Epstein-Barr virus-encoded microRNA BART7 downregulates major histocompatibility complex class I chain-related peptide A and reduces the cytotoxicity of natural killer cells to nasopharyngeal carcinoma

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Abstract. Evasion from natural killer (NK) cell surveillance enables cancer to proliferate and spread at the early stages. NK cells mediate specific cytolysis by activation of the triggering receptors on their cell surface, of which the communication between natural killer group 2, member D (NKG2D) and major histocompatibility complex class I chain-related peptide A (MICA) is a key regulatory axis. It has been indicated that cancer cells can reduce the surface expression of MICA, which thereby reduces the cytotoxicity of NK cells. In nasopharyngeal carcinoma (NPC), however, the underlying mechanism remains unclear. The present study indicated that MICA expression in NPC was regulated by TGFβ1. Furthermore, the human MICA gene was demonstrated to contain the c-Myc binding site in the promoter region. Notably, the results suggested that TGFβ1 upregulated MICA expression by promoting c-Myc expression. Additionally, the findings demosntrated that TGFβ1 expression in NPC was negatively controlled by Epstein-Barr virus-encoded microRNA BART7 (ebv-miR-BART7). In ebv-miR-BART7-expressing NPC, the TGFβ1/c-Myc/MICA regulatory axis was significantly inhibited. Notably, functional analysis indicated that NPC cells expressing ebv-miR-BART7 were less sensitive to the cytolysis mediated by NK cells. In conclusion, the present results revealed that ebv-miR-BART7-expressing NPC may impair NK cells recognition and activity, which suggests that targeting ebv-miR-BART7 may be a useful therapeutic strategy in NPC immunotherapy.

Introduction

Nasopharyngeal carcinoma (NPC) is a unique head and neck cancer as the pathogenesis of cancer is closely linked with Epstein-Barr virus (EBV). Guangdong province of southern China has the highest NPC incidence and EBV-associated NPC is the major histological type (1). EBV infection could be found in the normal individuals. In immunocompetent EBV carrier, EBV is in the latency 0 which does not trigger innate immune response (2). When the virus enters lytic phases, many viral gene products could interfere with the innate and adaptive response which protects the cells from detection and elimination by the immune effectors (3). EBV in NPC is in latency II stages. The characteristic viral gene products in EBV latency II can exert immunomodulatory functions and contribute to the immunoevasion to the host.

Natural killer (NK) cell is a cytotoxic effector in human innate system with the capability to kill tumor cells. Evasion from NK cell surveillance enables cancer to proliferate and spread at early stage (4). NK cells mediate target specific cytolysis by activation of the triggering receptors on their cell surface. Of which, the communication between immunoreceptor natural killer group 2, member D (NKG2D) and major histocompatibility complex class I chain-related peptide A (MICA) is a key regulatory axis. NKG2D is a c-type lectin-like receptor expressed on the NK cell surface (5). MICA is the NKG2D ligand expressed on transformed cells surface. NKG2D is an activating receptor of NK cells. Binding of MICA will trigger the activating signals and antitumor response of NK cells (6).

In NPC, it has been shown that the cytotoxic activity of NK cells in NPC patients were reduced in comparison with the normal individuals (7). NK cells can recognize cancer with high surface MICA expression. However, the cancer cells can suppress MICA expression which reduces their susceptibility to NK cell cytolysis. For NPC, the underlying mechanism remains unclear. MICA polymorphism (MICA-A9 and A5.1) is associated with the NPC risk in the Southern China male NPC patients (8,9). In liver cancer, it has been shown that MICA expression is regulated by microRNA (10). MicroRNA is short non-coding RNA which functions as post-transcriptional regulators to specific gene expression. In view of the close association between EBV and NPC, we speculate that EBV-encoded microRNA has a regulatory role on MICA expression in NPC. It has been shown that MICA expression could be upregulated by transforming growth

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factor β 1 (TGFβ1) (11). As our previous findings indicate that the EBV-encoded microRNA BART7 (ebv-miR-BART7) is a functional TGFβ1 suppressor (12), we hypothesized that NPC expressing ebv-miR-BART7 has lower MICA expression and has reduced sensitivity to NK cell cytolysis.

Materials and methods

Cell lines. NPC cell line HK1 was maintained in PRMI-1640 medium supplemented with 10% fetal bovine serum, 200 Unit/ml penicillin G sodium, 20 μg/ml streptomycin sulfate, and 0.5 μg/ml amphotericin B (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). NK92MI cells were incubated in Alpha Minimum Essential medium supplemented with 12.5% fetal bovine serum, 12.5% horse serum (both Gibco; Thermo Fisher Scientific, Inc.), 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid (all Sigma, St. Louis, MO, USA), 200 Unit/ml penicillin G sodium, 20 μg/ml streptomycin sulfate, and 0.5 μg/ml amphotericin B (all Gibco; Thermo Fisher Scientific, Inc.).

Recombinant TGFβ1 treatment. HK1 cells were treated by recombinant TGFβ1 (Thermo Fisher Scientific, Inc.) as previously described (12). HK1 was incubated with 0.5 ng/ml recombinant TGFβ1 for 72 h.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted with TRIzol (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA was converted to cDNA using High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.) following the supplier's guideline. RT-qPCR was carried out using FastStart Universal Probe Master on a LightCycler® 480 (both Roche Applied Science, Penzberg, Germany). The primers and probes were designed from Universal ProbeLibrary Assay Design Center (http://www.roche-applied-science.com/). Primers and probes were as follows: MICA-forward, 5'-GGCATCTTCCCTTTTGCAC-3'; MICA-reverse, 5'-GGACAGCACCGTGAGGTTAT-3'; MICA-probe, cat. no. 24; c-Myc-forward, 5'-GCTGCTTAGACGCTGGATTT-3'; c-Myc-reverse, 5'-TAACGTTGAGGGGCATCG-3'; c-Myc-probe, cat. no. 66; TGFβ1-forward, 5'-ACTACTACGCCAAGGAGGTCAC-3'; TGFβ1-reverse, 5'-TGCTTGAACTTGTCATAGATTTCG-3'; TGFβ1-probe, cat. no. 27. qPCR reaction was carried out at 95˚C for 10 min followed by 45 cycles of 95˚C for 15 sec and 60˚C for 1 min. Expression levels were calculated using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$).

BART7 mimic transfection. HK1 cells were transfected with 3 nM ebv-miR-BART7 mimic or negative control siRNA using Hiperfect transfection reagent (all Qiagen Inc., Valencia, CA, USA). After 72 h, the expression levels of TGFβ1, MICA and c-Myc were determined.

Immunocytochemistry (ICC). HK1 cells were seed on chamber slides and treated with recombinant TGFβ1 or transfected with ebv-miR-BART7 mimic. NPC cells were washed with PBS and fixed with 4% paraformaldehyde. Then, NPC cells were incubated with anti-MICA antibodies (Abcam, Cambridge, UK), followed by the addition of CF™488A Secondary Antibody Conjugates (Biotium, Inc., Freemont, CA, USA). Blue-fluorescent 4',6-Diamidino-2-Phenylindole (DAPI; Thermo Fisher Scientific, Inc.) was used to label nucleus.

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed using Magna ChIP™ A Chromatin Immunoprecipitation kit (EMD Millipore Billerica, MA, USA) following the manufacturer's protocol. In brief, HK1 cells were fixed with 1% paraformaldehyde and were lysed by cell lysis buffer. Then, cells were sonicated to shear cross-linked DNA to approximately 200-1,000 base pairs. The cell lysates were incubated overnight at 4˚C with protein A magnetic beads and anti-c-Myc antibodies or normal mouse IgG. Protein/DNA complex were eluted from the beads and free DNA was obtained by reverse cross-links of protein/DNA complex. The precipitated MICA promoter DNA was detected by RT-PCR. The primers spanning the c-Myc binding site of MICA promoter were as follows: Forward, 5'-GGTGGGATAGGGTGAGGAGA-3'; reverse, 5'-CCCCATCTGCTGAATGTCAC-3'. The RT-PCR products were analyzed by QIAxcel Advanced system (Qiagen Inc.), a capillary electrophoresis device.

NK cell cytotoxicity test. NK cell cytotoxicity test was performed using the xCELLigence system (13). HK1 cells were transfected with ebv-miR-BART7 mimic or negative control siRNA followed by seeded on the E16 plate. Then, NK92MI cells were added to NPC cells and survival of NPC cells was continuously monitored. The percentage of cytotoxicity was calculated from the formula: Percentage of cytotoxicity=[(cell index $_{\text{no effector}}$-cell index $_{\text{effector}}$)/cell index $_{\text{no effector}}$] x100%.

Statistical analysis. All results were shown as mean ± SD from 3 independent experiments. Student's t test was used to evaluate the differences between control and experimental groups. $P<0.05$ was considered statistically significant.

Results

TGFβ1 was implicated in MICA expression in NPC. To study whether TGFβ1 regulated MICA expression, NPC cells were first treated with recombinant TGFβ1. RT-qPCR analysis showed that MICA expression was transcriptionally un-regulated in HK1 cells (Fig. 1A). Consistently, subsequent immunostaining indicated that surface expression of MICA in HK1 were remarkably increased in response to TGFβ1 treatment (Fig. 1B).

C-Myc bound to the promoter region of MICA. To investigate the regulatory mechanism of MICA transcription, we performed promoter analysis and identified potential c-Myc binding site (CATGTG) in the promoter region of MICA gene (Fig. 2A). As shown in the ChIP assay, c-Myc protein could bind to the predicted c-Myc binding site located upstream of the transcription start site of MICA gene (Fig. 2B).

TGFβ1 activated c-Myc expression and the TGFβ1/c-Myc regulatory axis in NPC was inhibited by ebv-miR-BART7. To examine whether TGFβ1 could regulate c-Myc at

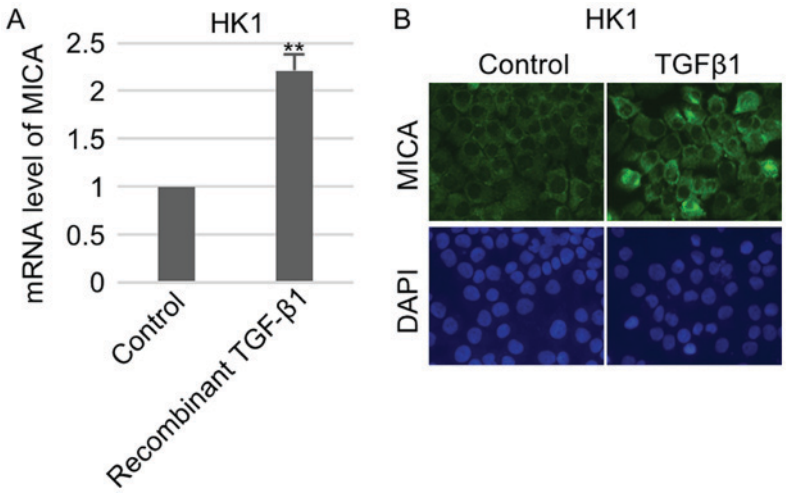

Figure 1. Recombinant TGFβ1 treatment enhanced the expression of MICA in NPC cells. (A) RT-qPCR analysis of MICA mRNA expression in NPC cells treated by recombinant TGFβ1. (B) Representative images of ICC showing the protein expression of MICA in NPC cells upon TGFβ1 treatment. Magnification, x400. The data were expressed as mean ± SD. **P<0.01 vs. control. TGFβ1, transforming growth factor β 1; MICA, major histocompatibility complex class I chain-related peptide A; NPC, nasopharyngeal carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

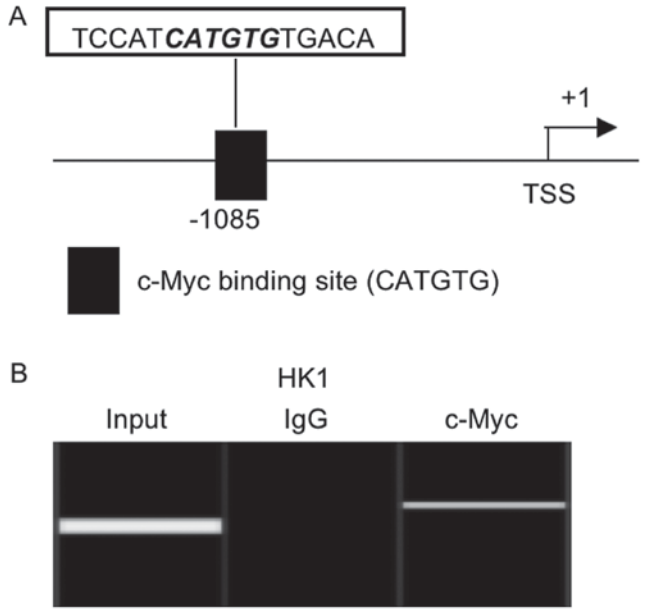

Figure 2. C-Myc bound to the promoter region of MICA. (A) Position and sequence of c-Myc binding site in MICA promoter. Position of c-Myc binding site was relative to TSS (+1). (B) ChIP analysis of the binding of c-Myc to the promoter region of MICA. Chromatin was incubated with normal IgG or anti-c-Myc antibodies followed by Reverse transcription-polymerase chain reaction analysis of precipitated MICA promoter region containing c-Myc binding site. The Reverse transcription-polymerase chain reaction products were analyzed by QIAxcel Advanced system. Input, 1% of total input lysate. MICA, major histocompatibility complex class I chain-related peptide A; TSS, translation start site; ChIP, chromatin immunoprecipitation.

transcriptional level, we examined the change of c-Myc expression in the TGFβ1-treated NPC cell lines. As shown in Fig. 3A, c-Myc expression was significantly increased in HK1 cells after treatment with recombinant TGFβ1. The expression of TGFβ1 was increased in HK1 cells treated with recombinant TGFβ1 (Fig. 3B). Recombinant TGFβ1 treatment did not affect the expression level of ebv-miR-BART7 (Fig. 3C). We have previously shown that ebv-mir-BART7 expression had a significant negative regulatory function on TGFβ1 expression in NPC (12). Thus, we anticipated that ebv-miR-BART7 expression in the NPC cells will also suppress c-Myc expression. To confirm our hypothesis, we transfected the NPC cell line with synthetic ebv-miR-BART7 microRNA and measured the change of TGFβ1/c-Myc expression. The expression of ebv-miR-BART7 was significantly increased in HK1 cells transfected with BART7 mimic, indicating the successful overexpression of ebv-miR-BART7 (Fig. 3D). NPC cells expressing ebv-miR-BART7 showed significant reduction of TGFβ1/c-Myc as compared with the mock transfectant (Fig. 3E and F).

MICA expression was remarkably reduced in ebv-miR-BART7-expressing NPC. To confirm the importance of ebv-miR-BART7 on MICA expression, changes of MICA expression in ebv-miR-BART7-expressing NPC cell line were performed. As shown in Fig. 4, ebv-miR-BART7 had a significant suppressive effect on MICA mRNA and protein expression in the NPC cell line (Fig. 4).

Ebv-miR-BART7-expressing NPC cells exhibited reduced sensitivity to NK cell cytolysis. To explore whether ebv-miR-BART7 expression will reduce the sensitivity of NPC cells to NK cell lysis, ebv-miR-BART7-expressing NPC cells and the mock transfectants were co-cultured with NK cell line NK92MI. When NPC cells reached their logarithmic growth phase, NK92MI cells were added to NPC cells at effector to target (E:T) ratio of 5:1. For HK1 cells, a significant lysis of target cells was observed at 20 h after the addition of NK92MI cells (Fig. 5). Ebv-miR-BART7-expressing HK1 cells were less lysed by NK92MI cells in comparison with control cells (Fig. 5), indicating that forced expression of ebv-miR-BART7 reduced the sensitivity of NPC cells to NK92MI cells.

Discussion

Reduced expression of major histocompatibility complex MHC class I protein on the cell surface is associated with the

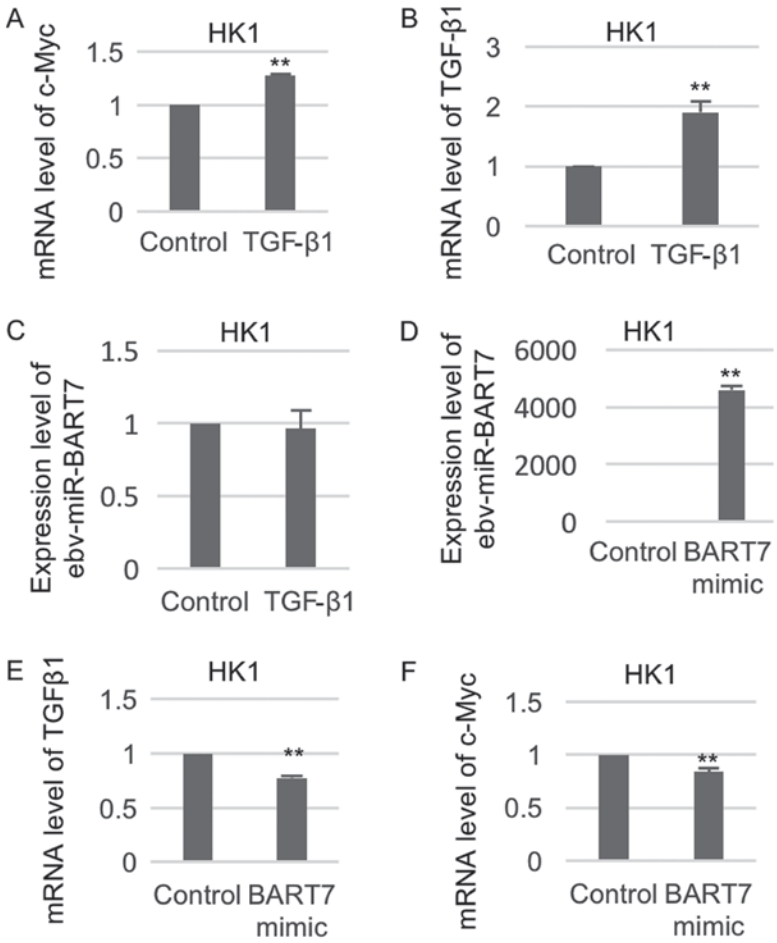

Figure 3. Ebv-miR-BART7 suppressed TGFβ1/c-Myc regulatory axis in NPC cells. (A) RT-qPCR analysis of c-Myc mRNA expression level in HK1 cells incubated with recombinant TGFβ1. (B) RT-qPCR analysis of TGFβ1 mRNA expression level in HK1 cells incubated with recombinant TGFβ1. (C) RT-qPCR analysis of ebv-miR-BART7 expression level in HK1 cells incubated with recombinant TGFβ1. (D) RT-qPCR analysis of ebv-miR-BART7 expression level in NPC cells transfected with ebv-miR-BART7 mimic. (E) RT-qPCR analysis of TGFβ1 expression level in NPC cells transfected with ebv-miR-BART7 mimic. (F) RT-qPCR analysis of c-Myc mRNA expression level in NPC cells transfected with ebv-miR-BART7 mimic. The data were expressed as mean ± SD. **P<0.01 vs. control. Ebv-miR-BART7, Epstein-Barr virus-encoded microRNA BART7; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TGFβ1, transforming growth factor β 1; NPC, nasopharyngeal carcinoma.

oncogenic transformation (14). MICA belongs to the MHC class I family and impairment of MHC class I expression is common in viral infected cancers. MICA can specifically bind to surface NKG2D on different immune cells such as NK cells and CD8αβ T cells and activates their immune functions (15,16). Therefore, suppressing MICA expression could prevent the viral infected cancer cells from immune attack.

TGFβ singling is important for maintaining the epithelial homeostasis and immunity (17). Disruption of TGFβ signaling is implicated in the pathogenesis of head and neck cancers (18). Recently, it was reported that TGFβ1 could inhibit mTOR activity of NK cells, leading to reduced metabolic activity and impaired cytotoxic activity of NK cells (19). TGFβ1 also reduced the expression of NK cell surface receptors including CD122 and IL-15RB, which might confer decreased metabolic activity (19). To identify the soluble factors released by B-acute lymphoblastic leukaemia (ALL) blasts that could impair function of NK cells, Rouce *et al* compared the levels of known soluble factors upregulated in other cancers between ALL blasts cultured with NK cells and healthy donor B cells cultured with NK cells (20). They found that TGFβ1 was the most notably increased soluble factor. Moreover, TGFβ1 suppressed the expression of activating receptors including NKG2D, NKp30 and NKp46 and enhanced the expression of inhibitory receptor NKG2A of NK cells, resulting in ALL-mediated NK cell dysfunction (20). We speculate that TGFβ can interrupt the interaction of NKG2D (on NK cell surface) and MICA in cancer patients in view of the fact that elevated TGFβ1 and reduced NKG2D expression on the NK cell surface is accompanying with cancer patients (21). It has been shown that TGFβ1 can suppress NKG2D expression on NK cells (22). Whether TGFβ1 has similar suppressing effects on MICA expression in NPC remains unclear. Promoter analysis indicated that MICA contains the binding site for the known TGFβ1-regulated transcriptional factor c-Myc (23). We first examined the effects of TGFβ1 on MICA expression in NPC. Our results demonstrated that TGFβ1 is a potent activator of MICA in NPC. Thus, suppressing TGFβ1 expression in NPC might provide protection against and evasion from NK cells. It has been reported that c-Myc could affect the response of tumor cells to the killing effect of NK cells. In tumor cell, c-Myc enhanced the expression of B7-H6 which was the surface expressed ligand for NKp30, an activating receptor of NK cells. Suppression of c-Myc impaired

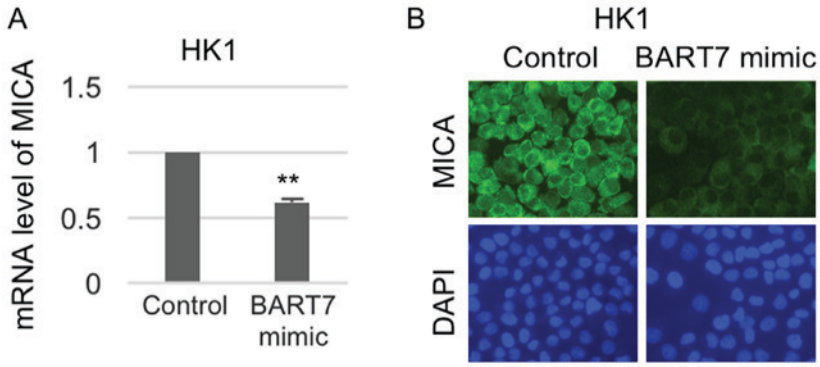

Figure 4. Ebv-miR-BART7 suppressed the expression of MICA in NPC cells. (A) RT-qPCR analysis of MICA mRNA expression in HK1 cells transfected with ebv-miR-BART7 mimic. (B) ICC analysis of MICA protein expression in NPC cells transfected with ebv-miR-BART7 mimic. Magnification, x400. The data were expressed as mean ± SD. **P<0.01 vs. control. Ebv-miR-BART7, Epstein-Barr virus-encoded microRNA BART7; MICA, major histocompatibility complex class I chain-related peptide A; NPC, nasopharyngeal carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ICC, immunocytochemistry.

the cytotoxic activity of NK cells activated by NKp30 (24). Likewise, we found that c-Myc could enhanced the expression of MICA, a ligand for another activating receptor NKG2D of NK cells.

The expression of MICA was much more affected by BART7 mimic in comparison with c-Myc and TGFβ1. This observation might be due to the presence of other mechanisms responsible for the regulatory role of ebv-miR-BART7 on MICA. Therefore, ebv-miR-BART7 modulated MICA expression partially through TGFβ1/c-Myc.

EBV infection is commonly found in the undifferentiated NPC. The viral gene products could be detected in the tumor cells with potent oncogenic functions in NPC development (25). At present, how EBV confers protection to NPC to escape from the attack of NK cells remains to be clarified. However, it has been shown that EBV can express different viral proteins to suppress the immune attack from other immune cells such as cytotoxic T cells. EBV-infected cells in the lytic phase could express BNLF2a to reduce transporter associated with antigen processing (TAP) function and expression of human histocompatibility leukocyte antigen (HLA) class I (26). EBV-expressed BGLF5 (EBV alkaline exonuclease) can impair T-cell recognition by interfering HLA class II immune responses (27).

Recent study has shown that EBV-encoded microRNA can inhibit recognition and immune attack of EBV-specific $CD8^+$ T cells by targeting peptide transporter subunit TAP2 (28). EBV-encoded microRNA could also modulate $CD4^+$ T cells response by targeting cytokine and MHC class II family expression (28). Comparatively, how does the EBV-infected cells evade from the immune attack of NK cells is less well understood. We have previously shown that ebv-miR-BART7 can suppress TGFβ1 in the NPC cells (12). Based on the new observation that TGFβ1 can un-regulate MICA expression in NPC, we speculate that ebv-miR-BART7 could play a part in the NPC immunity by weakening the interaction between NPC and NK cells. The NPC cell lines employed in the current study does not harbor EBV. Transfection of synthetic ebv-miR-BART7 results in a significant decrease in c-Myc and MICA indicating that EBV-associated NPC might escape from the surveillance of NK cells. This functional role is confirmed in the NPC/NK cells co-cultures and the results demonstrate the significance of ebv-miR-BART7 in NPC.

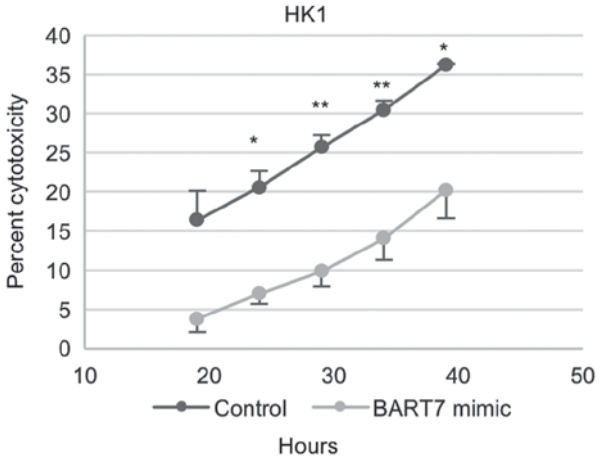

Figure 5. Forced expression of ebv-miR-BART7 reduced the sensitivity of NPC cells to NK92MI cells. NK cell cytotoxicity against NPC cells transfected with ebv-miR-BART7 mimic using xCELLigence technology. HK1 cells transfected with ebv-miR-BART7 mimic or negative control siRNA were seeded on E16 plate. NK92MI cells were added to NPC cells at effector to target (E:T) ratio of 5:1. Survival of NPC cells was continuously monitored. The percentage of cytotoxicity was calculated. The data were expressed as mean ± SD. *P<0.05, **P<0.01 vs. BART7 mimic. Ebv-miR-BART7, Epstein-Barr virus-encoded microRNA BART7; NPC, nasopharyngeal carcinoma.

In conclusion, our results show that ebv-miR-BART7 can reduce the susceptibility of NPC cells to NK cell lysis by reducing expression of MICA. As the activating role of MICA is well-studied and confirmed, we speculate that targeting the ebv-miR-BART7 expression in the NPC tissues can increase MICA and thereby increase the susceptibility of NPC to NK cells. Further studies are warranted to confirm the potential role of ebv-miR-BART7 in NPC immunotherapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TSW, WG and JYWC set up the hypothesis and designed the experiments. SC and MJZ performed the experiments and acquired the data. TSW, WG and JYWC analyzed and interpreted the data. TSW and WG wrote the manuscript. All authors read and approve the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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