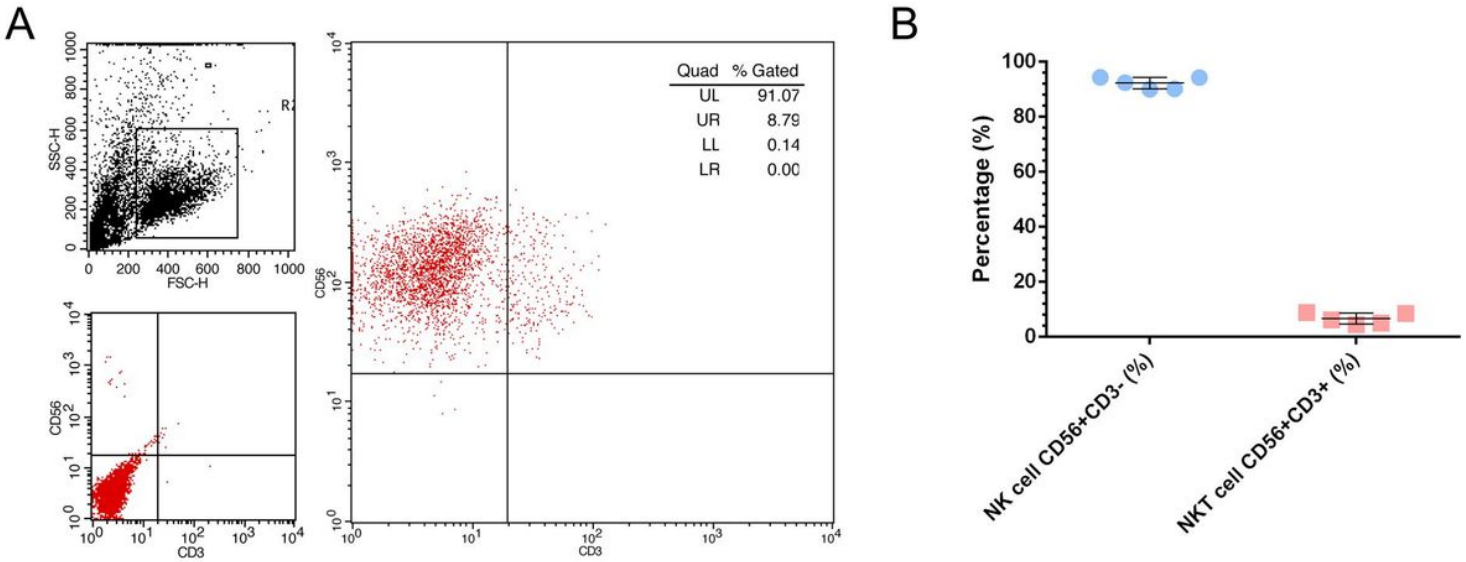

Figure 2

The proportion of NK cells

A: The representative flow cytometry data of NK cells ($CD56^{+}CD3^{-}$) proportion. B: The statistical results of NK cells ($CD56^{+}CD3^{-}$) and NKT ($CD56^{+}CD3^{+}$) cells proportion (n=5)

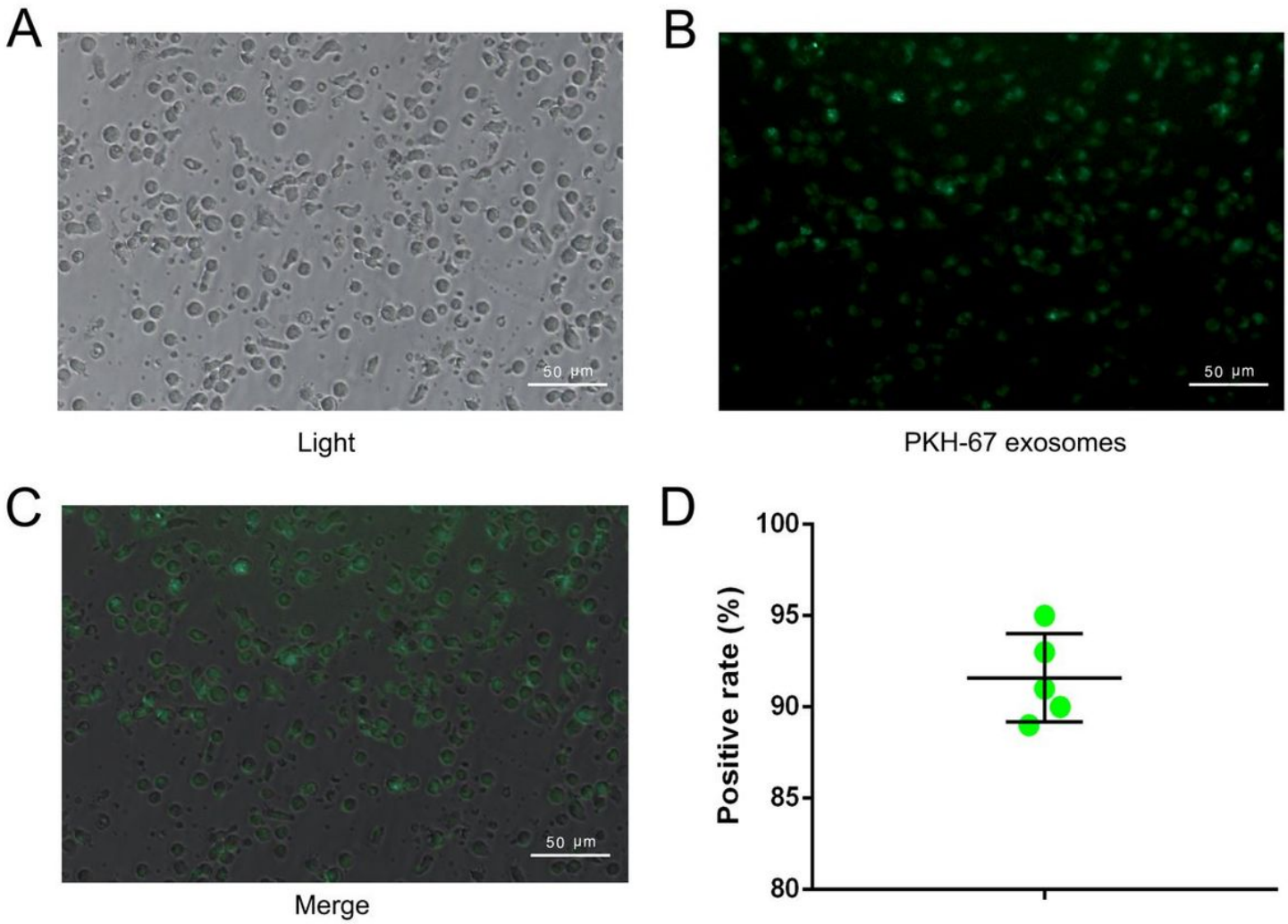

Figure 3

The exosomes uptake efficiency of NK cells

A: The NK cells in light field. B: The NK cells in fluorescence field, through uptake the PKH-67 stained T24 exosome, NK cell showed green fluorescent. C: The positive rate of NK cells which showed green fluorescent tested by flow cytometry (n=5).

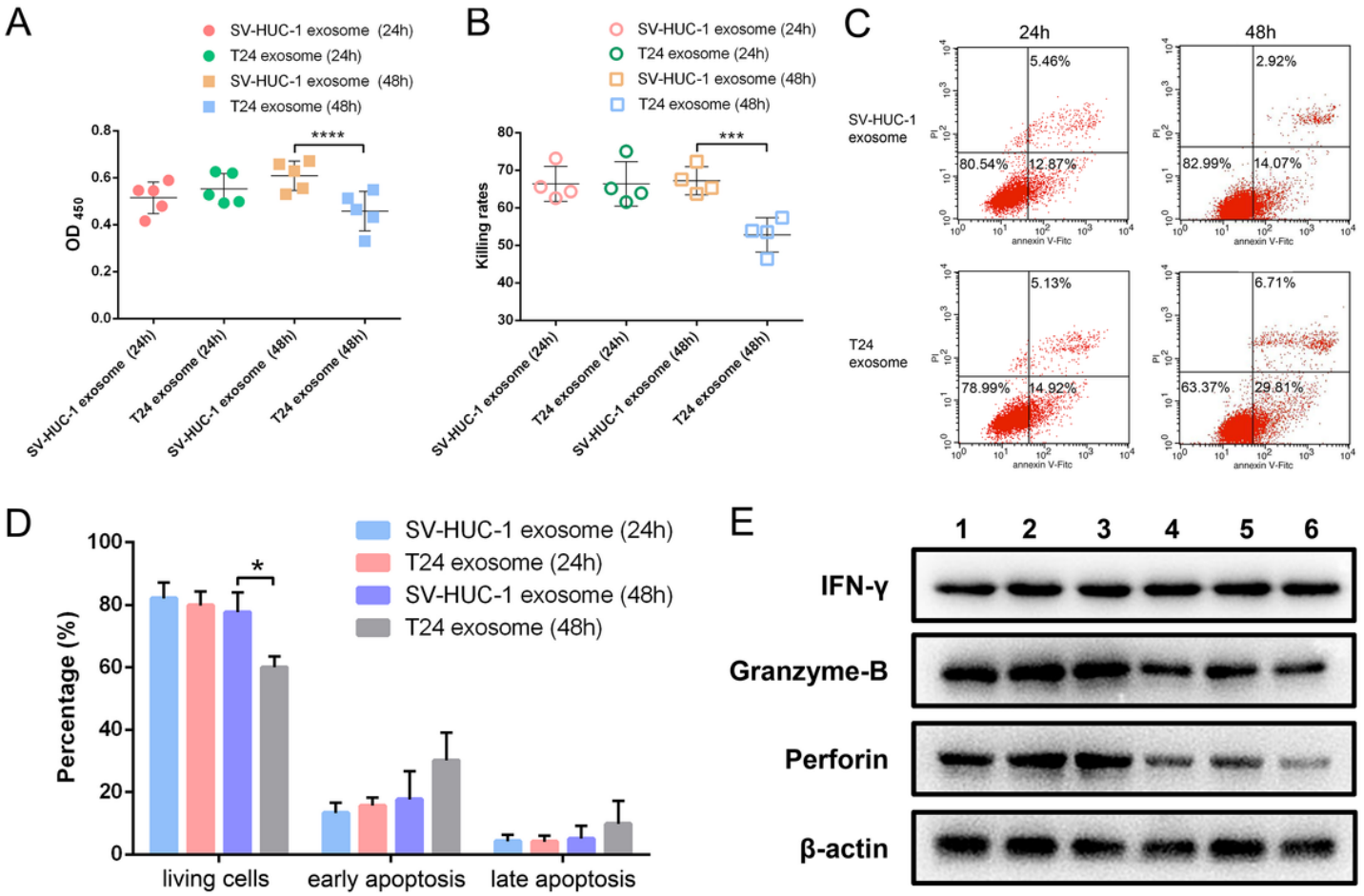

Figure 4

The effects of T24 exosome on NK cells viability, apoptosis, cytotoxicity and cytokines expression

A: The viability of NK cells after 24h and 48h T24 exosome treatment (n=5). B: The killing rate of NK cells after 24h and 48h T24 exosome treatment (n=4). C: The representative flow cytometry data of NK cell apoptosis. D: The bar graph showed the percentage of viable (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), and late apoptotic/necrotic (Annexin V^+/PI^+) cells in each group (n=5). T24 exosome treatment significant decrease the living NK cells. * *t* test, $p < 0.05$. E. Western blot analysis of cytokines (IFN-γ, perforin, granzyme-B) secretion of NK cells. Line 1-3, treated by T24 exosomes, line 4-6, treated by SV-HUC-1 exosomes. The expression of granzyme-B and perforin were decreased after T24 exosome treatment.

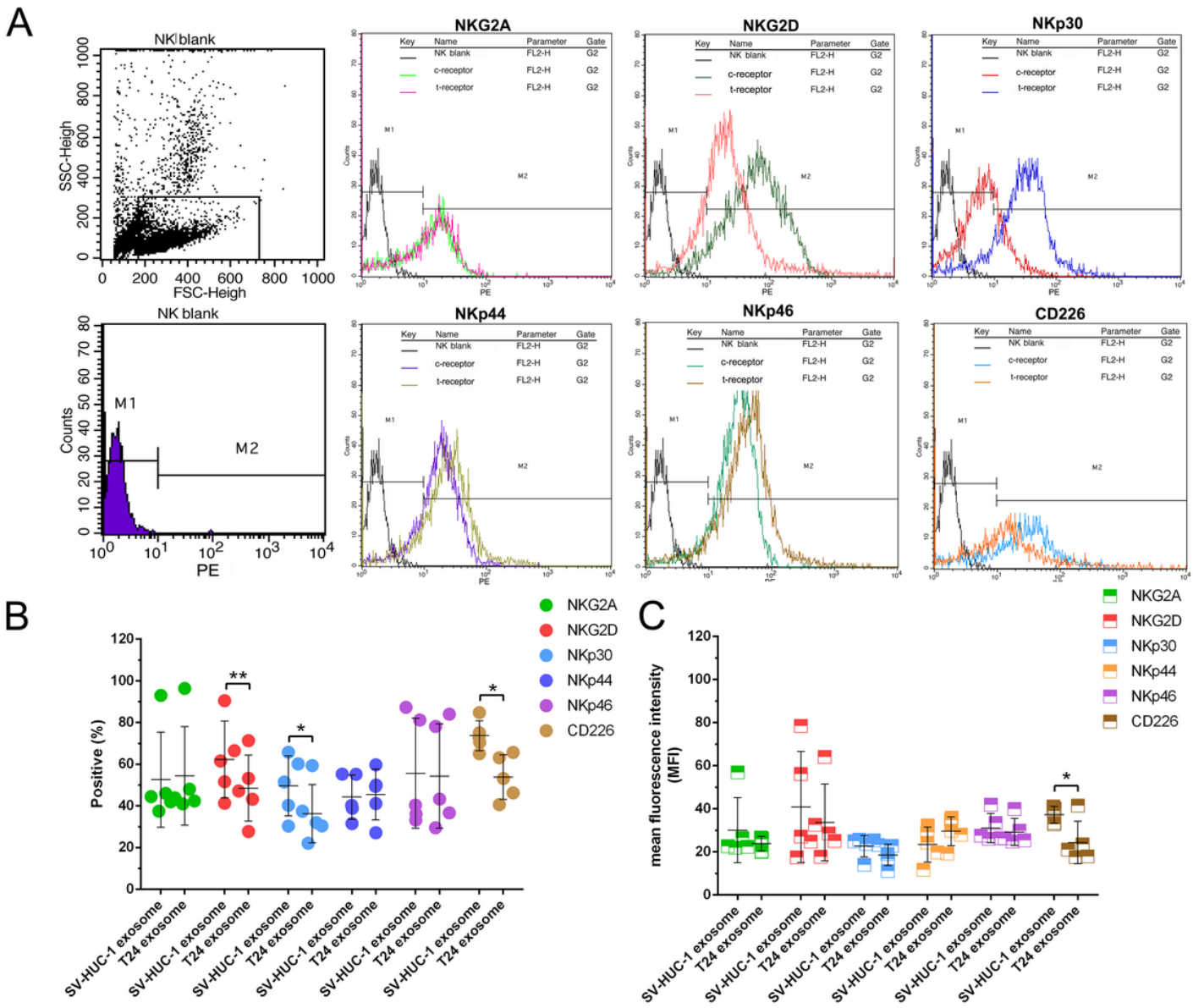

Figure 5

The effects of T24 exosome on the expression of NK cells functional receptors

A: The representative flow cytometry data of the expression of NK cells functional receptors (NKG2A, NKG2D, NKp30, NKp44, NKp46 and CD226). B: The bar graph showed the positive rate of NK cells functional receptors (n=5); C: The bar graph showed the mean fluorescence intensity (MFI) of NK cells functional receptors (n=5); The positive rate of NKG2D, NKp30 and CD226 were reduced after T24 exosome treatment; The MFI of CD226 was reduced after T24 exosome treatment as well. * *t* test, p <0.05; ** *t* test, p<0.01

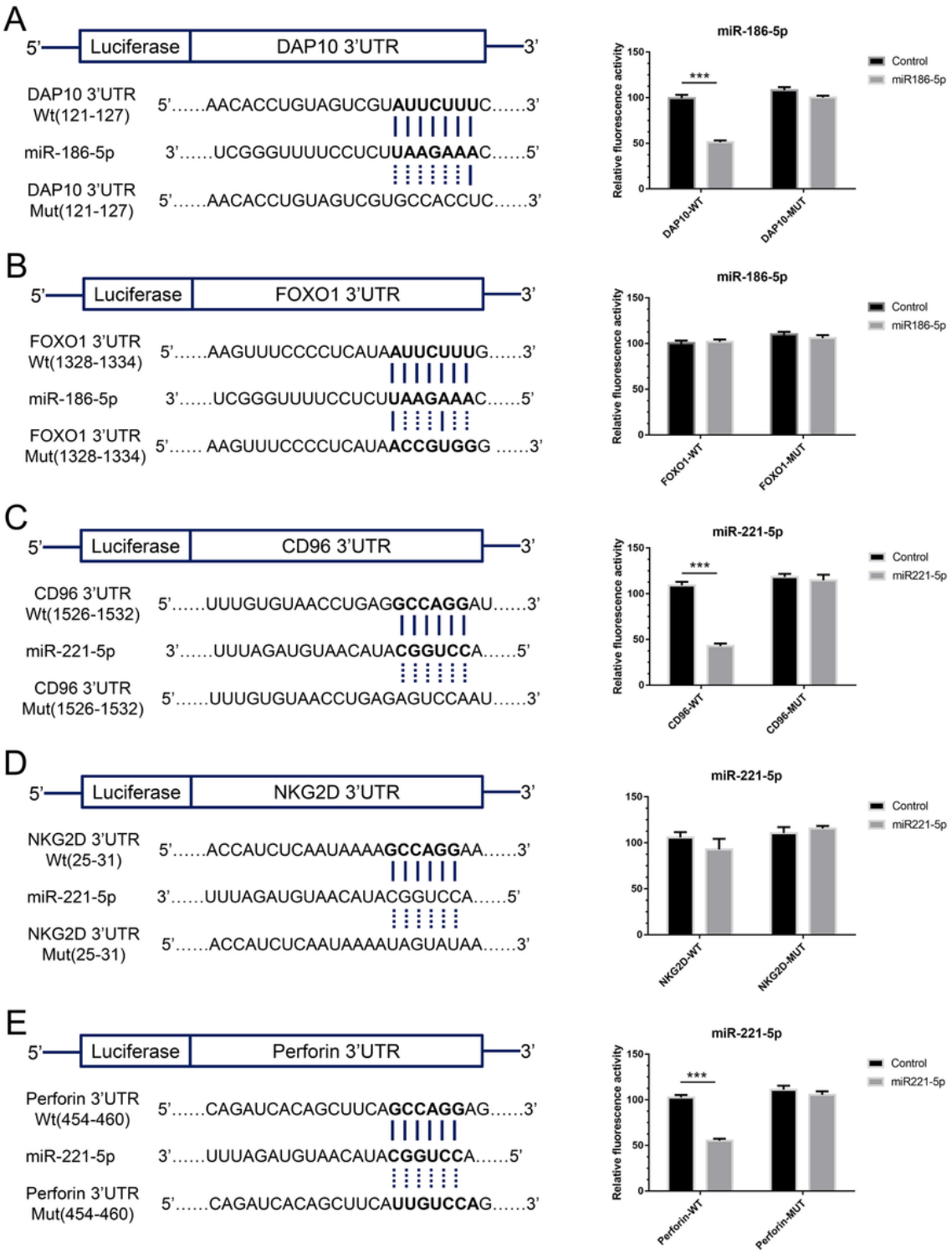

Figure 6

The pairing relationship of miRNAs and target genes

A: The binding site of miRNAs matched to the target genes. B: Dual-Luciferase reporter assay of miRNAs and target genes; The miR-186-5p targeted to DAP10; miR-221-5p targeted to perforin and CD96 were confirmed by reduced relative fluorescence. *** *t* test, $p < 0.001$

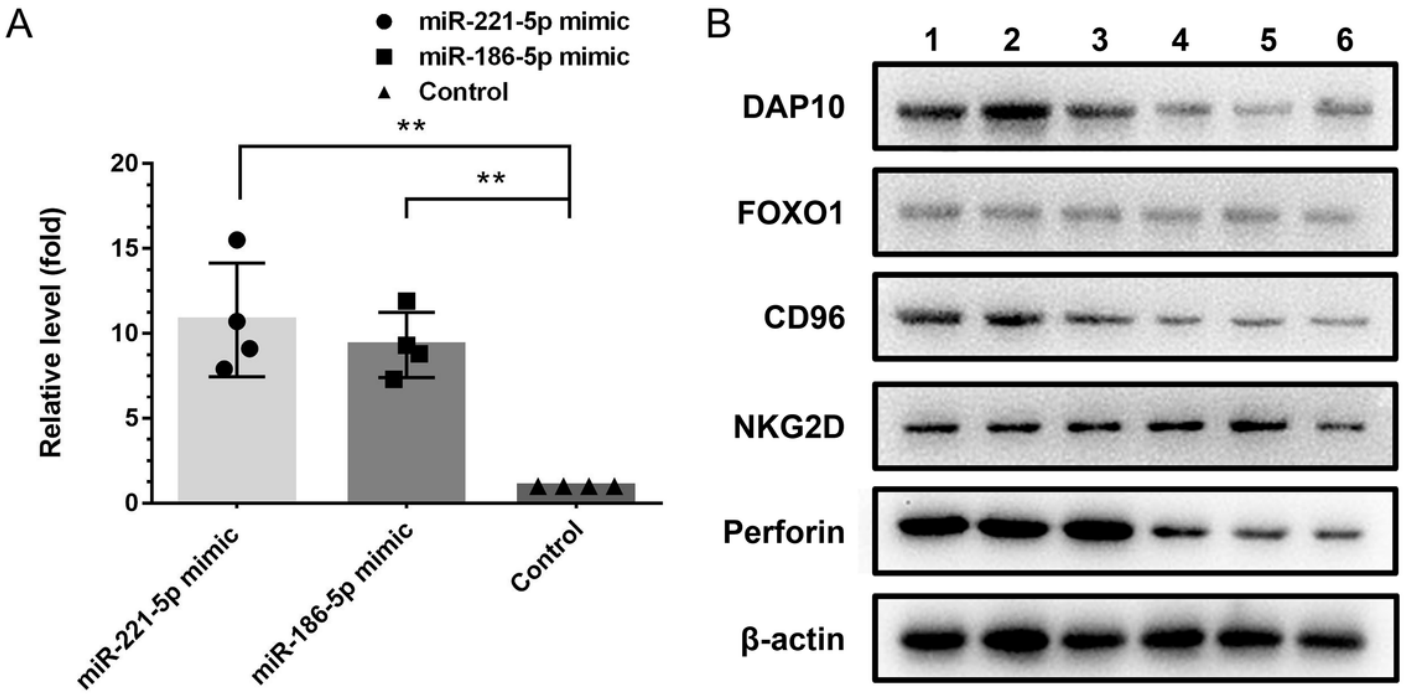

Figure 7

The transfection and interfere efficiency of miRNA mimics in NK cells

A: qPCR method to verify the transfection efficiency. It indicated the miR-186-5p and miR-221-5p have been effective transfected into NK cells B: Western blot analysis of the expression on the target genes (DAP10, Foxo1, CD96, NKG2D, perforin) in NK cells after mimic transfection; Line 1-3, treated by T24 exosomes, line 4-6, treated by SV-HUC-1 exosomes. The expression of DAP10, cd96 and perforin were decreased after mimic transfection. ** *t* test, $p < 0.01$

Supplementary Files

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- sfigure1.jpg