B7-H6 promotes the killing activity of NK cells against cervical cancer through the downstream ERK pathway of NKp30

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Research Article

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Abstract

OBJECTIVE

As a ligand of NKp30, B7 homolog 6 (B7-H6) is involved in the immune regulation of various tumors. The aim of this study was to clarify the effect of B7-H6 expressed in HeLa cells on NK cell killing function.

METHODS

The expression of B7H6 was changed in HeLa cells using short hairpin RNA. Furthermore the effect of B7-H6 on the killing function of NK cell was analyzed after cell co-culture. Flow cytometry was used to detect NKp30 expression, degranulation function, perforin (PFP) and Granzyme B (GZMB) secretion function of NK cells. Enzyme-linked immunosorbent assay (ELISA) was used to detect interferon-γ (INF-γ) production function. The cytotoxicity of NK-92 cells was determined using the CytoTox 96 Non-Radio active Cytotoxicity Assay. Western blotting (WB) detection was used to detect the ERK phosphorylation level in NK cells.

RESULTS

When NK-92 cells co-cultivated with HeLa cells with different expression levels of B7-H6, the expression of NKp30, NK-92 cell killing rate, PFP and INF-γ production, and degranulation function were correspondingly changed in NK cells, but there is no effect on GZMB production. After cell co-culture, ERK phosphorylation level in NK cells was increased gradually with the up-regulation of B7-H6 expression.

CONCLUSIONS

B7-H6 can enhance the killing function of NK cells to HeLa cells by activating the NKp30 downstream ERK signaling pathway.

Introduction

Imbalanced immune regulation plays an important role in the occurrence and development of tumors. In order to develop, tumors must successfully evade innate immunity. In studies related to immune regulation of cervical cancer [1], although there are a large number of studies involving T cells, dendritic cells, macrophages, neutrophils and a variety of inflammatory factors, and Pembrolizumab, which has limited clinical effect, has been approved by FDA as a PD-1/PDL-1 immune target blocker for second-line treatment of cervical cancer, but the involvement of innate immunity in the immune regulation mechanism and immune target drugs of cervical cancer is rarely reported.
NK cells are the main effector cells of the innate immune system. Decrease of NK cell killing activity in cervical cancer is one of the causes of immune escape[2]. Natural cytotoxic receptor (NCR) are the main activated receptors of NK cells, and NKp30 is a primary immunoglobulin in the NCR receptor family[3]. B7-H6, one of NKp30 ligand, is expressed on the surface of some tumor cells but not on normal cells[4–7]. Textor S et al. [8] found that in a variety of tumor cells such as SK-Mel-37 cells, A375 cells, Capan-1 cells and Raji cells, knockdown of B7-H6 expression can reduce the expression of NKp30 and affect the degranulation function of NK cells. Our preliminary study found that B7-H6 is differentially expressed in cervical lesions, promotes the progression of cervical cancer[9] and is related to clinical parameters of cervical cancer[10]. In this paper, we explored the effect of B7-H6 expressed on the surface of HeLa cells on NK cell killing function through NKp30.

**Materials and Methods**

**Cell lines**

Human cervical cancer cell line HeLa was obtained from the General Hospital of Tianjin Medical University (Tianjin, China). The HeLa cells were cultured into RPMI-1640 medium (cat. no. 31870074; Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (cat. no. F8318; SigmaAldrich; Merck KGaA), and maintained at 37˚C in a humidified atmosphere containing 5% CO2.

The NK-92 cell line was purchased from the BNCC Biotechnology Co (Beijing, China). The cells were cultured in vitro in special culture medium for NK-92 cells (cat. no. 5293/50; R&D Systems, Co.), consisting of MEMα, 0.2mM Inositol, 0.1mM β-mercaptoethanol, 0.02mM Folic Acid, 12.5% HS, 12.5% FBS, 1% P/S, supplemented with recombinant human interleukin-2 (IL-2) (cat. no. 5293/50; R&D Systems, Co.) in a ratio of 1:500, 1X L-glutamine (Invitrogen) and antibiotics (penicillin/streptomycin; Invitrogen).

The NK-92 cells were maintained at 37˚C in a humidified atmosphere containing 5% CO2.

**Cell co-culture**

The HeLa cells in each group were inoculated into 6-well plates at a density of $2 \times 10^5$ cells/well, and 1ml RPMI-1640 complete medium was added. Each 6-well plate was labeled, and the cell density and growing status were observed under a microscope. The cells were placed in an environment containing 5% CO2 at 37˚C for 6 hours. After HeLa cells were adhered to the wall, the cell culture medium was removed, and the NK-92 cells with the best growth condition were selected to inoculate into 6-well plates at a density of $1 \times 10^6$ cells/well, according to the ratio of NK-92 cells (effector cells) and HeLa cells (target cells), E:T, was 5:1. 2ml of special culture medium for NK-92 cells containing IL-2 was added into each well and cultured at 37˚C with 5%CO2 for 4h.

**Short hairpin RNA (shRNA) transfection**

The shRNA sequence of 28 µg/ml interfering with B7-H6 (shB7-H6) and a control shRNA sequence (shNC) were synthesized by Suzhou GenePharma Co., Ltd.. Meanwhile, we constructed the same
concentration overexpressed B7-H6 gene sequence (hB7-H6) and control sequence (hNC) by Suzhou GenePharma Co., Ltd., according to the full length of CDS region of human B7-H6 gene. Sequence details are shown in Table 1.

The pGLV3/H1/GFP lentiviral vectors (LV) LV3shRNA or LV3shRNANC (MOI, 10), and the EF-1α/GFP&Puro lentiviral vectors (LV) LV5-hB7-H6 or LV5-hNC (MOI, 10) were transfected into HeLa cells at 37°C, and screened with polybrene (1.5 µg/ml, 1:1,000; Suzhou GenePharma Co., Ltd.). Viral INFections were performed serially. The fluorescence microscope was used to observe whether fluorescence transfection efficiency was > 80% after 48h of transfection. Stable cell lines expressing gene sequences were selected with 2 µg/ml puromycin 72h after transfection.

<table>
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<th>Experiment Name</th>
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<th>Cat. No.</th>
<th>Gene Sequence(5′-3′)</th>
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<td>shRNA-1</td>
<td>190331CZ</td>
<td>CCCTGCTCTCCTAACAAGTT</td>
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<tr>
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<tr>
<td>hNC</td>
<td>no-load</td>
<td>D03007</td>
<td>–</td>
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</table>

Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was extracted from cultured cells using Trizol reagent (cat. no. 213407; Invitrogen; Thermo Fisher Scientific, Inc.). Purified RNA was then reversely transcribed to cDNA using RevertAid First Strand cDNA Synthesis Kit purchased from Thermo Fisher Scientific. Reverse transcription was performed. Next, RT-qPCR was performed with FastStart Universal SYBR Green Master Mix (cat. no. 04913850001; Roche Diagnostics) following the manufacturer’s instructions. An initial amplification using B7-H6 specific primers (Forward 5′TTTTCCATTCCATTGGTGGCCTA-3′ and reverse 5′TTTTCCATTCCATTGGTGGCCTA-3′) was done with denaturation, primer annealing, and primer extension. Data were normalized to the geometric mean of the housekeeping gene GAPDH (Forward 5′CTGGAACGTTAAGGACTGACA-3′ and reverse 5′AAGGGACTTCTGTAACA ATGCA-3′) to control the variability in expression levels.

Western blotting

The HeLa cells were lysed with RIPA lysis buffer (cat. no. BL504A; Biosharp; http://www.biosharp.cn/index/product/details/language/en/product_id/1783.html), and the protein concentration was determined using the QuickStart Bradford protein assay (BioRad Laboratories, Inc.). The NK-92 cell lysate consisted of 1ml PRIA lysis buffer, 10µl PMSF (cat. no. ST505; Beyotime Biotechnology, Inc.) and 10µl phosphatase (cat. no. P1048; Beyotime Biotechnology, Inc.) inhibitor. Equal amounts of protein (30 µg loaded per lane) were separated via 10% SDS-PAGE and transferred onto PVDF membranes (cat. no. P2938-1ROL; SigmaAldrich; Merck KGaA). The membranes were blocked at room...
temperature for 1 h with TBS-0.05% Tween-20 containing 5% skimmed milk powder (HeLa cells) or 5% BSA (NK-92 cells). The membranes of HeLa cell were subsequently incubated with an antiB7H6 rabbit antibody (1:1,000; cat. no. ab121794; Abcam) or an antiGAPDH antibody (1:5,000; cat. no. G8795; SigmaAldrich; Merck KGaA) at 4˚C overnight. The membranes of NK-92 cell were subsequently incubated with an antip44/42 ERK1/2 rabbit antibody (1:5,000; cat. no. 4695T; Cell Signaling Technology), antiphospho-p44/42 p-ERK1/2 rabbit antibody (Thr202/Tyr204) (1:5,000; cat. no. 4370T; Cell Signaling Technology) or an antiGAPDH antibody (1:5,000; cat. no. G8795; SigmaAldrich; Merck KGaA) at 4˚C overnight. Following primary antibody incubation, the membranes were incubated with a HRP-conjugated goat antirabbit (1:5,000; cat. no. abs20040; Absin Bioscience, Inc.) or goat antimouse secondary antibody (1:4,000; cat. no. abs20039; Absin Bioscience, Inc.) at 37˚C for 1 h. Protein bands were visualized using ECL reagent (cat. no. abs920; Absin Bioscience, Inc.). The ImageJ software (version no. v1.8.0; National Institutes of Health, Bethesda, MD) was used to quantify the WB data.

Flow cytometry

After co-culture, the NK-92 cells suspended in the culture medium were sucked out, and the NK-92 cells that were not co-cultured were placed into labeled centrifugal tubes respectively by groups, centrifuged at 1000Rpm for 3min, and the supernatant was discarded. The cells were cleaned twice in PBS. One part of each group of NK-92 cell samples was used for NK-92 cell membrane staining, and the other part was used for the staining of the encapsulated protein.

Fluorescence labeling of cell membrane antigen: The antibodies CD3-APC (cat. no. 47-0037042; eBioscience), CD56-FITC (cat. no. 304603; Biolegend), NKp30-PE /CY7 (cat. no. 25-4714-80; eBioscience) and CD107A-PE (cat. no. 328607; Biolegend) were added, and fully mixed, and incubated for 30min at room temperature under dark conditions. The cultured cells were PBS cleared and fixed with 4% paraformaldehyde fixative for machine detection and analysis. A single standard control was set for each sample.

Fluorescence labeling of intracellular antigen: First, DAPI was added and fully mixed, then cells were incubated for 20min at room temperature under dark conditions. Then, the supernatant was washed twice with PBS and centrifuged at 1500Rpm for 5min to remove it. Add 200μl 4% paraformaldehyde fixative, mix thoroughly, keep away from light for 30min at room temperature, wash with PBS twice, centrifuge at 1500Rpm for 5min, remove supernatant. Add 200μl breaking solution, centrifuge at 1500Rpm for 5min, repeat, then re-suspend the cells with 100μl breaking solution. Antibodies GZMB-PE (cat. no. 372208; Biolegend) and PFP-eFluor 450 (cat. no. 48-9994-42; eBioscience) were added into the suspended cells, and fully mixed, the NK-92 cells were incubated at room temperature under dark conditions for 30min. The incubated cells, PBS clear, suspended in 300μl PBS for machine detection and analysis.

Flow cytometry: NovoCyte D3000 flow cytometer was used to detect stained cells. Data analysis was performed by NovoExpress software (version 1.5, ACEA Biosciences, Inc.). NK cells were gated by CD3+ and CD56+.
Cytotoxicity assays

NK-92 cells were co-cultured with HeLa cells as described above according to the different effect target ratio: 5:1, 10:1 and 20:1. The cytotoxicity of NK-92 cells was determined using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (cat. no. G1780; Promega) according to the instructions.

Enzyme-linked immunosorbent assay (ELISA)

The INF-γ release ability of NK-92 cells was determined by ELISA. Dilute each reagent to working concentration according to kit (cat. no. PDIF50C; R&D Systems) instructions and configure INF-γ standard. NK-92 cells were centrifuged at 3000Rpm for 3min, and no cell supernatant was collected. Add 100µl trapping antibody to each well of the 96-well plate, seal the plate and incubate overnight at room temperature. Add 300µl of reagent dilution to each well, and incubate for 1h at room temperature. The sample well to be tested, standard well and blank well are set, and each well is set with 3 compound wells. Add 100µl sample to test sample well; Add 100µl standard to standard well; Add 100µl reagent dilution to blank well. The well plates were incubated at room temperature for 2h. Add 100µl INF-γ antibody to each well, incubate for 2h at room temperature. Add 100µl Streptavidin-HRP to each well, and incubate at room temperature away from light for 20 minutes. Add 100µl TMB substrate solution to each well, and incubate at room temperature, away from light for 20min. Add 150µl stop solution to each well to stop the reaction. Mix well until the color of the solution changes from blue to yellow. Measure the OD value of each well at 450nm with a microplate reader. Calibration was performed by subtracting the OD value of each standard and sample from the blank well. The standard concentration is the abscissa and the OD value is the ordinate. The ELISACalc software (ELISACalc Software, Inc.) is used to calculate the corresponding concentration of the sample according to the OD value of the sample.

Statistical analysis

The experimental data were processed by SPSS 22.0 statistical software (SPSS, Inc.). GraphPad Prism 8 (GraphPad Software, Inc.) is used to draw graphs and verify results. The data were shown as mean ± standard deviation (mean ± SD). One-way ANOVA analysis and Bonferroni test were used for comparison between multiple groups. Comparison between the two groups was performed by independent sample T test. Values of \( P < 0.05 \) were considered statistically significant. Significance was denoted as *, \( P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 \). All experiments were repeated three times, and the mean value of experimental results was taken.

Results

Knockdown or up-regulation of B7-H6 expression in HeLa cells

Fluorescence microscopy analysis of GFP expression showed that the infection efficiency of shB7-H6, shNC, hB7-H6 and hNC to HeLa cells were both greater than 80% (Fig. 1a). RTqPCR was used to analyze
the mRNA level of B7-H6 in HeLa cells. Compared with shNC results of 1.31 ± 0.04, the B7-H6 mRNA level of shB7-H6 was 0.24 ± 0.02 which decreased by 81%, ****P<0.0001 (Fig. 1b). The mRNA level of B7-H6 in hB7-H6 was 1.35 ± 0.21 and that in hNC was 0.84 ± 0.15. Compared with the hNC, the mRNA level of B7-H6 in hB7-H6 was increased by 61%, *P<0.05 (Fig. 1d). WB was used to analyze the expression of B7-H6 protein in HeLa cells. Compared with shNC result of 81934 ± 1848, the protein expression level of B7-H6 in shB7-H6 was 30541 ± 4374 which decreased by 63%, ****P<0.0001 (Fig. 1c). Compared with the hB7-H6 (98797 ± 6382), the protein expression level of B7-H6 in hB7-H6 was 55012 ± 3130 which increased by 80%, **P<0.01 (Fig. 1e).

**Effect of B7-H6 knockdown on NKp30 expression and NK cell killing function**

The positive rate of NK-92 cells expressing NKp30 was analyzed by flow cytometry (Fig. 2a). The results of NK-92 Only, NK-92 + shB7-H6 and NK-92 + shNC group were 0.41 ± 0.24%, 0.61 ± 0.15% and 1.64 ± 0.57%. After NK-92 + shB7-H6 co-culture, compared with NK-92 Only, the proportion of NK cells expressing NKp30 was slightly up-regulated, and the difference was not statistically significant. After co-culture of NK-92 + shNC, the proportion of NK-92 cells expressing NKp30 was significantly up-regulated, which was significantly higher than that of NK-92 Only and NK-92 + shB7-H6, both *P<0.05 (Fig. 2b).

We detected and analyzed the expression of CD107a on the surface of NK-92 cells to reflect the decapitation ability of NK-92 cells. By flow cytometry (Fig. 2a), the positive cell rate of CD107a in every group was detected respectively. The results in NK-92 Only, NK-92 + shB7-H6 and NK-92 + shNC group were 32.36 ± 2.11%, 40.07 ± 3.60% and 64.72 ± 3.96% respectively. Compared with NK-92 Only, NK-92 + shB7-H6 showed a slight up-regulation of CD107a expression, with no statistically significant difference. The rate of CD107a + cells in NK-92 + shNC was significantly up-regulated, significantly higher than that in NK-92 Only (****P<0.0001) and NK-92 + shB7-H6 (**P<0.001) (Fig. 2c).

The effect of B7-H6 on GZMB secretion of NK-92 cells was analyzed by intracellular staining. The flow cytometry detected the positive cell rate of GZMB in each group (Fig. 2a). The results in NK-92 Only, NK-92 + shB7-H6 and NK-92 + shNC group were 95.47 ± 3.84%, 92.77 ± 3.03% and 93.57 ± 3.30% respectively. There was no statistical difference on GZMB secretion capacity of NK-92 cells (Fig. 2d).

The effect of B7-H6 on PFP secretion of NK-92 cells was analyzed by intracellular staining. The flow cytometry detected the positive cell rate of PFP in each group (Fig. 2a). The results in NK-92 Only, NK-92 + shB7-H6 and NK-92 + shNC were 8.96 ± 0.61%, 2.76 ± 1.37% and 4.89 ± 3.16% respectively. Compared with NK-92 Only, the rate of NK-92 cells secreting PFP in NK-92 + shB7-H6 and NK-92 + shNC decreased significantly, both ****P<0.0001. However, when NK-92 + shB7-H6 was compared with NK-92 + shNC, the rate of NK-92 cells secreting PFP showed a trend corresponding to B7-H6. NK-92 + shNC compared with the NK-92 + shB7-H6, the positive rate were increased, **P<0.01 (Fig. 2e).

The effect of B7-H6 on INF-γ secretion of NK-92 cells was analyzed by ELISA in each group (Fig. 2f). The results of NK-92 Only, NK-92 + shB7-H6 and NK-92 + shNC were 4186.67 ± 189.03 pg/ml, 4402.67 ±
149.14 pg/ml and 5883.83 ± 433.56 pg/ml respectively. The NK-92 Only compared with the NK-92 + shB7-H6, there was no statistical difference. Compared with NK-92 + shNC, the concentration of INF-γ secreted in NK-92 Only and NK-92 + shB7-H6 were significantly decreased, both **P < 0.01.

NK-92 cells were co-cultured with each group of HeLa cells according to the different E:T at 5:1, 10:1 and 20:1 respectively, and then the killing rate of NK-92 cells was detected (Fig. 2g). After co-culture according to 5:1, the killing rate were 11.37 ± 1.07% (NK-92 + shB7-H6) and 18.00 ± 2.11% (NK-92 + shNC). After co-culture according to 10:1, the killing rate were 19.00 ± 2.00% (NK-92 + shB7-H6) and 32.00 ± 4.58% (NK-92 + shNC). After co-culture according to 20:1, the killing rate were 38.67 ± 1.53% (NK-92 + shB7-H6) and 32.71 ± 3.22% (NK-92 + shNC). Compared with the NK-92 + shNC, NK-92 + shB7-H6 reduced the killing rate of NK-92 cells at both 5:1 (**P < 0.01) and 10:1 (***P < 0.001). When the E:T was 20:1, compared with NK-92 + shNC, there was no statistical difference in the NK-92 + shB7-H6 group.

**Effect of B7-H6 up-regulation on NKp30 expression and NK cell killing function**

The positive cell rate of NK-92 cells expressing NKp30 was analyzed by flow cytometry (Fig. 3a). The results of NK-92 Only, NK-92 + hB7-H6 and NK-92 + hNC were 0.41 ± 0.24%, 3.81 ± 0.28% and 2.21 ± 0.25% respectively. Compared with NK-92 + hNC, NK-92 cells expressing NKp30 in NK-92 + hB7-H6 were significantly up-regulated, ***P < 0.001. In NK-92 Only, the percentage of NKp30 positive cells was significantly lower than that in NK-92 + hNC, ***P < 0.001 (Fig. 3b).

By flow cytometry, the positive cell rate of CD107a in every group were detected respectively. The results of NK-92 Only, NK-92 + hB7-H6 and NK-92 + hNC were 32.36 ± 2.11%, 73.31 ± 3.83% and 63.18 ± 3.55% respectively. Compared with NK-92 + hNC, the percentage of CD107a expressing cells in NK-92 + hB7-H6 was up-regulated, *P < 0.05. In NK-92 Only, the result was significantly lower than that in NK-92 + hNC, ****P < 0.0001 (Fig. 3c).

The flow cytometry detected the positive cell rate of NK-92 cell secreting GZMB in each group (Fig. 3a). The results of NK-92 Only, NK-92 + hB7-H6 and NK-92 + hNC were 95.47 ± 3.84%, 94.30 ± 3.65% and 95.90 ± 2.27% respectively. There was no statistical differenc (Fig. 3d).

The flow cytometry detected the positive cell rate of NK-92 cell secreting PFP in each group. The results of NK-92 Only, NK-92 + hB7-H6 and NK-92 + hNC were 8.96 ± 0.61%, 2.23 ± 0.30% and 1.01 ± 0.36% respectively. The positive cell rate in NK-92 + hB7-H6 and NK-92 + hNC both decreased significantly compared with NK-92 Only, ****P < 0.0001. However, compared with the NK-92 + hNC, the result was higher than that in NK-92 + hB7-H6, *P < 0.05 (Fig. 3e).

The ELISA detected the concentration of INF-γ secreted by NK-92 cell in each group. The results of NK-92 Only, NK-92 + hB7-H6 and NK-92 + hNC were 4186.67 ± 189.03 pg/ml, 6511.00 ± 234.72 pg/ml and 5494.33 ± 224.26 pg/ml respectively. Compared with NK-92 Only, the results in NK-92 + hNC (***P < 0.001)
and NK-92 + hB7-H6 (****P < 0.0001) were both significantly increased. The result in NK-92 + hB7-H6 group was significantly higher than that in NK-92 + hNC group, **P < 0.01 (Fig. 3f).

The killing rate of NK-92 cells was detected (Fig. 3g). After co-culture according to 5:1 E:T, the killing rate of NK-92 cells were 32.83 ± 2.65% (NK-92 + hB7-H6) and 19.34 ± 2.08% (NK-92 + hNC). After co-culture according to 10:1 E:T, the killing rate of NK-92 cells were 42.19 ± 2.89% (NK-92 + hB7-H6) and 29.30 ± 4.73% (NK-92 + hNC). After co-culture according to 20:1 E:T, the killing rate of NK-92 cells were 50.02 ± 1.99% (NK-92 + hB7-H6) and 43.12 ± 2.52% (NK-92 + hNC). At 5:1 and 10:1, compared with the NK-92 + hB7-H6, the cell killing rate of NK-92 in NK-92 + hNC were both decreased, both ***P < 0.001. And at 20:1, the killing rate of NK-92 cells in NK-92 + hNC group was also reduced compared with NK-92 + hB7-H6, *P < 0.05.

### Effect of B7-H6 on ERK pathway in NK-92 cells

According to the results of above co-culture experiment, we selected NK-92 Only, NK-92 + hNC and NK-92 + hB7-H6 groups, which had a significant changed on NKp30 expression and NK-92 cell killing ability, for the detection and analysis of NKp30 downstream ERK pathway. WB was used to detect the effect of B7-H6 on ERK phosphorylation.

First, we detected the expression of ERK and P-ERK in NK-92 Only cells, and the p-ERK/ T-ERK result was 0.10 ± 0.02. Afterwards, we analyzed the expression of ERK and P-ERK in NK-92 cells after co-culture in NK-92 + hNC and NK-92 + hB7-H6 groups. The results of P-ERK/T-ERK were 0.32 ± 0.05 and 0.84 ± 0.10.

Compared with NK-92 Only, the P-ERK/T-ERK increased significantly in NK-92 + hNC (*P < 0.05) and NK-92 + hB7-H6 (****P < 0.0001), and the increase in NK-92 + hB7-H6 was more obvious. Compared with NK-92 + hNC, the P-ERK/T-ERK in NK-92 + hB7-H6 also increased significantly, ***P < 0.001 (Fig. 4).

### Discussion

Worldwide, cervical cancer is the leading cause of cancer death among women in developing countries [11]. At present, for patients with persistent, recurrent and distant metastasis of cervical cancer, clinical options are very limited, with only combination chemotherapy including bevacizumab, or pembrolizumab/oritinib/entitinib selected based on genetic test results [12], and the clinical benefits are not optimistic [1]. Therefore, to find effective treatment for patients with advanced cervical cancer is an urgent problem to be solved. The development of cervical cancer is closely related to the imbalance between the host immune system response and tumor immune escape. Immunotherapy will become the treatment focus of advanced cervical cancer [2].

B7-H6 is a member of the B7 family. Our previous study found that B7-H6 expressed on the cervical cancer is expected to become a new biological therapeutic target for cervical cancer [9]. Interestingly, however, B7-H6, also known as NK cytotoxic receptor 3 ligand 1, can bind specifically to the surface active receptor NKp30 of NK cells through signaling motifs [13–14]. This junction plane constitutes the NKP30-B7-H6 complex [5]. NK cells are an important part of the body’s innate immunity and play an
important role in anti-infection and anti-tumor [15]. NK cell killing status is regulated by both surface activating receptors and inhibitory receptors [16]. Activation/inhibitory receptors bind to corresponding ligands to generate activation/inhibition signals, which activate/inhibit the killing function of NK cells through a cascade of downstream signals [17]. Once activated, NK cells recruit and activate other effectors by releasing cytotoxic enzymes and soluble chemokines and INF inflammatory factors, including PFP, GZMB, and INF-γ at the same time[18–19]. Many studies have proved [20–23] that the specific binding of B7-H6 expressed by tumor cells with NKp30 can activate the ability of NK cells to kill tumor cells and play an important role in the anti-tumor process. In related studies on drug intervention, the positive regulation of B7-H6 on NK cell activity mediated by NKp30 was also reflected. For example, Cao G et al. [21] found that cisplatin and 5-fluorouracil chemotherapy, radiotherapy, non-lethal heat shock and TNFa can induce the expression of B7-H6 in tumors, thus enhancing the sensitivity of tumors to NK cell cytotoxicity. Kellner C et al. [24] found that the combination of the recombinant immune ligand ULBp2:7D8 and B7-H6:7D8 can increase the NK cell-mediated killing effect of lymphoma. Interestingly, however, some studies [25, 26] have obtained opposite results, suggesting that B7-H6 can lead to immune escape of tumor cells through specific binding with NKp30, thus avoiding the protective barrier of the body’s first immune defense line. We study found that since the onset of cervical intraepithelial neoplasia, the expression of B7-H6 began to appear in cervical lesion tissues [9]. Therefore, it is speculated that B7-H6 not only plays role in the progression of cervical disease, but also mediates the body’s innate immunity to participate in the immune regulation of cervical lesion through its special association with NKp30.

In this study, we analyzed the effect of B7-H6 on NKp30 expression and NK cytotoxicity after co-culture. The resultes showed that B7-H6 could promote the up-regulation of NKp30 expression, increase the killing rate of NK-92 cells, enhance the degranulation function and the secretion capacity of INF-γ. The results of this study are similar to some recent findings which have suggested that the combination of B7-H6 and NKp30 can activate the killing function of NK cells [20]. For example, Phillips M et al. [22] proposed that B7-H6 can activate NK cell INF-γ secretion through NKp30, and further studied the novel synthetic peptide for tumors expressing B7-H6, which may be a promising lead for immunotherapy. PeKar L et al. [23] found that affinity mature B7-H6 can enhance NK cell-mediated lysis of tumor cells, and promote the release of INF-γ cytokines from bispecific immune oligomers through NKp30. Fiegler N et al. [27] found that in some tumors, after down-regulating the expression of B7-H6, NK cells' recognition ability to tumor cells was impaired. Schlecker E et al. [28] found that, in the study of human malignant melanoma, inhibition of B7-H6 protein hydrolysis and shedding into soluble B7-H6 can increase the expression level of B7-H6 on tumor cell surface, thus enhancing NKP30-mediated NK cell activation. However, our results also suggested that the B7-H6 had no effect on the GZMB secretion capacity of NK-92 cells. What was more interesting was the detection results of PFP after cell co-culture. These results suggest that the combined effect of multiple cytokines expressed by HeLa cells reduces PFP secretion capacity, but B7-H6 as an independent influencing factor can improve the PFP secretion capacity. The results of this study suggest that B7-H6 participate in the immune regulation of cervical cancer, promote the role of innate
immunity in cervical cancer surveillance. However, the effect of B7-H6 on NKp30 is weak, and the effect on various NK cytotoxic factors is different, which reason and significance need to be further analyzed.

Interestingly, some experiments have found the opposite results. For example, Mantovani S et al. [29] found that when NK cells were co-cultured with hepatocellular carcinoma cells expressing B7-H6, NKp30 on NK cells was significantly down-regulated, and this regulatory effect was eliminated when B7-H6 was silenced. Pesce S et al. [30] found that in ovarian cancer patients, soluble B7-H6 concentration was significantly correlated with the down-regulation of NKp30 expression, which led to the decrease of INF-γ release function and cytolysis ability of NK cells, and reduced the killing ability of NK cells to B7-H6-positive ovarian cancer cells. Ponath V et al. [25] found that after co-culture of isolated soluble B7-H6 with NK cells, the expression of NKp30 was down-regulated and the killing activity of NK cells decreased. Thomas P et al. [26] found that in cell lung cancer tissues and cell lines, the expression of B7-H6 has exceeded that of PD-L1, and the increased expression of B7-H6 gene is related to the decreased signal of activated natural killer cell gene. We speculate that B7-H6 is expressed in different tumor cells and has different regulatory effects on NK cells.

NKp30 binding with the corresponding ligand can induce NK cell killing function through immunoreceptor tyrosine-based activation motif (ITAM) [31], that is, the active receptor signal causes a series of phosphorylation reactions in NK cells. Multiple signal cascades can occur, one of which eventually leads to PI3K→Rac1→PAK1→MEK→ERK signal cascades, thus driving cytotoxicity of NK cells. In this study, further analysis of ERK phosphorylation in NK-92 cells after cell co-culture showed that up-regulation of NKp30 expression and increased NK cell killing ability were accompanied by increased ERK phosphorylation. This result was consistent with the findings of Teng R [18] that dephosphorylation and inactivation of ERK protein not only directly impaired NK cell killing ability, but also inhibited NK cell killing function by down-regulating NKp30 expression. These results suggest that the binding of B7-H6 with NKp30 may further affect the killing ability of NK cells by activating the downstream ERK pathway.

This study confirmed the existence of B7-H6/NKp30 immune regulatory axis in cervical cancer immune microenvironment, which can increase the killing effect of NK cells on cervical cancer cells. The results of this study broke the blind spot in the research of the inherent immune regulation of cervical cancer, brought a new idea for the research of cervical cancer immune targets, and provided a new molecular target for the development of cervical cancer immune drugs. However, this study still has some limitations, cervical cancer cell line selection is too simple, and the lack of animal tests for further verification, further improvement is needed in follow-up studies.

According to the results of our researches, on the one hand, the B7-H6 expressed by cervical cancer cells can promote cervical cancer development, on the other hand, B7-H6 can also stimulate the killing effect of NK cells to cervical cancer. These opposite effects of B7-H6 on cervical cancer bring great interest to the research work and highlights the value of B7-H6 in the study of cervical cancer. To further explore the mechanism of B7-H6 in the development of cervical cancer will certainly bring a new dawn for cervical cancer research and clinical treatment.
Declarations

Ethical Approval

This study was approved by the the second hospital of Tianjin Medical University ethics committee, and it is registered under number KY2022K019.

Competing interests

The authors declare that they have no competing interests including a financial or personal nature.

Authors' contributions

Ruimeng Guo and Ou Chai completed the overall design of the experiment and wrote the paper. Changying Li provided the overall technical support of the experiment. Yanying Xu provided some financial support. XueWang Guo prepared figure 1. Xueying Liu and Yu Xu prepared figure 3 2-4. All authors reviewed the manuscript.

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Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request

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Consent to participate

All participants signed an informed consent form to be part in the study.

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Figures
Figure 1

Verification of HeLa cell transfection

a. Fluorescence microscopy was used to analyze GFP expression in HeLa cells (×100), scale: 100 μm; b. Compared with shNC, the mRNA level of shB7-H6 was decreased; c. Compared with shNC, the protein expression level of shB7-H6 was decreased. d. Compared with hNC, the mRNA level of B7-H6 in hB7-H6 was increased. e. Compared with hNC, B7-H6 protein expression level in hB7-H6 was significantly up-regulated.
Figure 2

Effect of B7-H6 knockdown on NKp30 expression and NK cell killing function

a. Diagram of flow detection results. b. After cell co-culture, positive rate of NKp30 expression cells of NK-92+shB7-H6 was no different from NK-92 Only, and the result of NK-92+shNC was higher than that of NK-92 Only and NK-92+shB7-H6. c. The rate of CD107a positive cells after co-culture, NK-92+shB7-H6 was no different from NK-92 Only, and the result of NK-92+shNC was higher than that of NK-92 Only and NK-92+shB7-H6. d. NK-92 Only, NK-92+shNC and NK-92+shB7-H6, the positive rate of NK-92 cells secreting GZMB were no statistical difference. e. Compared with NK-92 Only, the positive rate of NK-92 cells secreting PFP of NK-92+shNC and NK-92+shB7-H6 were both decreased, but the result of NK-92+shNC was higher than that of NK-92+shB7-H6. f. After cell co-culture, the concentration of INF-γ secreted by NK-92 cells of NK-92+shB7-H6 was no different from NK-92 Only, and the result of NK-92+shNC was higher than that of NK-92 Only and NK-92+shB7-H6. g. The NK-92 cell killing rate of NK-92+shB7-H6 was lower than that of NK-92+shNC when E:T were 5:1 and 10:1. But there were no difference between the NK-92+shB7-H6 and NK-92+ shNC when E:T was 20:1.
Figure 3

Effect of B7-H6 up-regulation on NKp30 expression and NK cell killing function

a. Diagram of flow detection results. b. After cell co-culture, compared with NK-92+hNC, the positive rate of NKp30 expression cells of NK-92 Only was lower, and the result of NK-92+hB7-H6 was higher. c. After cell co-culture, compared with NK-92+hNC, the rate of CD107a positive cells of NK-92 Only was lower, and the result of NK-92+hB7-H6 was higher. d. In NK-92 Only, NK-92+hNC and NK-92+hB7-H6, the positive rate of NK-92 cells secreting GZMB were no statistical difference. e. Compared with the NK-92 Only, the positive rate of NK-92 cells secreting PFP both decreased in NK-92+hNC and NK-92+hB7-H6. However, compared with the NK-92+hB7-H6, the result in NK-92+hNC was increased. f. Compared with the NK-92 Only, the concentration of INF-γ in NK-92+hNC and NK-92+hB7-H6 were both increased. Compared with the NK-92+hNC, the concentration of INF-γ in NK-92+ hB7-H6 was also increased. g. Compared with the NK-92+hNC, the NK-92 cell killing rate in NK-92+hB7-H6 were both increased under three different E:T.
Figure 4

ERK phosphorylation in NK-92 cells after co-culture

Compared with NK-92 Only, the P-ERK/T-ERK in NK-92 cells were increased in NK-92+hNC and NK-92+hB7-H6, and the increase was more obvious in NK-92+hB7-H6.