

Regulation network and expression profiles of Epstein-Barr virus-encoded microRNAs and their potential target host genes in nasopharyngeal carcinomas

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Epstein-Barr virus (EBV) is associated with nasopharyngeal carcinoma (NPC) tumorigenesis. However, the mechanism(s) connecting EBV infection and NPC remain unclear. Recently, a new class of EBV microRNAs (miRNAs) has been described. To determine how EBV miRNAs control the expression of host genes, and to understand their potential role in NPC tumorigenesis, we profiled the expression of 44 mature EBV miRNAs and potential host genes in NPC and non-tumor nasopharyngeal epithelial tissues. We found that 40 EBV miRNAs from the BART transcript were highly expressed in NPC. Analysis of potential BART miRNA target genes revealed that 3140 genes and several important pathways might be involved in the carcinogenesis of NPC. A total of 105 genes with potential EBV miRNA binding sites were significantly downregulated, suggesting that EBV miRNAs may regulate these genes and contribute to NPC carcinogenesis. An EBV miRNA and host gene regulation network was generated to provide useful clues for validating of EBV miRNA functions in NPC tumorigenesis.

Epstein-Barr virus (EBV), microRNA (miRNA), nasopharyngeal carcinoma (NPC), gene regulate network

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Epstein-Barr virus (EBV), also known as human herpesvirus 4 (HHV-4), is a ubiquitous herpesvirus that asymptotically infects over 95% of the adult population worldwide, and sporadically induces infectious mononucleosis. In

the vast majority of cases, the virus establishes a lifelong latent infection without further complications. In certain circumstances, however, the virus has oncogenic potential. EBV is related to a broad spectrum of benign and malignant diseases, including tumors of B cell origin, such as Burkitt's and Hodgkin's lymphoma, tumors of epithelial cell origin,

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such as nasopharyngeal carcinoma (NPC) [1–5], and gastric cancer [6]. In tumor cells, EBV expresses a limited set of latent proteins, such as LMP1 [7], LMP2A [8], EBNA1–6, and a few non-coding RNAs (EBERs) [9]. EBV also expresses 44 mature microRNAs (miRNAs) from 25 miRNA precursors encoded by two primary transcripts (BHRF1 and BART) [2,10].

miRNAs represent a conserved class of small noncoding RNAs that are found in all higher eukaryotes and some DNA viruses [11–15]. These RNA molecules are 19–26 nt in length with partial homology to sequences in their target mRNAs [16–20]. miRNAs bind to complementary mRNA sequences, reducing the stability of the target mRNAs. This binding impairs translation and inhibits the expression of target genes in a small interfering RNA (siRNA)-like manner [16–18]. miRNAs are involved in regulating a broad range of biological processes [19–25], such as signal transduction pathways that are often dysregulated in cancers [26–29].

EBV miRNAs are expressed at various levels in tumor samples, including NPC, and different cell lines during viral latency and lytic growth [30,31]. The functions of EBV miRNAs in NPC are currently being explored by numerous researchers. Several genes involved in apoptosis, such as *PUMA*, *BIM*, and *TOMM22*, were identified as potential targets of various EBV miRNAs [32,33]. EBV BART miRNA targets, such as *IPO7*, *Dicer*, and *LMP2A*, participate in the immune evasion of NPC [31,34]. However, the biological effects and targets of EBV miRNAs have yet to be identified.

In this study, we established a comprehensive EBV miRNA profile in NPC. Host target genes, the targetome, of these miRNAs were predicted and an EBV miRNA and gene regulation network was generated. Our findings demonstrate the potential role of viral miRNAs in controlling host gene expression and oncogenesis in NPC.

1 Materials and methods

1.1 Clinical samples and cell lines

Ethics approval was obtained from the Ethics Review committees and the Institutional Review Boards of the Tumor Hospital and the Third Xiangya Hospital of the Central South University. NPC samples and non-tumor nasopharyngeal epithelial tissues were collected, and written informed consent was obtained from all patients. All 16 NPCs were non-keratinizing carcinoma (WHO II), according to WHO (1991) classification, including five samples for TNM stage II, five samples for stage III and six samples for stage IV. Five non-tumor nasopharyngeal epithelial tissues were obtained from patients with inflamed nasopharyngeal mucosa.

Two EBV-negative (HK-1 and NP69) and three EBV-

positive (Raji, C666-1, and B95-8) cell lines were grown in RPMI-1640 medium (Invitrogen, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS) (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (Invitrogen, Breda, the Netherlands). HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% FCS (Gibco).

1.2 RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Expression levels of EBV miRNAs were measured by qPCR using the miScript system (Qiagen) according to the manufacturer's instructions. The miRNAs were polyadenylated and then reverse-transcribed using oligo-dT primers that contain a universal tag at the 5' end. A subsequent PCR step was then conducted using a primer specific for the universal tag and a primer specific for each miRNA (Qiagen). Accumulation of the PCR product was monitored using SYBR green dye (Bio-Rad, CA, USA).

The cycle threshold (C_t) was defined as the number of PCR cycles required for a given reaction to reach an arbitrary fluorescence value within the linear amplification range. The change in C_t (ΔC_t) was determined for each individual miRNA compared with the C_t for C666-1 cells, with the change in ΔC_t ($\Delta\Delta C_t$) determined by adjusting for the difference in a control reaction in which the RNU6B small nuclear RNA was amplified. The $\Delta\Delta C_t$ for each sample was transformed into relative expression level, and then normalized and visualized with Genesis software [35].

To evaluate mRNA expression levels, qPCR assays were conducted using SYBR Premix Ex Taq™ (TaKaRa, Japan). We used the $2^{-\Delta\Delta C_t}$ to quantify mRNA transcripts, which were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). We used specific primers for GAPDH (5'-AAGGTGAAGGTCCGAGTCAAC-3' and 5'-GGGTC-ATTGATGGCAACAATA-3') and beta-transducin repeat containing E3 ubiquitin protein ligase (*BTRC*; 5'-GCTC-TATGCCAGGTCTCTGT-3' and 5'-GGGTTCCGCATT-ATTACAGTCTT-3').

1.3 *In silico* prediction of the EBV miRNA targetome in host cells

The seed region sequences of all EBV miRNAs were individually entered into TargetScan 4.1 (<http://www.targetscan.org>) [36] and RepTar (<http://bioinformatics.ekmd.huji.ac.il/reptar>) [37] to predict potential target genes for each miRNA. The overlapping potential target genes identified in both databases were retained for further analysis [38]. To determine the pathways shared by the target genes, the full list of predicted target genes was input into DAVID bioinformatics software (<http://david.abcc.ncifcrf.gov>) [39].

1.4 Identification of dysregulated host genes in NPC

Human gene expression profiles from 22 NPC biopsies and 10 non-tumor nasopharyngeal epithelial tissues have been previously reported [40,41]. Genes that were significantly dysregulated in NPC biopsies compared with the non-tumor samples were identified using SAM 3.0 [42].

1.5 Integrating analysis of EBV miRNA target genes and pathways

Significantly downregulated genes in NPC and lists of predicted EBV miRNA target genes were compared to identify overlap between the two groups. Genes belonging to both the EBV miRNA target gene list and the list of genes significantly downregulated in NPC were considered potential EBV miRNA target genes in NPC. EBV miRNAs and the potential target gene regulation network were analyzed and visualized using Pajek (<http://pajek.imfm.si>).

1.6 EBV miRNA mimics, plasmids and transient transfection

The syn-ebv-miR-BART10 miScript miRNA mimic was purchased from Qiagen. Allstars negative control (Qiagen) was used for miRNA transfection experiments.

We synthesized two single strands encoding the 3' untranslated region (UTR) of the *BTRC* gene that contained the EBV-miR-BART10 binding site, and a mutant control, in which eight bases in the binding site were substituted. The oligonucleotides used in these studies were as follows: the 3'-UTR of *BTRC*, 5'-CTAGTCCAACCAGCACAGC-TGGCGCTCTTAGCTCCTGATTGGTTGTGTGTTTTAT-TAAA-3' and 5'-AGCTTTTAATAAAACACACAACCA-ATCAGGAGCTAAGAGCGCCAGCTGTGCTGGTTGGA-3'; and the 3'-UTR of the mutant *BTRC*, 5'-CTAGTCCAACCAGCACAGCTGGCGCTCTTAGCTCCTGATTAA-AGTACGGTTTTATTAAA-3' and 5'-AGCTTTTAATAAAACCGTACTTTAATCAGGAGCTAAGAGCGCCAGCT-GTGCTGGTTGGA-3'. The sense and antisense strands of the oligonucleotides were annealed, digested with *Hind* III and *Spe* I, and ligated into the pmiR-Report luciferase vector (Ambion, Austin, TX, USA).

HEK293T cells were seeded in 24-well plates 24 h prior to transfection. The following day, 10 nmol L⁻¹ of syn-ebv-miR-BART10 miScript miRNA mimics along with 200 ng of reporter plasmid or negative controls were co-transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

1.7 Western blotting

Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes as previously described [17]. The

membranes were blocked with 5% non-fat milk and incubated with the appropriate primary antibody followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma, MO, USA). Primary antibody against BTRC was purchased from Cell Signaling (Danvers, MA, USA) and antibody against GAPDH was purchased from Sigma.

1.8 Luciferase assays

Luciferase activity was measured in cell lysates 24 h post-transfection using the Dual-Glo luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Renilla luciferase activity was used to normalize firefly luciferase activity.

2 Results

2.1 EBV miRNA profiles

The expression levels of 44 mature EBV-encoded miRNAs and two latent protein genes (LMP1 and EBNA1) were determined by qPCR in NPC tumor and non-tumor tissues, and in five cell lines. No EBV miRNAs or EBV latent protein genes were detected in the five non-tumor biopsies or in the two EBV-negative cell lines. Most of the 40 EBV miRNAs residing in the BART region and the latent protein genes were highly expressed in 14 of the 16 NPC samples and in the C666-1 cell line. Expression of these miRNAs was highest in stage III samples, and decreased in stage IV samples (Figure S1 in Supporting Information). No BHRF1 miRNAs were detected in the nasopharyngeal epithelium, tumor tissues, or non-tumor tissues. In contrast, lymphoma cells derived from humans and marmosets (Raji and B95-8) exhibited high levels of BHRF1 miRNAs but low levels of BART miRNAs (Figure 1).

2.2 Prediction of target genes and pathways for EBV miRNAs

TargetScan and RepTar were used to predict target genes. Because the EBV-encoded BHRF1 miRNA cluster was not expressed in NPC, only the BART miRNA cluster was considered for further analysis. The 40 mature BART miRNA sequences were obtained from miRBase and input into TargetScan and RepTar to generate two separate lists of predicted target genes. Using TargetScan, 5569 target genes were predicted, and RepTar predicted 6494 genes. A total of 3140 genes were identified by both bioinformatics tools. Among these 3140 genes, BART18-5p had the highest number of targets (148 genes), whereas BART8, BART13*, and BART19-3p had no potential target host genes.

To determine which pathways were commonly targeted by BART miRNAs, the 3140 target genes were subjected to functional clustering analysis using DAVID. The results showed that BART miRNAs target a wide range of path-

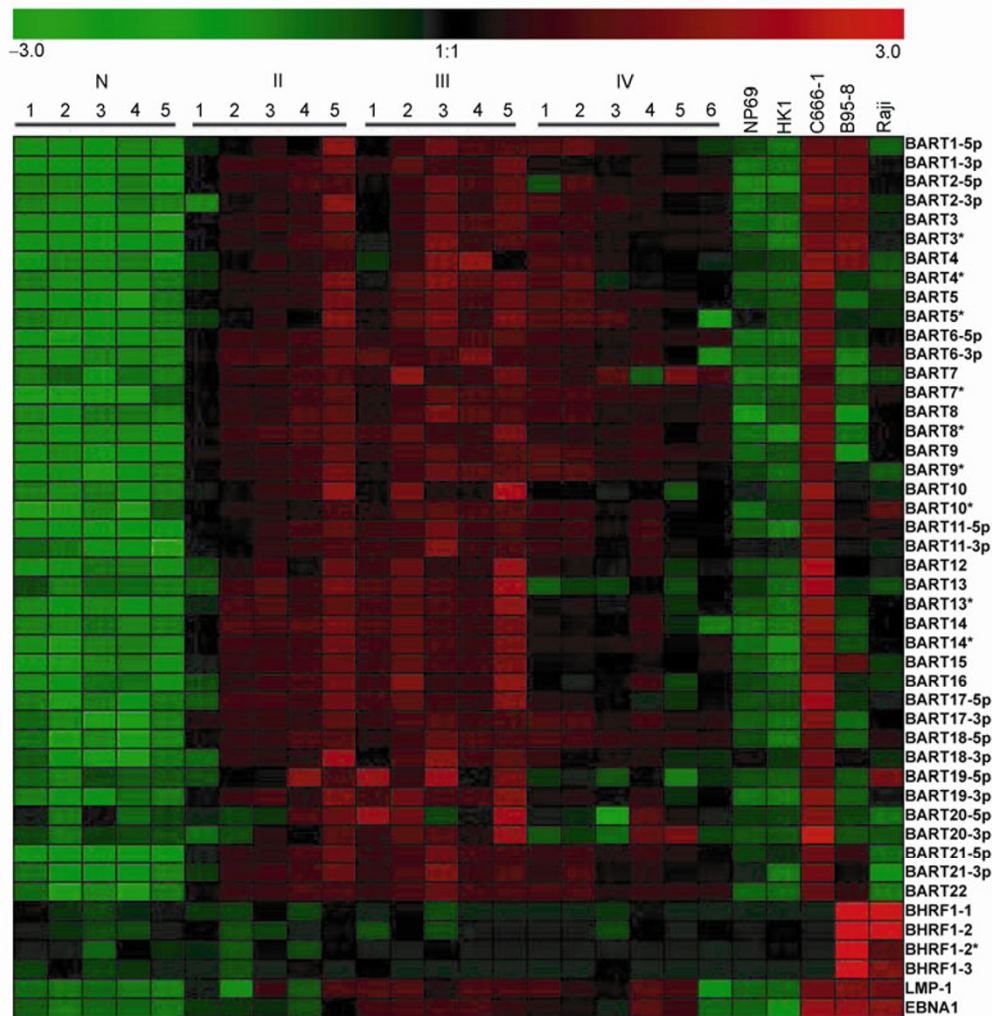


Figure 1 Heatmap of EBV-encoded miRNAs in biopsies and cell lines. The gene expression profile of the 44 EBV-encoded mature miRNAs and the two mRNAs encoding LMP-1 and EBNA1 in five non-tumor nasopharyngeal epithelium tissues (N, EBV negative), five clinical stage II NPC biopsies (II), five stage III NPC biopsies (III), five stage IV NPC biopsies (IV), two EBV-negative nasopharyngeal epithelium cell lines named NP69 (immortalized normal nasopharyngeal epithelium) and HK1 (NPC) and three EBV-positive cell lines named C666-1 (NPC), Raji (Burkitt's lymphoma) and B95-8 (marmoset B-cell), were detected by qPCR and then normalized and visualized by Genesis software. Red represents high expression and green represents low or absent expression. Note that the first stage II NPC biopsy and the last NPC stage IV biopsy were EBV negative.

ways, with the five most significant pathways listed in Table 1. These include axon guidance, cancer pathways, the WNT signaling pathway, regulation of the actin cytoskeleton, and adherens functions.

2.3 Integrated analysis of the EBV targetome and the NPC transcriptome

The SAM test was used to compare the transcriptomes of NPC and non-tumor tissues. We found that 732 genes were significantly dysregulated in NPC, including 270 upregulated and 462 downregulated genes (Figure 2). The 462 downregulated genes were compared with the 3140 potential EBV miRNA target genes. We found that 105 genes were both downregulated in NPC and predicted as potential

targets of EBV miRNAs by TargetScan and RepTar (Figure 3).

2.4 EBV miRNA and target gene regulation network

For the 105 genes that were downregulated in NPC and targeted by EBV miRNAs, 175 miRNA:mRNA target pairs were identified. BART22 had the highest number of potential targets (37 genes), followed by BART8* (23), BART18-5p (12), BART9 (11), and BART14 (11). These five EBV miRNAs regulated 94 of the 175 (53.4%) miRNA:mRNA target pairs (Table 2). Certain genes, such as *ATRX*, *BTRC*, *CTBP2*, *FOXP1*, and *NFIB*, were predicted to have several EBV miRNA binding sites in their 3'-UTRs (Table 3). To illustrate the complexity of the regulatory re-

Table 1 The five top targeted EBV miRNA pathways in host cells

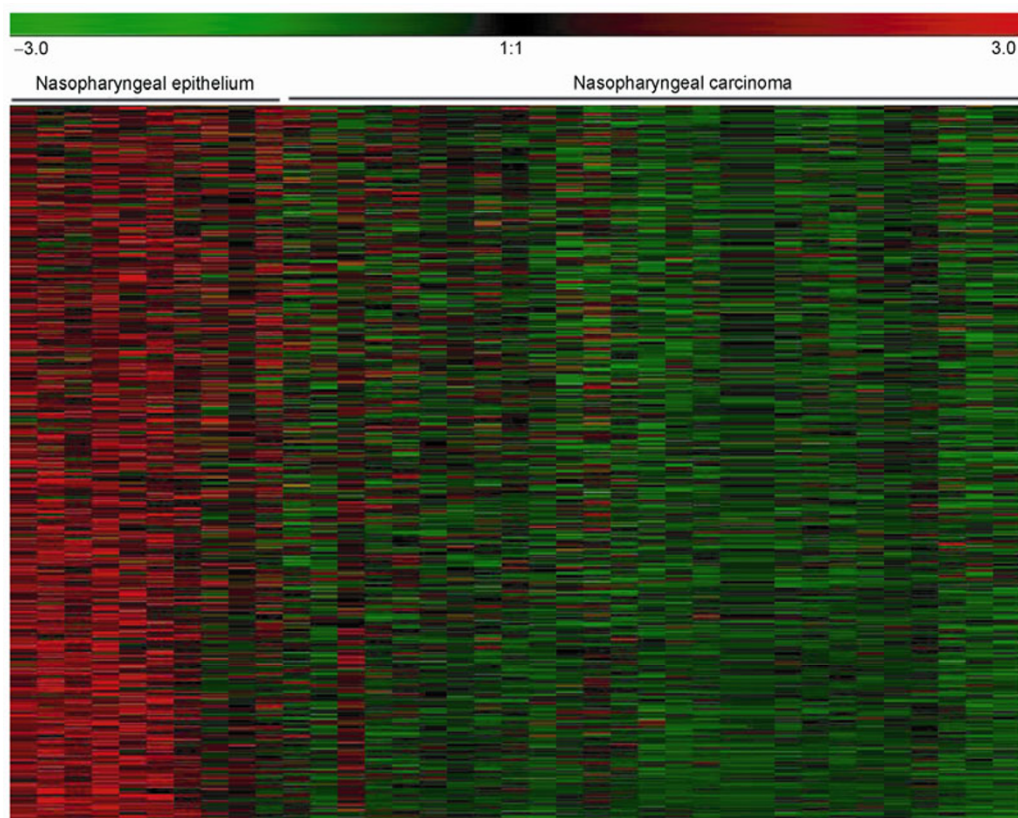
KEGG pathway	Count	P-value
Axon guidance	53	1.30×10^{-10}
Pathways in cancer	98	2.30×10^{-9}
Wnt signaling pathway	53	7.80×10^{-8}
Regulation of actin cytoskeleton	67	2.20×10^{-7}
Adherens function	32	7.80×10^{-7}

Table 2 The five EBV miRNAs with the highest number of target genes

miRNA	Genes
BART22	<i>ADCY2, ATP9A, ATRX, C2orf24, CALM1, CD47, CP, CTBP2, CTSS, EPAS1, F11R, FAM8A1, FOXP1, INSR, IPO11, KIAA0895, KIF21A, KLHL5, MCRS1, MLL5, NEBL, NFIA, NFIB, NPAS2, OSBPL10, PCDHA5, PCMI, PHF17, PLD2, PPP2R5A, SFRS5, SORT1, SYTL2, TMF1, TOB2, TUSC2, UBE2J1</i>
BART8*	<i>ABCE1, ARHGAP5, ASXL2, ATRX, C9orf5, CLASP2, CRY2, CTBP2, CTNBN1, DNER, GFPT1, IL1RN, KIAA0895, KPNA3, MAP6, NFIB, OXTR, PCMI, PFN2, PLCB4, STK39, TLE4, TRIB2</i>
BART18-5p	<i>BTRC, C9orf5, CLASP2, EAF1, EPAS1, KIAA0895, KIF3B, KLHL2, MAP1A, NUCB2, STK39, TNFRSF21</i>
BART9	<i>CLASP2, DAG1, EPAS1, EPHA2, FAM8A1, LACTB, MCOLN3, MYRIP, NFIA, NFIB, SLC39A9</i>
BART14	<i>ABCE1, ALDH1A2, ATRX, CENTD1, EFHC1, FOXP1, NFIB, PPP2R5A, SLC16A2, TUSC2, UBP1</i>

Table 3 The five most likely host genes targeted by EBV miRNAs

Gene	miRNAs
<i>ATRX</i>	BART4*, BART8*, BART14, BART20-5p, BART22
<i>BTRC</i>	BART4, BART4*, BART6-3p, BART10, BART18-5p, BART19-5p
<i>CTBP2</i>	BART8*, BART10*, BART13, BART22
<i>FOXP1</i>	BART11-5p, BART11-3p, BART12, BART14, BART15, BART17-5p, BART19-5p, BART22
<i>NFIB</i>	BART8*, BART9, BART9*, BART10, BART14, BART22

**Figure 2** The heatmap of 462 down-regulated host genes in NPC and non-tumor tissues. The SAM test was applied to compare the whole-genome wide mRNA expression profile of NPC ($n=27$) and nasopharyngeal epithelium tissues ($n=10$). 732 genes were significantly dysregulated in NPC, including 270 up-regulated genes and 462 down-regulated genes. 462 down-regulated genes' expression profile were normalized and visualized by Genesis software. Red represents high expression and green represents low or absent expression.

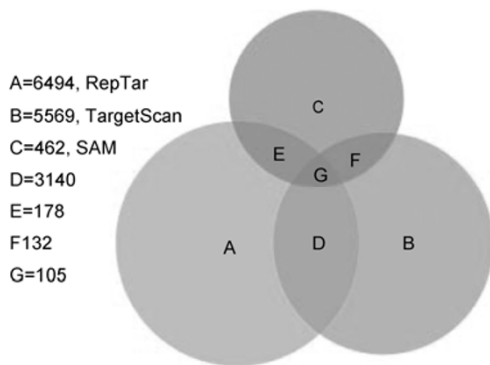


Figure 3 Venn diagram of the EBV miRNA target genes predicted using two types of software and the significantly down-regulated genes in NPC identified using SAM analysis. Forty mature BART miRNA sequences were obtained from miRBase and input onto TargetScan and RepTar to predict their target genes. A total of 6494 human target genes were predicted by RepTar (A), and 5569 genes were predicted by TargetScan (B). A total of 3140 genes were identified by both bioinformatic tools (D). To integrate the analysis of the EBV targetome and transcriptome of NPC, 462 down-regulated genes in NPC that were found using SAM 3.0 from the whole genome gene expression profile data of 10 non-tumor nasopharyngeal epithelia and 22 NPC (C) were compared to the EBV miRNA target gene list. A total of 105 genes that are down-regulated in NPC were also predicted as potential targets of EBV miRNAs by both TargetScan and RepTar (G).

relationships between EBV miRNAs and host genes, their regulatory network was visualized using Pajek (Figure 4).

2.5 Low expression of *BTRC* and negative correlation with EBV-miR-BART10

Expression of *BTRC* was significantly low in the mRNA

expression profiles of NPC biopsies (Figure 5A). To date, there have been no reports of a mechanistic link between the *BTRC* gene and NPC tumorigenesis. Using qPCR assays, we measured the expression of *BTRC* and EBV-miR-BART10 in another group of clinical biopsies. Our qPCR results verified that *BTRC* was significantly downregulated in NPC samples (Figure 5B), while EBV-miR-BART10 was upregulated (Figure 5C) and negatively correlated with *BTRC* gene expression (Figure 5D).

2.6 EBV-miR-BART10 downregulated *BTRC* expression

To determine whether *BTRC* is an authentic EBV-miR-BART10 target, we transiently expressed EBV-miR-BART10 mimics in HEK293T cells (Figure 6A). After 24 h, EBV-miR-BART10 mimics significantly reduced expression of endogenous *BTRC* mRNA (Figure 6B). Similarly, expression of *BTRC* protein level was also decreased (Figure 6C). These results confirmed that EBV-miR-BART10 modulated expression of the *BTRC* gene.

2.7 EBV-miR-BART10 targets the 3'-UTR of the *BTRC* gene

TargetScan, RepTar, miRanda and RNAhybrid all predicted six EBV-miR-BART10 binding sites in the 3'-UTR of *BTRC*. The predicted binding site was around nt 3751 of the *BTRC* 3'-UTR, had the lowest MFE ($-25.7 \text{ kcal mol}^{-1}$) and exhibited the best complementarity to EBV-miR-BART10 (Figure 7A). To validate the recognition of this

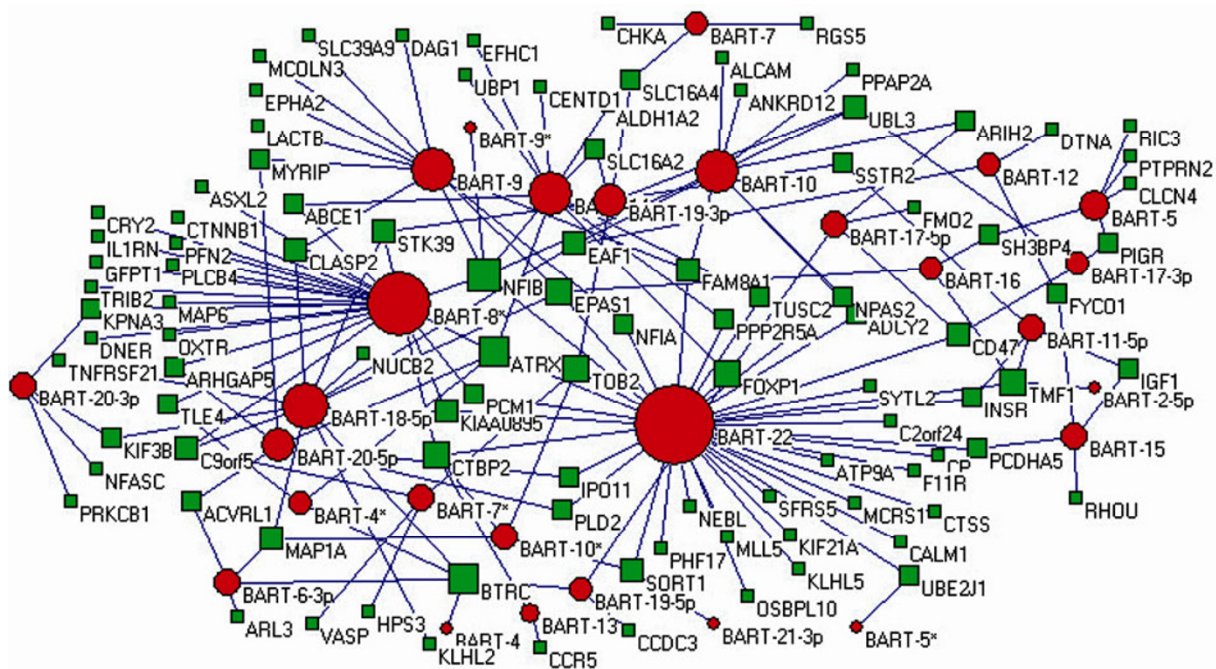


Figure 4 Potential regulating network of EBV miRNAs and their host cellular target genes. The red circles represent EBV-encoded miRNAs, and the green squares represent host target genes; the size of each shape represents the number of regulating relationships.

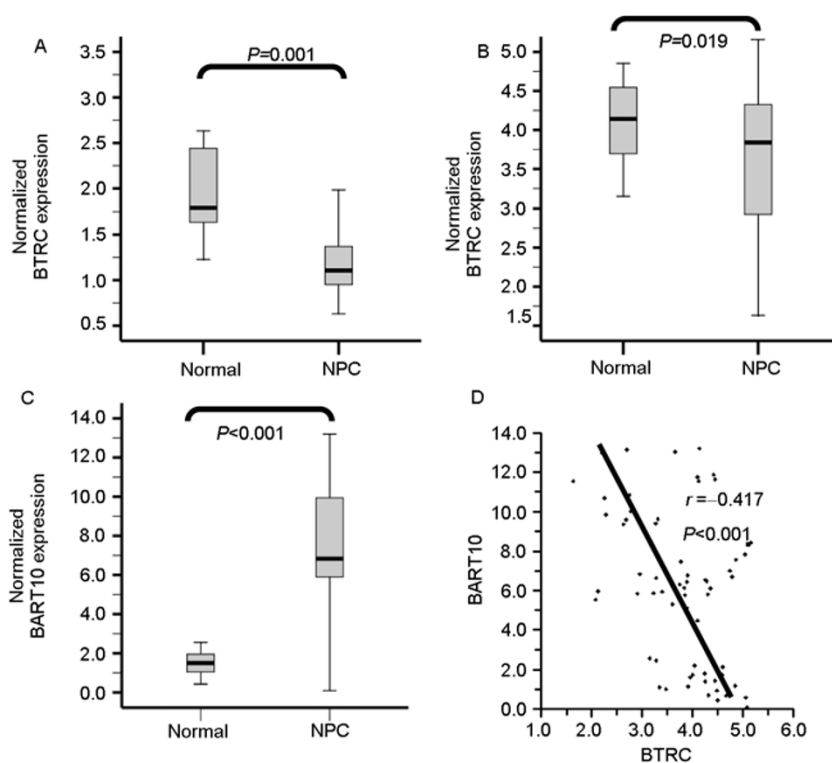


Figure 5 The expression of EBV miR-BART10 and *BTRC* in NPC samples. A, The normalized expression of *BTRC* in our previous reported gene expression profile data of NPC and normal nasopharynx epithelia tissues. B, Real-time PCR results of *BTRC* expression in another group of clinical biopsies verified that *BTRC* was significantly down-regulated in NPC samples. C, Real-time PCR measured the EBV miRNA BART10 expression in the same samples of Figure 5B, and found that BART10 was significantly up-regulated in NPC biopsies. D, We compared the *BTRC* and BART10 expression data in Figure 5B and C, and found that BART10 and *BTRC* expression was negatively correlated.

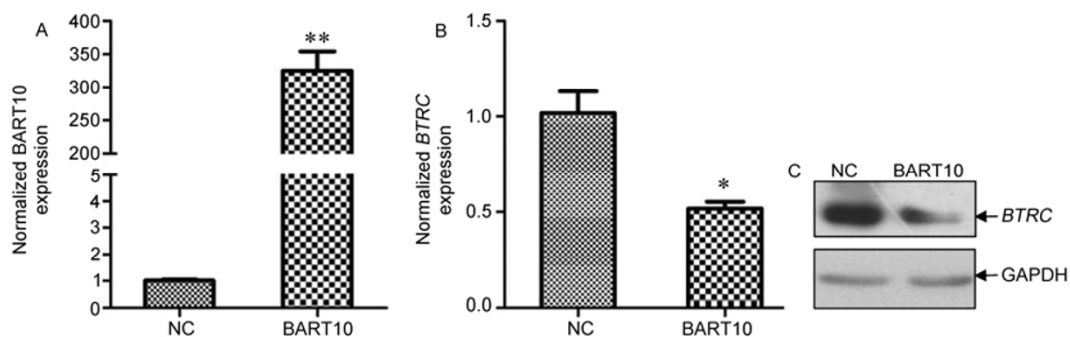


Figure 6 EBV miRNA BART10 down-regulated *BTRC* expression. A, EBV miRNA BART10 mimics was transfected in HEK 293T cells, allstars negative control (NC) was used. After 24 h, BART10 expression level was confirmed by real-time PCR, the expression of *RNU6B* small nuclear RNA was measured and used for data normalization. **, $P < 0.01$. B, Real-time PCR analysis of *BTRC* mRNA expression in HEK293T cells transfected with EBV miRNA BART10 or negative control (NC) mimics, GAPDH was selected as a control. BART10 mimics significantly down-regulated the endogenous *BTRC* mRNA expression. *, $P < 0.05$. C, Western blotting analysis of *BTRC* protein expression in HEK293T cells transfected with EBV miRNA BART10 or negative control (NC) mimics, GAPDH was selected as a control. BART10 mimics significantly down-regulated the endogenous *BTRC* protein expression.

site by EBV-miR-BART10, luciferase assays were conducted. The target site in the *BTRC* 3'-UTR and a mutant control were synthesized and cloned into a pmiR-Report luciferase vector (Figure 7B). The pmiR-report constructs were co-transfected into HEK293T cells with 10 nmol L^{-1} EBV-miR-BART10 mimics or all-star negative control for 24 h. Co-transfection with EBV-miR-BART10 mimics resulted in a significant reduction of luciferase activity. No

inhibition was observed when the *BTRC* 3'-UTR binding site was mutated (Figure 7C).

3 Discussion

The risk factors for NPC include both environmental and genetic factors. However, how these risk factors interact is

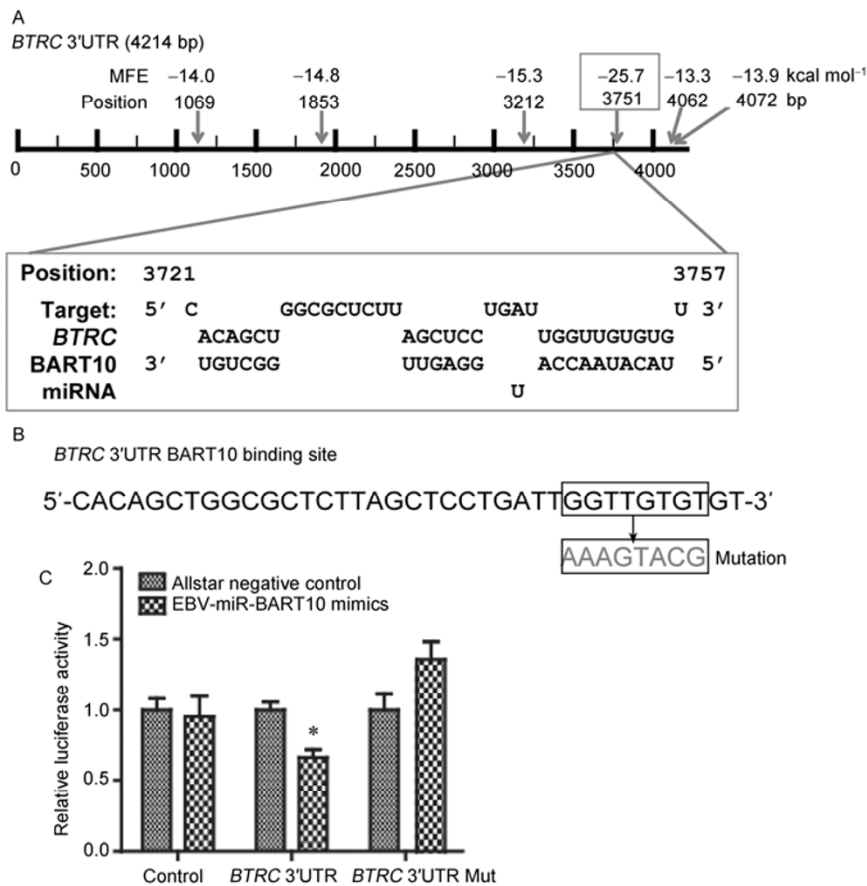


Figure 7 EBV miRNA BART10 target to the 3'-UTR of *BTRC* mRNA. A, There are six EBV miRNA BART10 binding sites located within the 3'-UTR of human *BTRC* mRNA. The positions and their minimal free energy (MFE, kcal mol⁻¹) of the EBV miRNA BART10 target sites located within the *BTRC* 3'-UTR are schematically presented. The predicted binding site around the 3751 bp of the *BTRC* 3'-UTR has the lowest MFE (-25.7 kcal mol⁻¹) and exhibits the best complementarity to miRNA BART10. B, The binding site of EBV miR-BART10 in *BTRC* 3'-UTR around 3751 bp was mutated by replacing eight bases in the seed sequence. Synthesized oligonucleotides containing wild type or mutated BART10 binding site were inserted into the luciferase report plasmids. C, HEK293T cells were co-transfected with 10 nmol L⁻¹ EBV miR-BART10 mimics or allstar negative control, and 200 ng pmiR-Report-*BTRC* 3'-UTR, pmiR-Reporter-*BTRC* 3'-UTR mutant or empty pmiR-Report vector (as control) plasmids. EBV miR-BART10 significantly down-regulates *BTRC* 3'-UTR reporter activity in HEK293T cells. *, $P < 0.05$.

not well understood [43–49]. EBV is a typical environmental oncogenic virus of NPC that can transform cells and subsequently induce cell proliferation and tumor growth. The identification of virus-encoded miRNAs and their host target genes provides an ideal model for investigating the interaction between environmental and genetic risk factors in NPC, and will potentially generate new insights into viral transformation. Previous studies identified 44 miRNAs in the EBV genome [10]. Detailed characterization of the miRNA profiles in NPC will help elucidate the contribution of these miRNAs to cancer pathogenesis. Expression of EBV-encoded miRNAs in NPC and other EBV-positive cells were profiled using qPCR assays, and the results showed that NPC and lymphoma cells have distinct EBV miRNA expression profiles, and that all miRNAs from the BART cluster are expressed in NPC tissues. BHRF1 miRNAs are highly expressed in lymphoma cells, while BART miRNA expression is low or absent, suggesting that the

BHRF1 and BART clusters of EBV miRNAs possibly play different roles and function via different mechanisms to transform cancers derived from epithelial or B-cell lineages [50]. The expression of BART miRNAs was highest in clinical stage III samples, with levels in stage IV samples somewhat lower. These findings support the “hit-and-run” viral oncogenesis hypothesis [51,52].

Determination of how EBV-encoded miRNAs control the expression of host genes and the elucidation of molecular mechanisms for EBV miRNAs in NPC carcinogenesis requires comprehensive identification of all host target genes for these miRNAs. Identifying this targetome has been a very difficult task. To enhance the accuracy of miRNA target prediction, TargetScan and RepTar were used in this study. Only genes predicted by both programs were included in the target gene pool, which resulted in a targetome of 3140 potential genes. DAVID analysis suggested that many cytoskeletal and adherens-related genes

were regulated by EBV miRNAs, implying that EBV miRNAs most likely regulate the invasion and metastasis of tumors. Consistent with other studies, the WNT signaling pathway [4,53] was also identified as an important downstream cascade regulated by EBV miRNAs [54].

Because the predicted EBV miRNA target genes might not be expressed in NPC, transcriptome data was analyzed for identification of downregulated genes and compared with the predicted EBV miRNA targetome. A total of 105 genes were predicted to contain EBV miRNA target sites in their 3'-UTR, with expression patterns negatively correlated with those of EBV miRNAs. These 105 genes are the most likely EBV miRNA-regulated host genes in NPC development.

The relationship between host genes and EBV miRNAs is complex. The 105 genes identified are potentially targeted by 28 EBV miRNAs. Some miRNAs, such as BART22, BART8*, and BART18-5p, may target multiple genes, while some genes may be targeted by several miRNAs. In total, 175 miRNA:mRNA target pairs were identified. These results imply that synchronous and synergistic expression and regulation of EBV miRNAs and host genes may cooperate to transform nasopharyngeal epithelium cells. Among the five host genes with the largest number of binding sites for EBV-encoded miRNAs, *BTRC* and *alpha thalassemia/mental retardation syndrome X-linked (ATRX)* are tightly associated with viral infection.

The *BTRC* gene encodes the β -TrCP1 F-box protein, which is an E3 ubiquitin protein ligase that targets various substrates such as β -catenin, $\text{I}\kappa\text{B}\alpha$, ATF4, CDC25A, CD4, and Snail [55–61]. β -TrCP1 is essential for many aspects of tumorigenesis [62,63]. β -TrCP1 interacts with HIV-1 Vpu and regulates HIV release by mediating the degradation of its substrates. Additionally, Tang et al. found that the EBV LMP1-95-8 variant reduced the levels of endogenous β -TrCP2/HOS via phosphorylated $\text{I}\kappa\text{B}\alpha$, suggesting that LMP1-95-8 is a pseudo-substrate of the SCF β -TrCP/HOS E3 ubiquitin ligase and that interaction between LMP1 and HOS restricts the extent of LMP1-induced NF- κ B signaling [64].

ATRX is a component of the PML nuclear body (PML-NB), which is the nuclear localization site of many DNA viruses, including herpes simplex virus 1 (HSV-1), human cytomegalovirus and adenovirus 5 [65]. *ATRX* is also associated with herpesvirus infections. Upon entering the host cell nucleus, the herpesvirus genome must deal with intrinsic antiviral resistance that restricts viral gene expression and replication. The EBV major tegument protein, BNRF1, interacts with the host nuclear protein Daxx in PML-NBs and disrupts the formation of the Daxx-*ATRX* chromatin remodeling complex. This would indicate that *ATRX* plays a role in the regulation of early EBV infection [66]. Jurak et al. [67] found that *ATRX* is a target of HSV-1

miR-H1 and HSV-2 miR-H6, suggesting that *ATRX* may repress lytic viral gene expression through its miRNAs.

In this study, we selected the EBV-miR-BART10:*BTRC* target pair for experimental manipulation. We found that EBV-miR-BART10 could efficiently repress *BTRC* expression levels, and that this was dependent on the *BTRC* 3'-UTR. The reduction of *BTRC* by EBV-miR-BART10 possibly results in decreasing degradation of its substrates, including $\text{I}\kappa\text{B}\alpha$ and β -catenin, ATF4, CDC25A, CD4, and Snail [56–61]. This could regulate the development of NPC. Elucidating the function of this miRNA should assist in identifying new mechanisms by which EBV infection leads to transformation and malignancy.

This report provides the first EBV miRNA and host mRNA regulation network. Our results provide a starting point for systematically identifying and clarifying the essential miRNAs encoded by EBV and the regulated signaling pathways for these miRNAs during NPC development. Interfering with overexpressed miRNAs or the artificial expression of the mRNAs downregulated by these miRNAs might be useful for NPC therapy in the future [68–73]. We postulate that these miRNAs, or mRNAs, most likely act as biomarkers for NPC diagnosis or prognosis.

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Supporting Information

Figure S1 All of 40 EBV encoded BART cluster miRNAs expression profiling in different clinical stages of NPC samples and normal tissues. N, Normal; II, clinical stage II; III, clinical stage III; IV, clinical stage IV.

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