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Epstein-Barr virus-coded miR-BART13 promotes nasopharyngeal carcinoma cell growth and metastasis via targeting of the *NKIRAS2*/NF-κB pathway

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ABSTRACT

Based on analysis of Epstein-Barr virus (EBV) BART microRNA expression profiles, we previously reported that EBV-encoded miR-BART13 is upregulated in nasopharyngeal carcinoma (NPC) plasma specimens. However, the effects and molecular mechanisms of miR-BART13 in NPC remain largely unknown. We found that miR-BART13 was significantly upregulated in NPC tissue specimens. Ectopic expression of miR-BART13 promoted NPC cell proliferation, epithelial mesenchymal transition, and metastasis *in vitro*, and facilitated xenograft tumor growth and lung metastasis *in vivo*. Molecularly, NF-κB inhibitor interacting Ras-like 2 (*NKIRAS2*), a negative regulator of the NF-κB signaling, was identified to be a direct target of miR-BART13 in NPC cells, and *NKIRAS2* mRNA and protein expression was inversely correlated with miR-BART13 in NPC tissues, respecitvely. Furthermore, the NF-κB signaling pathway was activated by miR-BART13. By rescued experiments, reconstitution of *NKIRAS2* expression abrogated all the phenotypes upregulated by miR-BART13, and attenuated activity of NF-κB signaling pathway activated by miR-BART13 in NPC cells. Our findings indicated the newly identified miR-BART13/*NKIRAS2*/NF-κB signaling axis may provide further insights into better understanding of NPC initiation and development, and targeting of this pathway could be further studied as a therapeutic strategy for NPC patients.

1. Introduction

Nasopharyngeal carcinoma (NPC) shows unbalanced geographic distributions occurring the most in Southern China and Southeast Asia [1]. NPC development is associated with a combination of different risk factors, like Epstein-Barr virus (EBV) infection, genetic susceptibility, and environmental aspects [2,3]. Clinically, NPC is usually treated with radiotherapy if tumor resides locally due to its anatomical complexity and high radiosensitivity, while advanced disease was treated with intensity-modulated radiotherapy (IMRT) and combination with chemotherapy [4]. The 5-year overall NPC survival rate has substantially improved from approximately 50%–80% for the last decades [5,6], whereas there were 20%–30% NPC patients developing local recurrence or distant metastasis [6], leading to a median survival of approximately 20 months [7]. Thus, further investigation of the underlying molecular mechanisms of NPC development and progression could lead to early detection, prevention, and treatment strategies to effectively control NPC clinically.

microRNAs (miRNAs) are a group of small non-coding RNAs (21–25 nucleotides in length) in human genome and function to regulate the targeting gene expression through post-transcriptional degradation of the targeting transcripts and/or inhibition of the protein translation [8]. Accumulating evidence demonstrated that aberrant miRNA expression was a hallmark of human cancer development and progression [9]. As the most important risk factor in NPC, the EBV was the first shown to encode and express different miRNAs [10], BamHI fragment A

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rightward transcript (BART) and BamHI fragment H rightward reading frame 1 (BHRF1) during viral replication cycle [11]. BART miRNAs could be detected in all EBV latent infection and abundantly expressed in NPC lesions with a total of 22 BART pre-miRNAs and possible 44 mature miRNAs [12,13], whereas few EBV proteins, such as EBV nuclear antigen (EBNA) and latent membrane protein 1/2 (LMP1/2), could be found in NPC cells [14], suggesting that these BART miRNAs may be associated with NPC carcinogenesis and progression.

BART miRNAs could function as negative regulators to target both viral and host genes to retain virus latency or promote NPC development [15]. For example, some BART miRNAs were shown to inhibit expression of their own viral gene, like viral DNA polymerase BALF5 [16], EBNA2 [17], to maintain EBV in a certain state of latency in case of being recognized and eliminated by the host cells. BART miRNAs might also regulate diversified cell responses, such as immune escape [18,19], cell apoptosis [20,21], tumor growth [22–25] and metastasis [24–27] through targeting of the host gene expression. Hence, more comprehensive investigation is required to make a full clarification of the role of other BART miRNAs in the tumorigenesis and development of NPC. Our previous study revealed that the circulating EBV-related miR-BART13 could serve as a potential serological biomarker for NPC early detection and treatment response [28]. Level of plasma miR-BART13 was high in NPC patients compared with that of healthy individuals or non-NPC tumor patients before treatment and reduced in NPC patients after radiotherapy [28]. Level of plasma miR-BART13 before treatment was associated with NPC N classification and clinical stage [28]. In a recent study, it was also demonstrated that extracellular vesicles-bound miR-BART13 in circulation could be used to distinguish NPC from other head & neck cancers and individuals with asymptomatic EBV-infections [29]. However, to our knowledge, the biological effect and molecular mechanism of miR-BART13 in NPC have not yet been elucidated.

In this study, we first assessed level of miR-BART13 in tissue samples, and then investigated the effect of miR-BART13 expression on regulation of NPC cell proliferation and invasion *in vitro* and tumorigenesis and metastasis in nude mouse models. Finally, the underlying molecular events in miR-BART13-regulated changes in NPC cell phenotypes and gene expression were explored. By this way, the study aimed to expand our understanding the molecular mechanisms that regulated the development and progression of NPC, and may provide a reference for the development of novel treatment strategies against NPC in the future.

2. Materials and methods

2.1. Cell lines and tissue specimens

Human NPC CNE-1 cell line and 293T cell were purchased from Wuhan Institute of Cell Biology, China Center for Type Culture Collection, and NPC SUNE-1 cell line was obtianed from Sun Yat-sen University Cancer Center (Guangzhou, China), while NPC C666-1 cell line was a kindly gift of Prof. George S.W. Tsao of the University of Hong Kong, and all grown in Roswell Park Memorial Institute medium-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Australia). In addition, 36 freshly frozen NPC and 25 normal nasopharyngeal (NP) tissues were obtained from Fujian Cancer Hospital, Fujian Medical University Cancer Hospital (Fuzhou, China) between January 2012 and December 2012. Furthermore, 36 corresponding formalin-fixed, paraffin-embedded samples from such patients with NPC were also collected. Any chemotherapy or radiotherapy before biopsy was not given to the patients. This study was approved by the Institutional ethic Review Board of Fujian Cancer Hospital, Fujian Medical University Cancer Hospital (#2015-010-02) and a written informed consent was obtained from each patient.

2.2. Nude mouse tumor cell xenograft growth and lung metastases models

An animal protocol of this study was approved by the Institutional Animal Care and Use Committee (IACUC) of Fujian Cancer Hospital, Fujian Medical University Cancer Hospital (Fuzhou, China). In brief, female BALB/c nude mice with 4–7 weeks old and 20–25 g in body weight were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). For nude mouse tumor xenograft model, stable miR-BART13 or negative control SUNE-1 cells were grown and subcutaneously injected into mouse dorsal flank region (1×10^6 cells in 200 µl PBS). Tumor xenografts were monitored daily and the size was measured every three days. After 15 days, mice were sacrificed and tumor xenografts were dissected, weighted, and subjected to tissue processing and hematoxylin and eosin (H&E) staining for histological observation.

For nude mouse tumor lung metastasis, stable miR-BART13-luciferase or negative control infected SUNE-1 cells were grown and injected into mouse tail vein (5×10^5 cells in 100 µl PBS). Five weeks later, we injected the VivoGlo™ Luciferin, *in vivo* Grade (Promega, Madison, WI, USA) at 150 mg/kg into the abdominal cavity of each mouse, and the mice underwent bioluminescence imaging using Caliper IVIS Lumina II (Caliper Life Sciences, Hopkinton, MA, USA) to determine the luminescent value of the mouse lungs. After that, the mice were sacrificed to resect the lung tissues for fixation in 10% formalin, tissue processing, embedded in paraffin, and sectioning. Each of 5 µm-thick tissue sections was stained with hematoxylin and eosin (H&E). The presence of macroscopic and microscopic metastatic lesions in each mouse lung was accounted and averaged.

2.3. iTRAQ analysis

An iTRAQ 8-plex assay using NPC cell samples (C666-1-CTRL cells vs. C666-1-BART13 sponge cells) were analyzed in a previous study [30] on a QSTAR-Pular iHybrid Mass Spectrometer (AB Sciex). Date was analyzed using Protein Pilot Software (Version 4.0, AB Sciex). For iTRAQ analysis, a multiple of differential proteins between the two cell groups was set as more than 1.3-fold. A P value of less than 0.05 was of statistical significance.

2.4. Western blot

Total cellular and nuclear proteins were extracted using the protein extraction kits from Millipore (Billerica, MA, USA) and Western blot was performed according to our previous study [31]. The antibodies used were against human PCNA (#13119), Survivin (#2808T), Vimentin (#5741T), N-cadherin (#13116T), ZEB1 (#3396T), p-NF-κB (p65) (#3033S), β-actin (#4967), all were from Cell Signaling Technology (Danvers, MA, USA). E-cadherin (ab15148), *NKIRAS2* (ab154020), NF-κB(p65) (ab32536), LaminB1 (ab16048), all were from Abcam (Cambridge, MA, USA). IκBβ (sc-74451) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). β-actin and LaminB1 were used as protein loading controls.

2.5. Statistical analysis

All data were summarized as mean ± standard deviation (SD) and analyzed using two tailed Student *t*-test with SPSS 17.0 software (SPSS, Chicago, IL, USA), while Spearman's correlation test was performed to analyze the association of miR-BART13 with *NKIRAS2*, *TIAL1*, and *TOPBP1* mRNA expression in tissue specimens, respectively. A P value < 0.05 was considered as statistically significant.

2.6. Other procedures

Protocols for other procedures were described in the Supplementary Material.

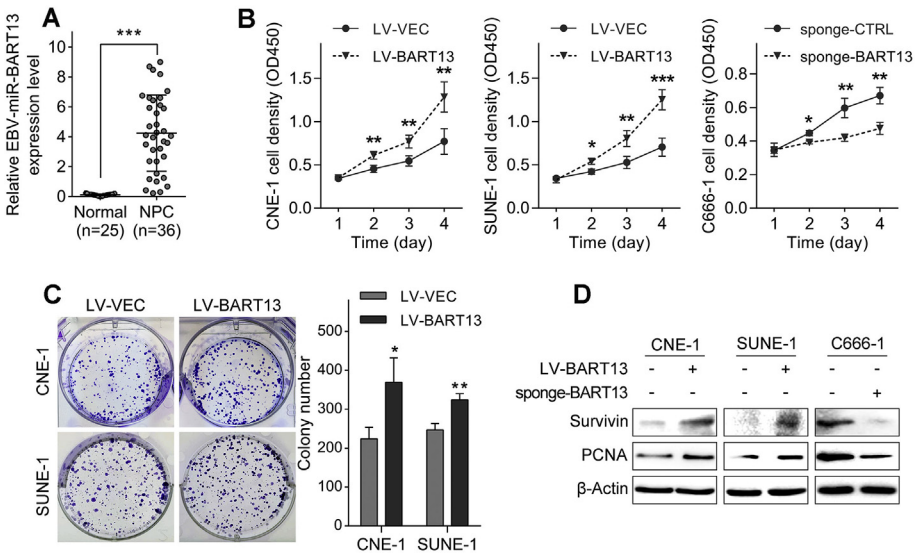

Fig. 1. EBV-miR-BART13 is upregulated in NPC tissue specimens and promotes NPC cell proliferation *in vitro*. A. Analysis of miR-BART13 levels in normal nasopharyngeal (NP) tissues (n = 25) and NPC tissues (n = 36) by RT-qPCR. U6 was used as an internal control. B and C. miR-BART13 overexpression or knockdown on the proliferation of NPC cells was assessed by the CCK8 assay (B) and the colony formation assay (C). D. Western blot analysis of the effects of miR-BART13 overexpression or knockdown on NPC cell proliferation-associated proteins. Values are mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results

3.1. miR-BART13 is upregulated in NPC tissue specimens and promotes NPC cell proliferation in vitro

As shown in Fig. 1A, miR-BART13 level was upregulated in 36 freshly frozen NPC biopsy specimens compared with that of 25 NP specimens ($P < 0.001$). To investigate the biological effects of miR-BART13 overexpression or knockdown on NPC proliferation, lentiviruses carrying miR-BART13, miR-BART13 sponge, or negative controls were firstly infected into NPC CNE-1, SUNE-1, and C666-1 cells, respectively. Then tumor cell viability and colony formation capabilities were significantly increased (Fig. 1B and C) with upregulated PCNA and Survivin expression (Fig. 1D) after miR-BART13 expression in CNE-1 and SUNE-1 cells. In contrast, miR-BART13 knockdown in C666-1 cells had opposite effects on tumor cell proliferation and protein expression (Fig. 1B and D).

3.2. miR-BART13 promotes NPC cell EMT and metastasis in vitro

To further investigate the biological effects of miR-BART13 on NPC development, the assays of NPC cell EMT, migration and invasion capacity *in vitro* were performed. Firstly, CNE-1 and SUNE-1 cells with miR-BART13 overexpression changed their morphology characterized by relative loss of cell-to-cell adhesions but gain of spindle-like phenotype, whereas miR-BART13 knockdown in C666-1 cells led to partial loss of the EMT-related cell morphology (Fig. 2A). In addition, the migration and invasion capacity of CNE-1 and SUNE-1 cells after miR-BART13 overexpression was increased dramatically (Fig. 2B, C and D), while knockdown of miR-BART13 using microRNA sponges greatly decreased the migration and invasion capacity in C666-1 cells (Fig. 2B, C and D). Finally, miR-BART13 overexpression decreased expression of epithelial marker (E-cadherin) but increased expression of mesenchymal markers (Vimentin, N-cadherin, and ZEB1) in CNE-1 and SUNE-1 cells (Fig. 2E). In contrast, miR-BART13 knowdown in C666-1 cells reversed expression of EMT-related proteins by Western blot (Fig. 2E).

3.3. miR-BART13 promotes NPC cell growth and metastasis in vivo

To further investigate the effects of miR-BART13 on NPC cell growth and metastasis *in vivo*, xenograft growth assays and lung metastasis via tail vein in nude mice were performed. Firstly, it was found that miR-BART13 overexpression in SUNE-1 cells faciliated tumor cell xenograft formation and growth *in vivo* (Fig. 3A). In addition, lung metastasis via tail vein *in vivo* demonstrated that miR-BART13 overexpression in SUNE-1 cells could promote the lung metastases in nude mice. It was shown that the bioluminesence value of lung nodules was significantly higher in the miR-BART13 overexpression nude mice than in the control nude mice (Fig. 3B), while the average size of macroscopic lung nodules was also larger in the miR-BART13 overexpression nude mice (Fig. 3C). The H&E stained lung tissues showed that the number and size of NPC cell lung metastasis were significantly larger in the miR-BART13 overexpression nude mice as well (Fig. 3D).

3.4. NKIRAS2 is inversely correlated with miR-BART13 in NPC specimens

To explore the underlying molecular mechanisms by which miR-BART13 promotes NPC malignant behaviors, the publicly available database (NPInter, Datebase URL: https://www.bioinfo.org/NPInter/keyword.htm) and the iTRAQ detection were employed to predict miR-BART13 target genes. Based on the NPInter datebase, 38 potential target genes of miR-BART13 was found. By the iTRAQ analysis, 250 proteins were upregulated by more than 1.3-fold in C666-1 cells infected with lenti-miR-BART13 sponge compared with those infected with lenti-vector sponge. Finally, 3 genes including *NKIRAS2*, *TIAL1*, and *TOPBP1* were found through combinational analysis of the NPInter database and the iTRAQ detection (Fig. 4A). We then analyzed these 3 potential target genes mRNA expression in the clinical specimens. It was found that only the inverse correlation between miR-BART13 and *NKIRAS2* was detected in NPC tissues (Fig. 4B and C). Therefore, we chose *NKIRAS2* for further work. It was found that *NKIRAS2* mRNA expression was markedly lower in NPC than in NP tissues (Fig. 4C, $P < 0.001$). Furthermore, *NKIRAS2* protein expression was heterogeneous with low, medium, and high expression in paraffin-embedded NPC specimens by immunohistochemistry (IHC) examination (Fig. 4D). Finally, the correlation analysis showed that miR-BART13 was also inversely associated with *NKIRAS2* protein expression (Fig. 4E, $r < -0.6$, $P < 0.01$).

3.5. NKIRAS2 is a direct target of miR-BART13 in NPC cells

Since NIKRAS2, nuclear factor kappa B (NF-κB) inhibitor interacting Ras-like 2, is a negative regulator of the NF-κB pathway and aberrant expression or activity of the NF-κB pathway altered inflammation response and NPC development [32–34], and is inversely correlated with miR-BART13 in NPC specimens in our current study, a series of assays were performed to identify whether it is a direct target of miR-BART13 in NPC. Firstly, the luciferase reporter assay was performed to assess

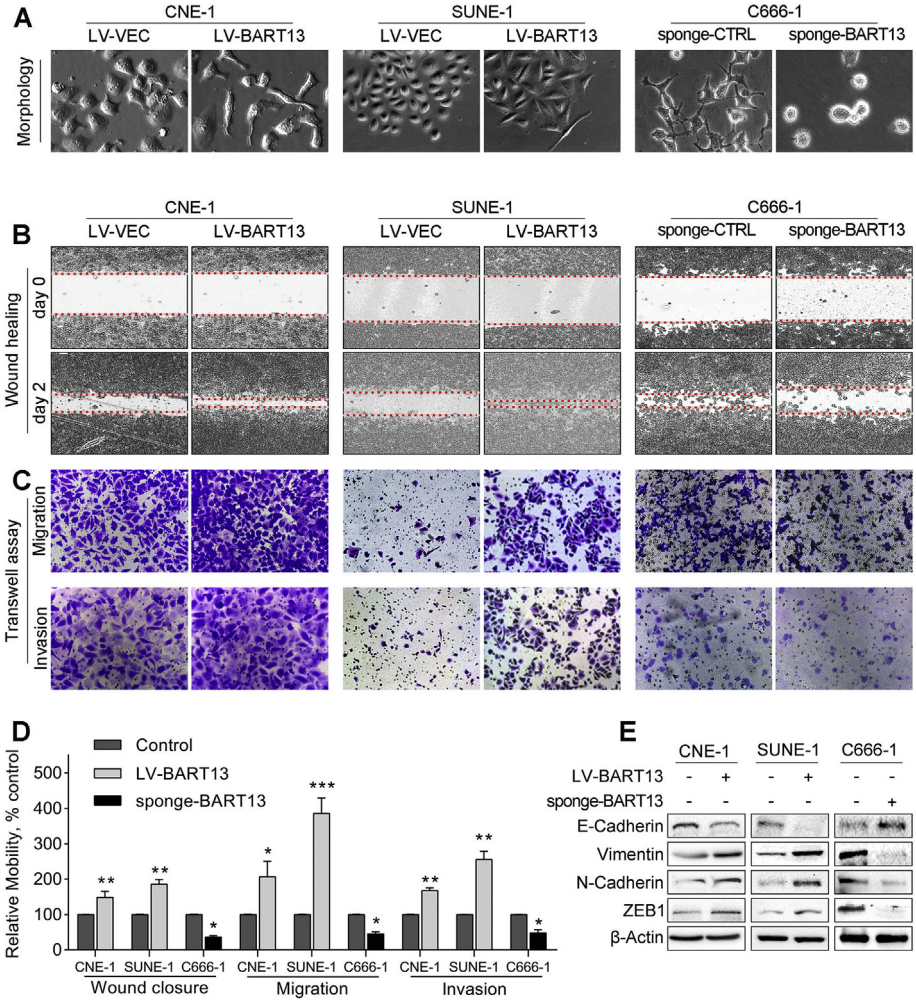

Fig. 2. EBV-miR-BART13 promotes NPC cell EMT, migration, and invasion *in vitro*. A-D. miR-BART13 was overexpressed or knocked down in the indicated cell lines. A. Phase-contrast microscopy was used to observe the morphology of the indicated cells. Magnification, × 100. B. The wound healing assay was used to detect cell migration. C. The Transwell migration and invasion assay were used to detect cell migration and invasion. D. The relative mobility of the indicate cells was measured by the wound healing and Transwell assays. Data are presented as mean ± SD; $*P < 0.05$; $**P < 0.01$; $**P < 0.001$. E. EMT markers were measured by Western blot.

whether *NKIRAS2* can be negatively regulated by miR-BART13 (Fig. 5A). After co-transfection with lv-miR-BART13 or lv-miR-BART13 sponge, the luciferase activity of wide-type 3'UTR reporter gene decreased or increased significantly, whereas that of the mutant reporter gene was not influenced (Fig. 5B). Finally, RT-qPCR and Western blot verification also demonstrated that *NKIRAS2* mRNA and protein expression could be negatively regulated by miR-BART13 in the indicated NPC cells (Fig. 5C).

3.6. miR-BART13 regulates activity of the NF-κB signaling pathway in NPC cells

Next, the effect of miR-BART13 on *NKIRAS2*-regulated NF-κB signaling pathway in NPC cells was investigated. As shown in Fig. 5D, after overexpression of miR-BART13 in CNE-1 or SUNE-1 cells, expression of IκBβ protein was downregulated, but expression of p-NF-κB (p65), nuclear NF-κB (p65) was all upregulated compared with the control cells. In contrast, C666-1 cells after miR-BART13 knockdown had inverse data on expression of these proteins. These data indicated that miR-BART13 could activate the NF-κB signaling pathway by inhibition of IκBβ expression, phosphorylation of NF-κB (p65), and thus promotion of NF-κB (p65) nuclear localization.

3.7. NKIRAS2 mediates miR-BART13-induced NPC cell proliferation, EMT, migration, invasion, and NF-κB signaling

Since *NKIRAS2* is a negative regulator of the NF-κB pathway, we assessed whether *NKIRAS2* mediated miR-BART13-induced NPC cell proliferation, EMT, and metastasis through activation of the NF-κB signaling. Firstly, the SUNE-1 cells stably overexpressing miR-BART13 were infected with *NKIRAS2* without its 3'UTR (lenti-*NKIRAS2*) or negative control (lenti-vector). It was found that the ectopic *NKIRAS2* expression greatly reduced tumor cell viability (Fig. 6A), colony formation (Fig. 6B) and tumor cell proliferation-associated proteins (Fig. 6C) compared with the control cells. Furthermore, *NKIRAS2* overexpression changed the morphology of miR-BART13-overexpressing SUNE-1 cells, including relative gain of cell-to-cell adhesions but loss of spindle-like cell phenotype (Fig. 6D), in addition to reduced the migration and invasion capacity (Fig. 6E, F and G). Finally, *NKIRAS2* overexpression also upregulated expression of epithelial marker (E-cadherin) and downregulated expression of mesenchymal markers (Vimentin, N-cadherin, and ZEB1) (Fig. 6H). These findings indicated that *NKIRAS2* was the functional target of miR-BART13 and that *NKIRAS2* overexpression significantly reversed miR-BART13-induced cell growth and metastasis *in vitro*.

Thereafter, the effects of *NKIRAS2* on activity of the NF-κB signaling

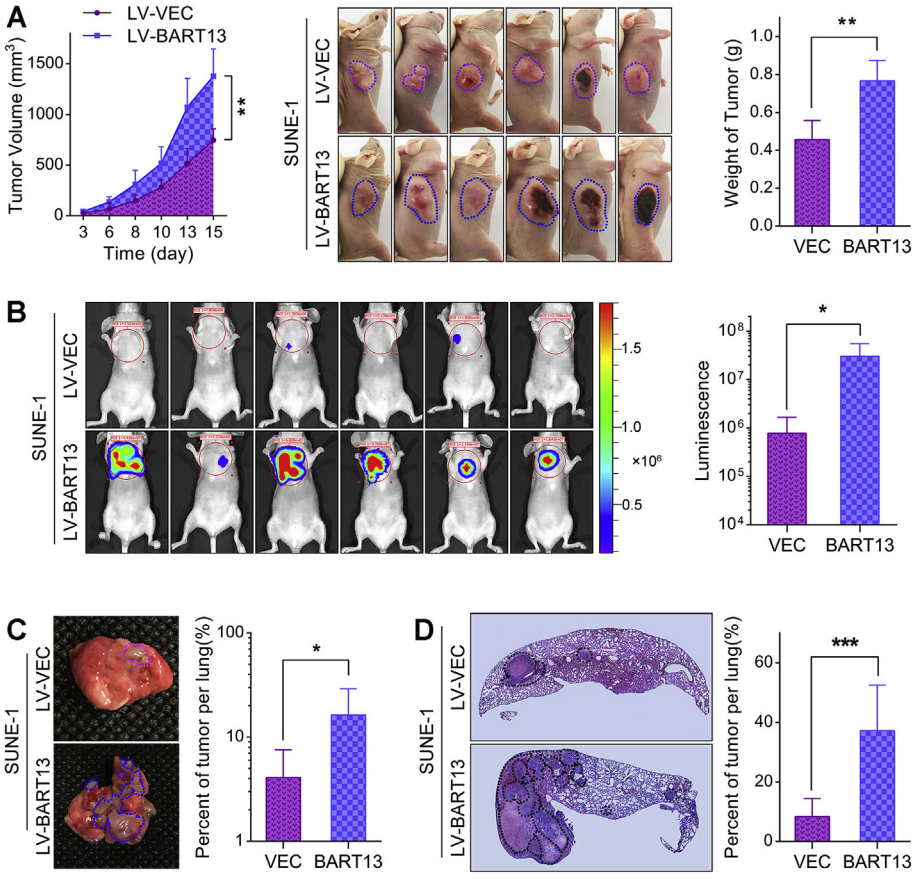

Fig. 3. EBV-miR-BART13 promotes NPC cell growth and metastasis *in vivo*. A. Effects of miR-BART13 overexpression in SUNE-1 cells on xenograft growth in nude mice. It showed the nude mouse tumor growth curves (left), tumor xenograft assay (middle), and tumor weight (right) ($n = 6$). B-D. Effects of miR-BART13 overexpression in SUNE-1 cells on lung metastasis from tail vein in nude mice. B. Bioluminescence imaging was performed 5 weeks after injection. ($n = 6$) C. Representative images of macroscopic lung metastatic nodules (left) and quantification of the percentage of metastatic nodules on the surface of the lungs (right). The purple and blue quoits indicate the metastatic nodules. D. Representative images of lung cross sections stained with H&E (left) and pathological quantitation of lung metastatic nodules (right). Data are summarized as mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

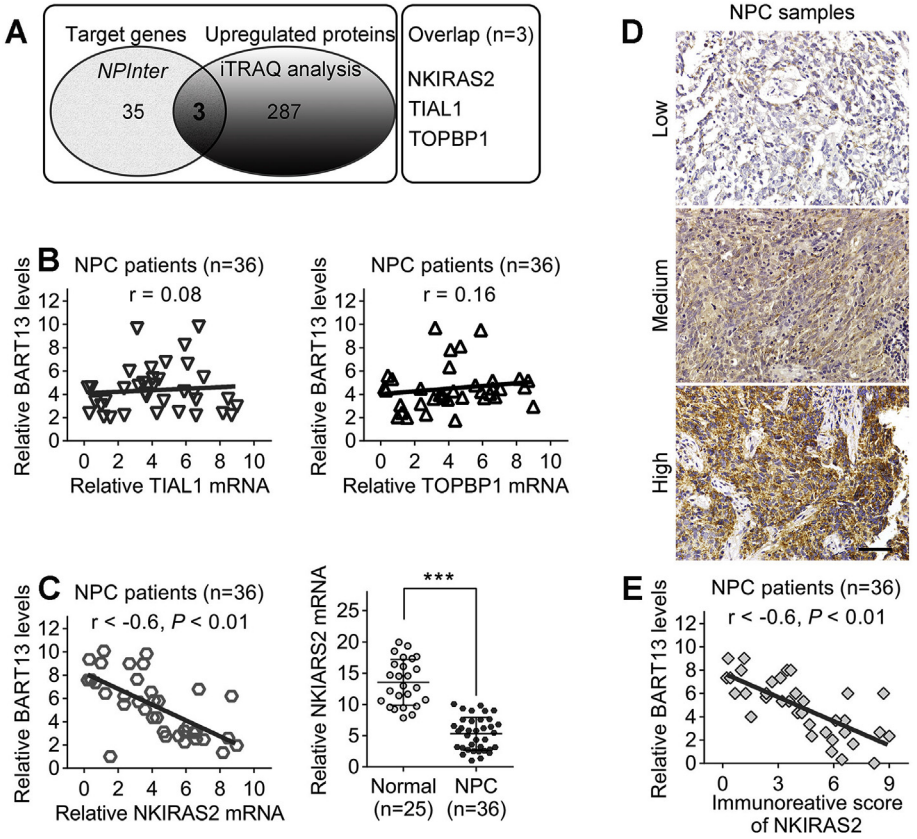

Fig. 4. *NKIRAS2* is inversely correlated with EBV-miR-BART13 in NPC specimen. A. The screening method for identifying the potential target genes of miR-BART13.38 target genes were found in the NPInter database, while 250 proteins were up-regulated by more than 1.3-fold after miR-BART13 was knocked down in C666-1 cells. Through combinational analysis, 3 potential target genes including *NKIRAS2*, *TIAL1*, and *TOPBP1* were found. B. *TIAL1* and *TOPBP1* mRNA expression was not correlated with miR-BART13 in 36 freshly frozen NPC specimens, respectively ($r = 0.08$ and $r = 0.16$). C. *NKIRAS2* mRNA expression was inversely correlated with miR-BART13 expression in NPC tissues (left, $r < -0.6$, $P < 0.01$), and markedly lower in NPC than in normal tissues (right, $P < 0.001$). D. *NKIRAS2* protein expression was detected by immunohistochemistry (IHC) examination in paraffin-embedded NPC specimens. The IHC staining score 0–3 indicated low, the score 4–7 indicated medium, and the score 8–12 indicated high expression. E. *NKIRAS2* protein expression was inversely correlated with miR-BART13 expression in NPC tissues ($r < -0.6$, $P < 0.01$).

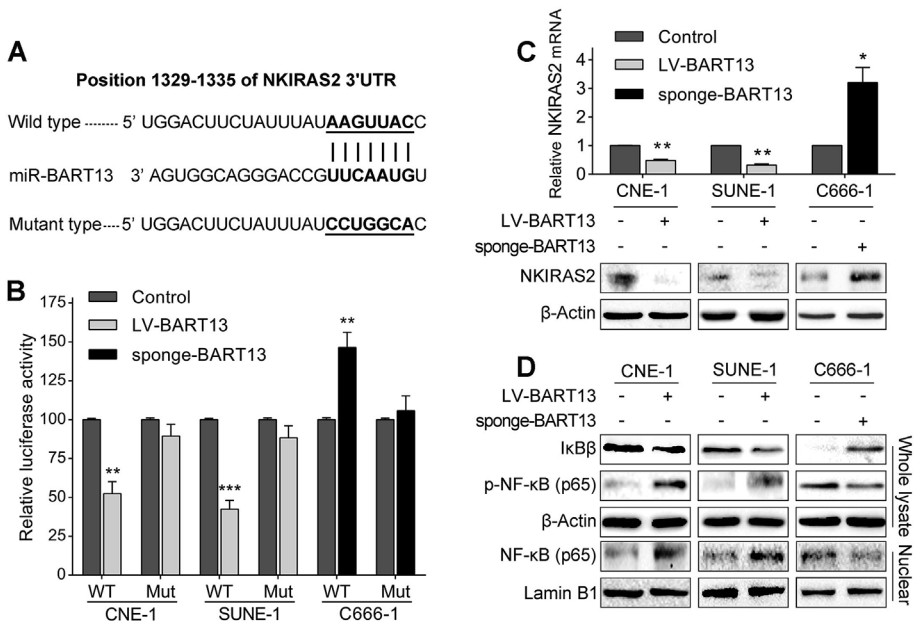

Fig. 5. EBV-miR-BART13 directly targets *NKIRAS2* expression and activates the NF-κB signaling pathway in NPC cells. A. Sequence alignment of wild-type and mutant *NKIRAS2* 3′-UTR with miR-BART13. B. Relative luciferase activity of NPC cells after co-transfection with the wild-type (WT) or mutant (Mut) *NKIRAS2* 3'UTR reporter genes and miR-BART13 overexpression/knockdown or control. C. Quantification of *NKIRAS2* mRNA by RT-qPCR (upper) or *NKIRAS2* protein expression by Western blot (lower) in the indicated cells. D. Western blot analysis of the NF-κB signaling pathway-associated proteins activated by miR-BART13 in NPC cells. Values are mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

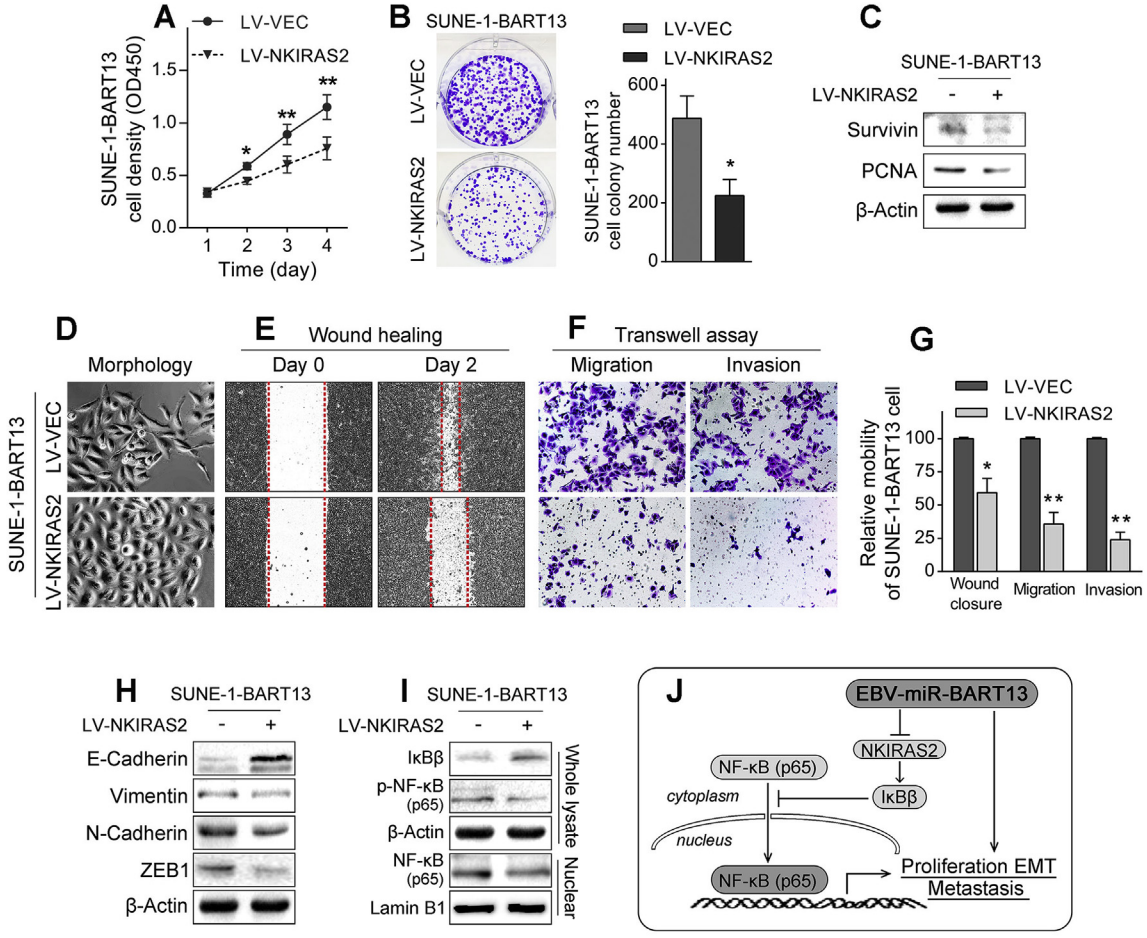

Fig. 6. *NKIRAS2* is involved in miR-BART13-regulated NPC cell growth, EMT, migration, invasion, and NF-κB signaling pathway activity. A. CCK-8 assay. Stably miR-BART13 overexpressing SUNE-1 cells were grown and infected with *NKIRAS2* (lenti-*NKIRAS2*) or negative control (lenti-vector) and then subjected to cell viability assay. B. Colony formation assay. The duplicated cells were subjected to colony formation assay. C. NPC cell proliferation-associated proteins were measured by Western blot. D. Phase-contrast microscopy images. E. Wound healing assay. F. Transwell migration and invasion assay. G. The relative mobility of the indicate cells was measured by the wound healing and Transwell assays. H. EMT markers was measured by Western blot. I. NF-κB signaling pathway-associated proteins were measured by Western blot. J. Illustration of the miR-BART13-mediated pathway in NPC cell proliferation, EMT, and metastasis. Values are mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$.

activated by miR-BART13 were assessed as well. As shown in Fig. 5I, *NKIRAS2* overexpression upregulated expression of IκBβ proteins, but downregulated the levels of phosphorylation of NF-κB (p65), nuclear NF-κB (p65) proteins in SUNE-1-miR-BARTA13 cells after *NKIRAS2* overexpression. Taken together, miR-BART13 was able to inhibit *NKIRAS2* expression to suppress expression of IκBβ proteins, but increase levels of phosphorylated NF-κB (p65) and nuclear NF-κB(p65) proteins, and in turn to activate NF-κB (p65) transcriptional activity to induce NPC cell proliferation, EMT, migration, and invasion (Fig. 6J).

4. Discussion

In the present study, the role of EBV-coded miR-BART13 in NPC initiation and progression was explored and then the underlying molecular events in NPC cells was investigated. It was found that miR-BART13 expression was upregulated in NPC tissues. miR-BART13 expression was shown to promote NPC cell proliferation, EMT, and metastasis *in vitro* and facilitated tumor cell xenograft growth and metastasis *in vivo*. We also provided evidence that miR-BART13-promoted tumor growth and metastasis were through directly targeting of a putative tumor suppressor *NKIRAS2* to subsequently activate the NF-κB signaling pathway. Thus, our current study represents the first to reveal the oncogenic activity of miR-BART13 in NPC initiation and progression, and further study will assess whether targeting of miR-BART13 as a strategy in control of NPC.

The EBV is confirmed to be an oncogenic virus in NPC in addition to Burkitt's lymphoma, Hodgkin's lymphoma, nasal NK/T lymphoma, and gastric cancer [35,36]. The EBV is one of the most common viruses to infect most world populations, indicating that the EBV induction of human cancer is a very rare event; thus, a variety of other influential factors could enhance the EBV carcinogenesis activity in humans, like genetic susceptibility, gene mutations, and/or EBV-activated NF-κB signaling [37–39]. In the current study, we focused on BART miRNAs, one cluster of EBV-encoded miRNAs, have recently emerged their importance in regulation of numerous cell biological functions because these miRNAs were highly expressed in EBV-associated malignancies [40,41]. To date, a total of 44 BART miRNAs have been identified and their aberrant expression in NPC suggests their role in NPC development and progression [15]. Our previous study revealed high miR-BART13 expression in EBV-positive NPC cell lines and in the extracellular supernatants [28]. In NPC patients, plasma miR-BART13 level was markedly increased and associated with lymph node metastasis and advanced clinical stage [28]. Our current study further confirmed this notion and further showed that oncogenic activity of miR-BART13 in NPC. It was found that the upregulation of miR-BART13 expression promoted NPC cell proliferation, EMT and metastasis *in vitro* and xenograft tumor growth and lung metastasis *in vivo*. In line with our current observations of miR-BART13 in NPC, previous studies also showed other EBV-encoded miRNAs, such as miR-BART9 and miR-BART7, were reported to induce NPC growth and metastasis [24,26,42]. Furthermore, a recent study demonstrated that ectopic miR-BART6-3p expression could reverse EMT phenotype, and suppress tumor cell migration and invasion of NPC [43]. These data together further indicated that aberrant miRNA expression, including miR-BART13, did have the ability to promote NPC carcinogenesis and progression.

BARTs miRNA exert their functions through binding to 3′-UTR of their host target genes [15]. In the current study, the combinational analysis of the NPInter database and the iTRAQ detection was used to identify miR-BART13 potential targeting genes. Finally, 3 potential targeting genes including *NKIRAS2*, *TIAL1* and *TOPBP1* were identified. However, only the mRNA expression of *NKIRAS2*, instead of *TIAL1* and *TOPBP1*, had an inversely associated with miR-BART13. Furthermore, *NKIRAS2* mRNA was markedly lower in NPC than in normal tissues, and *NKIRAS2* protein expression was inversely correlated with miR-BART13. Therefore, *NKIRAS2* was possibly a target gene of miR-BART13. Previous study has shown that *NKIRAS2* was expressed in different normal tissues but lost in human cancers [44]. Our current study also found that *NKIRAS2* was partially lost in NPC, which may due to the EBV infection that could induce the activity of the NF-κB signaling in NPC [36,45,46]. In addition, *NKIRAS2* has been repeatedly reported to interfere with proteosomal degradation of inhibitor of NF-κB β (IκB-β) [32,47,48] and then negatively regulate activity of the NK-κB signaling pathway. In contrast, inhibition of *NKIRAS2* by another miRNA, miR-125b, also showed to induce the activity of the NF-κB pathway and antagonized Temozolomide anti-glioma effects *in vitro* [33]. We thus, focused on *NKIRAS2* in our current study and our current study confirmed *NKIRAS2* as the direct target gene of miR-BART13 in NPC cells with different experimental settings. Furthermore, ectopic *NKIRAS2* expression abrogated the stimulatory effects of miR-BART13 on NPC cells using the rescue experiments, including NPC proliferation, EMT, migration, and invasion, indicating that *NKIRAS2* could be a putative tumor suppressor or possesses an anti-NPC activity (Fig. 6). Thus, further study is needed to delineate NPC oncogenesis by focusing on EBV miR-BART13 targeting of *NKIRAS2*.

The NF-κB signaling is constitutively activated in NPC and regulates cell proliferation, apoptosis, and invasion [45,46]. Our current study identified that miR-BART13 regulated activity of the NF-κB signaling pathway in NPC cells. The NF-κB contains two subunits, p50/p65, to interact with the inhibitors of NF-κB (such as IκBα, IκBβ, and IκBε), leading to its translocation from the cytoplasm in inactive state to the cell nuclei as an active state of the protein [49,50]. In this study, it was observed that ectopic miR-BART13 expression in NPC cells downregulated levels of IκBβ proteins, whereas it increased expression of p-NF-κB(p65) and nuclear NF-κB(p65) proteins to therefore, activate the NF-κB signaling in NPC cells. In contrast, knockdown of miR-BART13 expression in C666-1 cells inhibited constitutive activity of the NF-κB signaling pathway. These results suggested that NF-κB signaling pathway could be activated by miR-BART13 in NPC. However, ectopic *NKIRAS2* expression attenuated the activation effects of miR-BART13 on the NF-κB signaling pathway in NPC cells in our rescue experiments (Fig. 6J). Therefore, we may conclude that miR-BART13 activated NF-κB signaling in NPC was through directly targeting *NKIRAS2*, to promote NPC cell proliferation, EMT, migration, and invasion *in vitro* and NPC cell xenograft growth and lung metastasis in nude mice.

In conclusion, our findings demonstrated that miR-BART13 expression was consistently upregulated and had an inverse association with *NKIRAS2* expression in NPC tissues. In addition, miR-BART13 can promote tumor growth and metastasis in NPC *in vitro* and *in vivo* by targeting *NKIRAS2*/NF-κB signaling pathway. Thus, targeting of this newly identified miR-BART13/*NKIRAS2*/NF-κB signaling axis may provide evidence for developing a targeting therapeutic strategy for NPC patients.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.01.022.

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