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miR-9 modulates the expression of interferon-regulated genes and MHC class I molecules in human nasopharyngeal carcinoma cells

Fei Gao [a], Zun-Lan Zhao [a], Wen-Tao Zhao [a], Quan-Rong Fan [a], Sheng-Chun Wang [a], Jing Li [a], Yu-Qing Zhang [a], Jun-Wen Shi [a], Xiao-Lin Lin [a], Sheng Yang [a], Rao-Ying Xie [a], Wei Liu [b], Ting-Ting Zhang [a], Yong-Liang Sun [a], Kang Xu [c,*], Kai-Tai Yao [a,*], Dong Xiao [a,b,*]

[a] Cancer Research Institute, Southern Medical University, Guangzhou 510515, PR China
[b] Institute of Comparative Medicine & Laboratory Animal Center, Southern Medical University, Guangzhou 510515, PR China
[c] Department of General Surgery, Sun Yat-sen Memorial Hospital of Sun Yat-sen University, Guangzhou 510120, PR China

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ABSTRACT

The functions of miR-9 in some cancers are recently implicated in regulating proliferation, epithelial–mesenchymal transition (EMT), invasion and metastasis, apoptosis, and tumor angiogenesis, etc. miR-9 is commonly down-regulated in nasopharyngeal carcinoma (NPC), but the exact roles of miR-9 dysregulation in the pathogenesis of NPC remains unclear. Therefore, we firstly used miR-9-expressing CNE2 cells to determine the effects of miR-9 overexpression on global gene expression profile by microarray analysis. Microarray-based gene expression data unexpectedly demonstrated a significant number of up- or down-regulated immune- and inflammation-related genes, including many well-known interferon (IFN)-induced genes (e.g., IFI44L, PSMB8, IRF5, PSMB10, IFI27, PSB9_HUMAN, IFIT2, TRAIL, IFIT1, PSB8_HUMAN, IRF1, B2M and GBP1), major histocompatibility complex (MHC) class I molecules (e.g., HLA-B, HLA-C, HLA-F and HLA-H) and interleukin (IL)-related genes (e.g., IL20RB, GALT, IL7, IL1B, IL11, IL1F8, IL1A, IL6 and IL7R), which was confirmed by qRT-PCR. Moreover, the overexpression of miR-9 with the miRNA mimics significantly up- or down-regulated the expression of above-mentioned IFN-inducible genes, MHC class I molecules and IL-related genes; on the contrary, miR-9 inhibition by anti-miR-9 inhibitor in CNE2 and 5–8F cells correspondingly decreased or increased the aforementioned immune- and inflammation-related genes. Taken together, these findings demonstrate, for the first time, that miR-9 can modulate the expression of IFN-induced genes and MHC class I molecules in human cancer cells, suggesting a novel role of miR-9 in linking inflammation and cancer, which remains to be fully characterized.

1. Introduction

Some microRNAs (miRNAs) which have been experimentally validated to function as oncomirs or tumor suppressor miRNAs are known to be involved in multiple aspects of cancer initiation and progression, including the regulation of cell growth, survival, angiogenesis and metastasis, etc. [1–3]. miR-9, initially demonstrated to function in neurogenesis, has been confirmed to be implicated in cancer [4]. miR-9 is under-expressed in many types of cancers, including breast cancer [5,6], colon cancer [7] and melanoma [8], all of which is indicative of a tumor suppressor potential, whereas miR-9 is over-expressed in brain cancer [9,10] and Hodgkin's lymphoma [11], suggesting oncomir activity for miR-9 in these two cancers. Some evidence indicates that the functions of miR-9 in cancer cells are recently implicated in regulating proliferation [6,8,9,11], epithelial–mesenchymal transition (EMT), invasion and metastasis [8,12,13], apoptosis [6], and tumor angiogenesis [12,13], etc. miR-9 is commonly down-regulated in nasopharyngeal carcinoma (NPC) [14], however, the contribution of miR-9 to the pathogenesis of NPC remains to be characterized.

Therefore, we firstly used miR-9-expressing CNE2 cells to determine the effects of miR-9 overexpression on global gene expression profile by microarray analysis. Microarray-based gene expression data unexpectedly demonstrated a significant number of up- or down-regulated immune- and inflammation-related genes, including many well-known interferon (IFN)-induced genes, such as IFI44L, PSMB8, IRF5, PSMB10, IFI27, IFIT2, TRAIL, IFIT1 and IRF1, indicating the strong relationship between miR-9 and inflammation in cancer. Furthermore, miR-9 enhances interleukin (IL)-2 and IFN-γ production in activated human CD4(+) T cells by repressing Blimp-1 [15]. Our findings and recently published reports strongly suggest a novel role of miR-9 in linking inflammation and cancer, which remains to be fully characterized.

* Corresponding authors. Address: Cancer Research Institute, Southern Medical University, Guangzhou 510515, China. Fax: +86 20 61648225 (D. Xiao, K.-T. Yao).
E-mail addresses: xukang1995@yahoo.com (K. Xu), Yaokaitai@yahoo.com.cn (K.-T. Yao), Xiao_d@hotmail.com (D. Xiao).

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These above-mentioned findings and information encouraged us to fully investigate whether miR-9 was really implicated in regulating the expression of IFN-related genes, major histocompatibility complex (MHC) class I molecules and IL-related genes in NPC cells.

2. Materials and methods

2.1. Cell line and cell culture

Human NPC cell lines (i.e., CNE2 and 5–8F) were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) in a humidified incubator with 5% CO_2 at 37 °C.

2.2. Establishment of miR-9-expressing CNE2 cells

Lentiviral expression clones for miR-9 (pEZX-MR03-miR-9; Catalog No.: HmiR0017-MR03) or a control miRNA (pEZX-MR03; Catalog No.: CmiR0001-MR03) were purchased from Genecopoeia (Guangzhou, China), while the lentiviral packaging plasmids (psPAX2 and pMD2.G) were kindly provided by Dr. Didier Trono (University of Geneva, Geneva, Switzerland). To produce viruses expressing empty vector or miR-9, pEZX-MR03 or pEZX-MR03-miR-9 along with packaging plasmids psPAX2 and pMD2.G were transfected into HEK293T cells (maintained in 10% FBS) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction. The packaged lentiviruses expressing empty vector and miR-9 were named LV-con and LV-miR-9, respectively. 48 h after transfection, virus supernatant was harvested, and then used to infect CNE2 cells, followed by 2 weeks exposure to 2 μg/ml puromycin (GIBCO) to kill non-infected cells. The successful overexpression of miR-9 was verified by quantitative Real-Time PCR (qRT-PCR).

2.3. miRNA transient transfection

The human miR-9 mimics, a nonspecific miRNA control (i.e., mimics control), anti-miR-9 inhibitor and a nonspecific anti-miR inhibitor control were all purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). MiRNAs were transiently transfected into cells at a working concentration of 100 nmol/L using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's procedure. Cells for total RNA isolation were usually harvested 48 h after oligonucleotide treatment.

2.4. RNA isolation, reverse transcription and qRT-PCR

For miRNA and mRNA analyses, total RNA from NPC cells was extracted with Trizol Reagent (TaKaRa) according to the protocol provided by the manufacturer. Total RNA was reversely transcribed with the PrimeScript RT reagent Kit (TaKaRa). The expression levels of mature miRNA were determined by SYBR Green quantitative PCR amplifications performed on the Stratagene Mx3000P Real-Time PCR system (Agilent Technologies, Inc., USA). U6 was used for normalization. Expression of mRNA analysis was performed using SYBR Green Master Mix (TaKaRa) as described, GAPDH was used for normalization. The primers used for the amplification of the indicated genes were listed in Table S1–S4. All samples were normalized to internal controls and fold changes were calculated through relative quantification ($2^{-\Delta\Delta Ct}$).

2.5. mRNA microarray analysis

Expression microarray analysis was carried out with commercially available Affymetrix Human Gene U133 Plus 2.0 array according to the Affymetrix standard protocol, which carries 47,000 transcripts representing 38,500 well-characterized human genes. All the hybridization procedures and data analysis were performed by Capital Bio Corp. (Bejing, China). Total RNA samples were isolated from NPC cells (CNE2 cells) using Trizol reagent (Invitrogen). Briefly, total RNA was used to synthesize cDNA in an in vitro transcription reaction, and then cDNA was fluorescently labeled by Cy5 or Cy3-CPT with Klenow enzyme. Labeled cDNA was then hybridized to Affymetrix Human Gene U133 Plus 2.0 arrays. Hybridization was processed at 45 °C, with rotation for 16 h (Affymetrix GeneChip Hybridization Oven 640). Chips were then washed and stained in the Affymetrix Fluidics Station 450. Hybridization signals were scanned with a Lux-Scan3.0 scanner (Capital Bio. Corp., Beijing, China). The resultant images were digitized with Genepix Pro 6.0 software (Axon Instruments, Foster City, CA, USA).

2.6. Statistical analysis

Data were presented as mean ± SEM unless otherwise indicated of at least three independent experiments. Statistical analysis was performed using a SPSS 13.0 software package. Statistical significance was assessed by the Student's *t*-test ($^{*}p < 0.5$; $^{\#}p < 0.01$).

3. Results

3.1. Over-expression of miR-9 in human NPC cells

In order to study the roles of miR-9 dysregulation in the pathogenesis of NPC, two methods were employed to over-express miR-9 in human NPC cells. First, miR-9-expressing CNE2 cells were generated as described in Materials and methods. Level of miR-9 transgene in infected CNE2 cells was evaluated by qRT-PCR. As shown in Fig. 1B, miR-9 level in CNE2 cells harboring miR-9 transgene was significantly higher than that in CNE2 cells harboring empty vector. Next, miR-9 mimics and control RNA (cnt RNA, mimics control) were transiently transfected into CNE2 and 5–8F cells. As shown in Fig. 1C, the level of miR-9 in CNE2 and 5–8F cells transfected with miR-9 mimics was much higher than that in CNE2 and 5–8F cells transfected with control RNA.

Moreover, the level of miR-9 in CNE2 and 5–8F cells transfected with anti-miR-9 inhibitor was much lower than that in CNE2 and 5–8F cells transfected with negative control inhibitor, indicating that anti-miR-9 inhibitor efficiently down-regulated the endogenous miR-9 expression in CNE2 and 5–8F cells.

3.2. Global analysis of gene expression differences in miR-9-expressing CNE2 cells

miR-9 is known to regulate RNA expression levels of multiple genes and we reasoned that the overexpression experiments should modify mRNA expression patterns in the cells. Therefore, we used miR-9-expressing CNE2 cells mentioned above to determine the effects of miR-9 overexpression on global gene expression profile by microarray analysis as described in the methods. Our results demonstrated that 208 genes were significantly down-regulated, and 245 genes were significantly up-regulated in LV-miR-9-infected CNE2 cells compared with control (data not shown). Unexpectedly, among 453 significantly changed genes, we found a significant number of differentially expressed genes (e.g., IFN-inducible genes, MHC class I molecules and IL-related genes) involved in regulating immune and inflammatory responses (Figs. 2A, 3A, 4A, and Table S5). Moreover, some immune- and inflammation-related genes exhibiting a fold change of more than 1.5 and less than two were also shown in Figs. 2A, 3A, 4A, and Table S5. Detailed information, such as gene names, gene functions

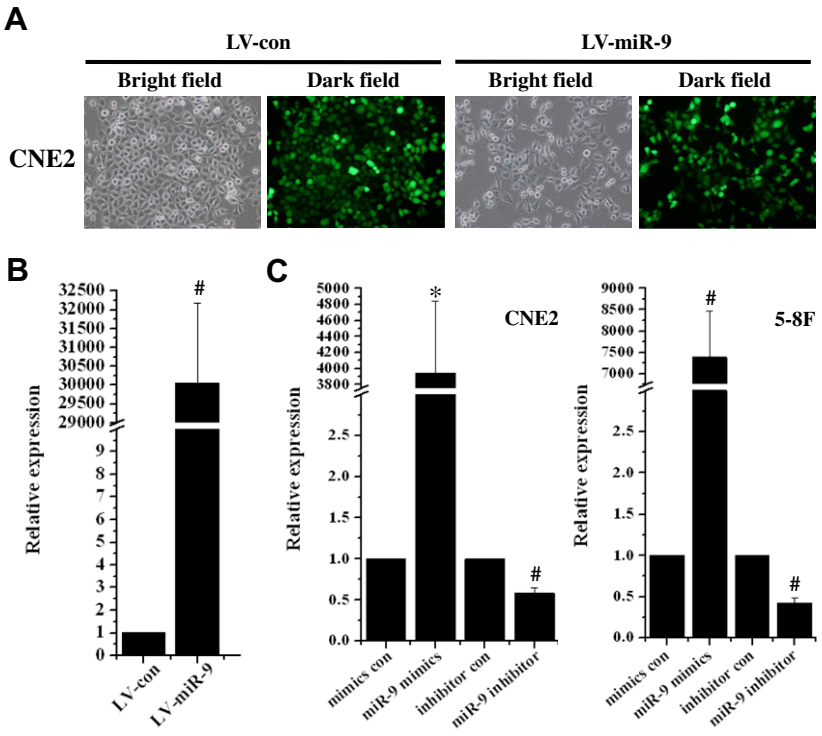

Fig. 1. miR-9 overexpression and down-regulation of endogenous miR-9 expression in human nasopharyngeal carcinoma (NPC) cells. (A–B) Generation of stable NPC cell line expressing EGFP and miR-9 transgenes. A: EGFP assay under inverted fluorescence microscopy; B: the detection of miR-9 expression in CNE2 cells carrying miR-9 transgene by qRT-PCR. (C) qRT-PCR-based analysis of the expression levels of miR-9 after CNE2 and 5–8F cells were transfected with miR-9 mimics (100 nM) or anti-miR-9 inhibitor (100 nM) for 48 h.

and fold changes, is summarized in Table S5. The up- and down-regulated genes derived from microarray data were submitted to DAVID online tool (http://david.abcc.ncifcrf.gov/home.jsp) for gene ontology (GO) annotation. Table 1 lists the GO terms representing biological process related with immune and inflammatory responses. Array results were validated by qRT-PCR for selected transcripts (Figs. 2B, 3B and 4B).

3.3. miR-9 altered IFN-regulated gene expression in NPC cells

As mentioned above, microarray analysis of miR-9-expressing CNE2 cells showed the induction of many IFN-regulated target genes (e.g., IFI44L, PSMB8, IRF5, PSMB10, IFI27, PSB9_HUMAN, IFIT2, TRAIL, IFIT1, PSB8_HUMAN, IRF1 and B2M) (Fig. 2A and Table S5), which was confirmed by qRT-PCR analyses (Fig. 2B).

To fully explore the effects of miR-9 on IFN-regulated target genes, CNE2 and 5–8F cells were transiently transfected with miR-9 mimics and anti-miR-9 inhibitor, respectively. As shown in Fig. 2C and D, the results of qRT-PCR demonstrated that miR-9 mimics significantly up-regulated the expression of some IFN-regulated genes (i.e., IFI44L, PSMB8, IRF5, PSMB10, IFI27, IFIT2, TRAIL, IFIT1, IRF1, B2M and GBP1) in CNE2 and 5–8F cells ($P < 0.05$ or 0.01), and down-regulated the expression of some IFN-regulated genes (i.e., ISG20 and AIM2) in CNE2 or 5–8F cells ($P < 0.05$ or 0.01), respectively, while anti-miR-9 inhibitor correspondingly decreased the expression of miR-9 mimics-upregulated IFN-related genes (i.e., IFI44L, PSMB8, IRF5, PSMB10, IFI27, IFIT2, TRAIL, IFIT1, IRF1, B2M and GBP1) in CNE2 and 5–8F cells ($P < 0.05$ or 0.01), and increased the expression of miR-9 mimics-downregulated gene (i.e., ISG20 and AIM2) in CNE2 or 5–8F cells ($P < 0.01$), separately. Collectively, the most significant alteration after miR-9 overexpression in NPC cells was the increased expression of genes involved in IFN induction, including IFI44L, PSMB8, IRF5, PSMB10, IFI27, IFIT2, TRAIL, IFIT1, IRF1, B2M and GBP1.

3.4. Induction of the expression of MHC class I molecules by miR-9 in NPC cells

As shown in Fig. 3A and Table S5, microarray analysis of miR-9-expressing CNE2 cells showed the up-regulated expression of MHC class I molecules (HLA-B, HLA-H, HLA-C, HLA-F, Q8WW48_HUMAN, NP_001004349.1, Q6ZUW0_HUMAN and O19682_HUMAN) and TAP1 gene [encoding antigen peptide transporter 1, as ATP-binding cassette (ABC) transporter]. qRT-PCR of HLA-B, HLA-F and TAP1 confirmed the microarray changes (Fig. 3B).

Furthermore, when CNE2 and 5–8F cells were transiently transfected with miR-9 mimics, the expression of MHC Class I molecules (such as HLA-B and HLA-F) and TAP1 was significantly up-regulated, while CNE2 and 5–8F cells transfected with anti-miR-9 inhibitor indicated the decreased expression of HLA-B, HLA-F and TAP1 (Fig. 3C and D). In summary, miR-9 plays a significant role in regulating the expression of MHC Class I molecules.

3.5. miR-9 overexpression altered IL-related gene expression in NPC cells

As indicated in Fig. 4A and Table S5, the microarray data derived from miR-9-expressing CNE2 cells demonstrated the significantly altered expression in human IL-related genes (for example, IL20RB, GALT, IL7, IL1B, IL11, IL1F8, IL1A, IL6 and IL7R), which was further confirmed by qRT-PCR analyses (Fig. 4B).

To explore the effects of both miR-9 up-regulation and endogenous miR-9 down-regulation on IL-related genes, CNE2 and 5–8F cells were transiently transfected with miR-9 mimics or anti-miR-9 inhibitor, respectively. As shown in Fig. 4C, the results of qRT-PCR illustrated that miR-9 mimics significantly up-regulated the expression of some IL-related genes (i.e., IL20RB, GALT, IL7) in CNE2 cells ($P < 0.05$ or 0.01), while other IL-related genes (i.e., IL1B, IL11, IL1F8, IL1A, IL6 and IL7R) were remarkably

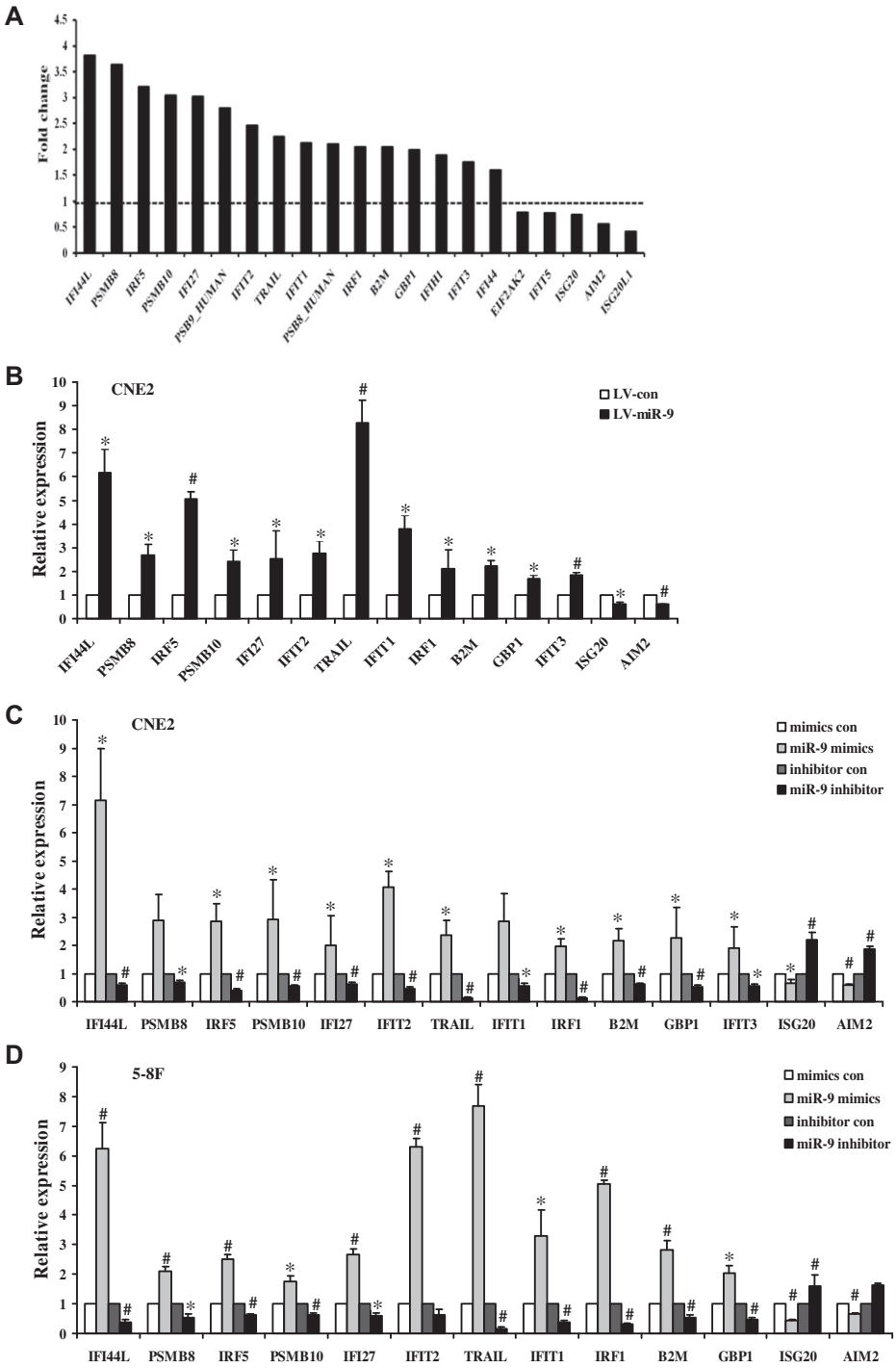

Fig. 2. miR-9 modulated IFN-related gene expression in NPC cells. (A) Graph illustrating the fold change in gene expression of representative differentially IFN-regulated genes between LV-miR-9-infected CNE2 cells to LV-con-infected CNE2 cells. miR-9-expressing CNE2 cells and control cells were analyzed by Affymetrix arrays. The horizontal dashed line marks a fold change of 1 (no change). (B) qRT-PCR confirmed the increased or decreased mRNA expression of IFN-regulated genes in LV-miR-9-infected CNE2 cells. Messenger RNA expression levels of IFI44L, PSMB8, IRF5, PSMB10, IFI27, IFIT2, TRAIL, IFIT1 and IRF1, relative to reference gene expression (GAPDH) in LV-miR-9-infected CNE2 cells were measured by qRT-PCR to confirm the results of microarray experiments (shown in Fig. 2A). (C–D) miR-9 regulated IFN-related gene expression in CNE2 and 5–8F cells. CNE2 cells and 5–8F cells were transfected with miR-9 mimics (100 nM) or anti-miR-9 inhibitor (100 nM) for 48 h, followed by evaluating IFN-inducible genes via qRT-PCR as described in Materials and methods.

downregulated by miR-9 mimics in CNE2 cells ($P < 0.05$ or 0.01). Conversely, anti-miR-9 inhibitor correspondingly decreased the expression of IL20RB, GALT and IL7 in CNE2 cells ($P < 0.05$ or 0.01), and increased the expression of IL1B, IL11, IL1F8, IL1A, IL6 and IL7R in CNE2 cells ($P < 0.05$ or 0.01), respectively. Similar results were observed in another human NPC cell line 5–8F (Fig. 4D). Collectively, the most significant alteration after miR-9 overexpression was the decreased expression of IL-related genes, including IL1B, IL11, IL1F8, IL1A, IL6 and IL7R.

4. Discussion

In the last few years, miRNAs have emerged as a class of gene expression regulators highly involved in a variety of biological

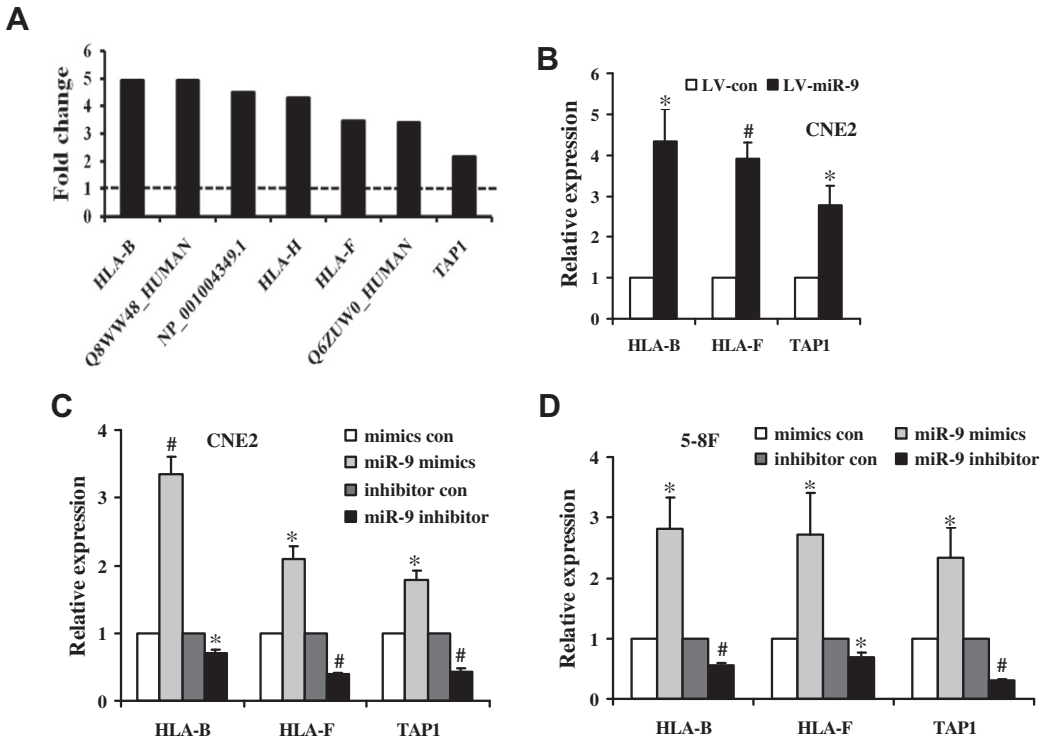

Fig. 3. Overexpression of miR-9 enhanced the expression of MHC class I molecules in NPC cells. (A) Graph illustrating the fold change in the expression of representative differentially MHC class I molecules between LV-miR-9-infected CNE2 cells to LV-con-infected CNE2 cells. For other details see legend to Fig. 2A. (B) qRT-PCR verified the increased mRNA expression of MHC class I molecules in LV-miR-9-infected CNE2 cells. Messenger RNA expression levels of HLA-B, HLA-F and TAP1, relative to reference gene expression (GAPDH) in LV-miR-9-infected CNE2 cells were measured by qRT-PCR to confirm the results of microarray experiments (shown in Fig. 3A). (C–D) miR-9 altered MHC class I molecule expression in CNE2 and 5–8F cells. CNE2 cells and 5–8F cells were transfected with miR-9 mimics (100 nM) or anti-miR-9 inhibitor (100 nM) for 48 h, followed by evaluating MHC class I molecule expression via qRT-PCR as described in Materials and methods.

and pathological processes (http://en.wikipedia.org/wiki/MicroRNA). More and more recent evidence indicates that miRNAs (e.g., miR-155, miR-146, miR-16, miR-106, let-7e and miR-19) also play crucial roles in immunity and inflammation in various physiological and pathological processes [16–22].

Recent evidence illustrates that miR-9 has been highly involved in immunity and inflammatory diseases [11,15,23,24]. miR-9 was induced in monocytes and neutrophils during the immune responses induced by LPS stimulation or the proinflammatory cytokines TNF-α and IL-1β, and one of the validated miR-9 targets is NFKB1/p50/p105, further supporting a pivotal role of this miRNA in the regulation of inflammation [23,25]. Moreover, downstream of NF-kB are the numerous genes (e.g., cytokine IL-6) that it transcriptionally induces as part of the inflammatory program [17,25]. Recent research demonstrates that the activation of CD4(+) T cells up-regulated the expression of miR-9 which ultimately resulted in an increase in the secretion of the pro-inflammatory cytokines IL-2 and IFN-γ in activated human CD4(+) T cells by repressing Blimp-1 [15], while IL-2 and IFN-γ in turn enhanced immune reactions in inflammatory disorders as Asthma bronchiale [26] or multiple sclerosis [27]. miR-9 overexpression in chondrocytes suppressed IL-1β-induced TNF-α and MMP-13 secretion, indicating that miR-9 may function as a negative regulator of inflammation also in chondrocytes, in addition to immune cells [24]. In Hodgkin lymphoma, miR-9 inhibition modulated genes implicated in B- & T-cell activation and inflammation & cytokine signaling, while in Hodgkin lymphoma, miR-9 inhibition reduced the secretion of cytokines (i.e., TNF-α, CCL-5, IL-6 and IL-5) from L428 and L540 cells [11]. In this study, we showed that miR-9 modulated the expression of IL-related genes (including IL20RB, GALT, IL7, IL1B, IL11, IL1F8, IL1A, IL6 and IL7R) in NPC cells, while we found for the first time that miR-9 could up-regulate the expression of IFN-induced genes (e.g., IFI44L, PSMB8, IRF5, PSMB10, IFI27, IFIT2, TRAIL, IFIT1 and IRF1) and MHC class I molecules (e.g., HLA-B, HLA-H, HLA-C and HLA-F) in human cancer cells, which has never been yet reported in other physiological and pathological processes. Therefore, these above-mentioned findings from other investigators and this study strongly support that like the classic miR-155 and miR-146, LPS-responsive miR-9 is highly associated with immune response and inflammatory diseases.

Inflammation not only works as a tumor-promoting agent but also influences other steps of tumorigenesis by inducing DNA damage, angiogenesis, invasion and metastasis. For example, the silencing of the interferon regulatory factor IRF7 in breast cancer cells promoted bone metastasis through immune escape [28]. In Hodgkin lymphoma, the massive infiltration of immune cells and fibroblasts in the tumor provided important survival and growth signals to tumor cells [11]. The inhibition of miR-9 which was overexpressed in Hodgkin lymphoma decreased the production of cytokines (i.e., TNF-α, CCL-5, IL-6 and IL-5) from L428 and L540 cells of Hodgkin lymphoma followed by an impaired ability to attract normal inflammatory cells, and then impairing tumor outgrowth in vivo [11]. As mentioned above, both over-expression and inhibition of miR-9 in CNE2 and 5–8F cells modulated the expression of IFN-inducible genes, MHC class I molecules and IL-related genes, while our recent research has implicated miR-9 that are translationally down-regulated in NPCs in the regulation of the proliferation, EMT, invasion and metastasis of NPC (see our upcoming papers for more details). Our findings and recently published reports strongly suggests that modulating the expression of immune- or inflammatory-related genes by miR-9 in NPC might contribute to the pathogenesis of NPC, which remains to be fully characterized.

In summary, we have shown that miR-9 can modulate IL-related gene expression in NPC cells, and we have found for the first

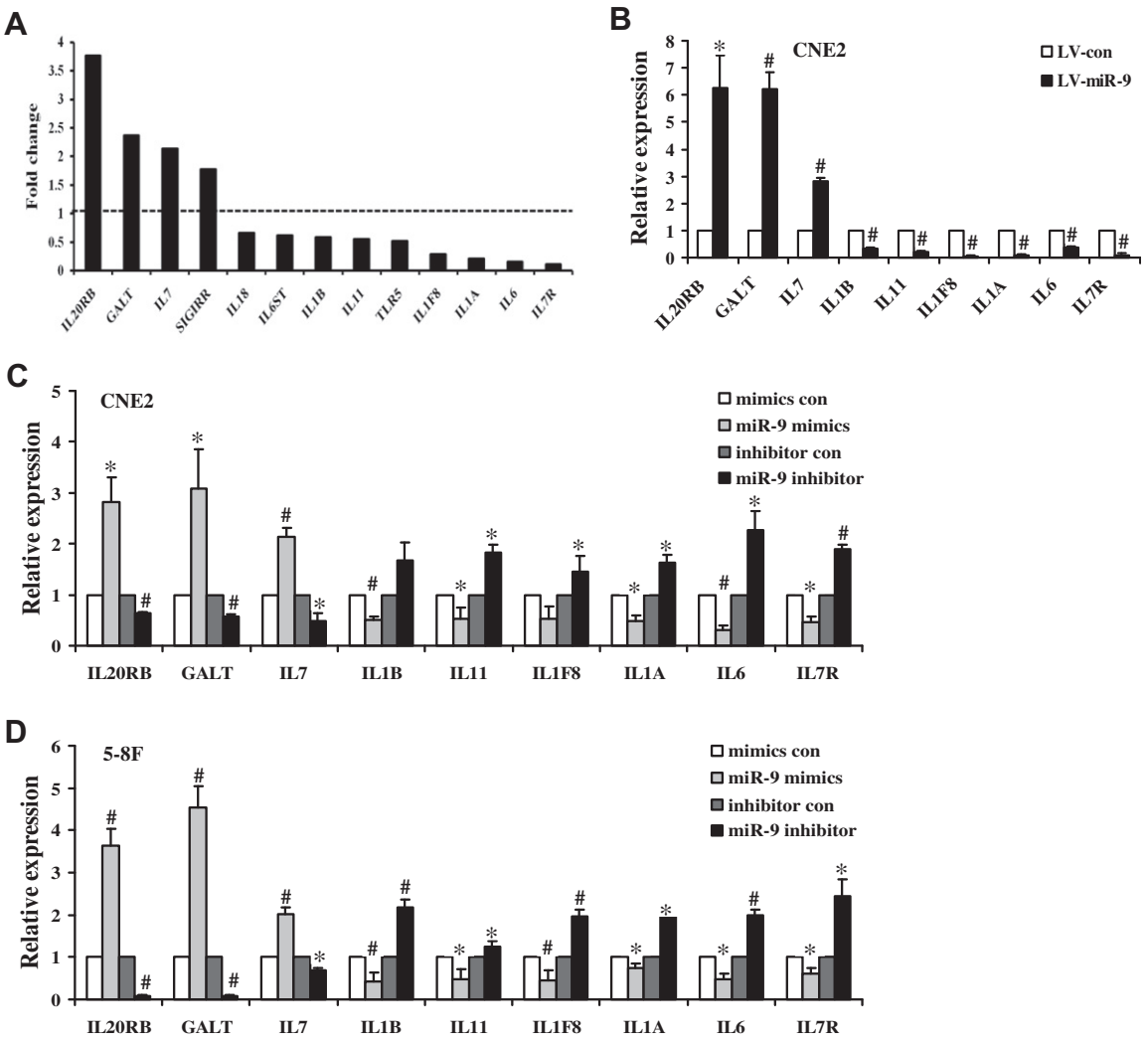

Fig. 4. miR-9 overexpression enhanced or reduced the IL-related gene expression in NPC cells. (A) Graph illustrating the fold change in gene expression of representative differentially IL-related genes between LV-miR-9-infected CNE2 cells to LV-con-infected CNE2 cells. For other details see legend to Fig. 2A. (B) qRT-PCR confirmed the increased or decreased mRNA expression of IL-related genes in LV-miR-9-infected CNE2 cells. mRNA expression levels of IL20RB, GALT, IL7, IL1B, IL11, IL1F8, IL1A, IL6 and IL7R, relative to reference gene expression (GAPDH) in LV-miR-9-infected CNE2 cells were measured by qRT-PCR to confirm the results of microarray experiments (shown in Fig. 4A). (C–D) miR-9 regulated the mRNA expression of IL-related genes in CNE2 and 5–8F cells. CNE2 cells and 5–8F cells were transfected with miR-9 mimics (100 nM) or anti-miR-9 inhibitor (100 nM) for 48 h, followed by evaluating IL-related gene expression via qRT-PCR as described in Materials and methods.

Table 1
Gene ontology analysis of differentially expressed genes with immune and inflammation from LV-miR-9-infected CNE2 cells to LV-con-infected CNE2 cells.

GO ID	Biological process	Count	*P*-value
GO:0006955	Immune response	25	8.65E-27
GO:0002474	Antigen processing and presentation of peptide antigen via MHC	10	9.02E-18
GO:0019882	Antigen processing and presentation	10	3.61E-15
GO:0006953	Acute-phase response	4	4.99E-08
GO:0006954	Inflammatory response	7	1E-07

time that miR-9 overexpression and inhibition alter the expression of well-known IFN-induced genes and MHC class I molecules in human cancer cells, but the physiological and pathological consequences of the altered expression of immune- or inflammatory-related genes by miR-9 in NPC remain unclear. Future studies are needed to fully clarify the potential contribution of miR-9 to the proliferation, EMT, invasion, metastasis or tumor angiogenesis of NPC by playing key roles in modulating genes implicated in immune and inflammation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.097.

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