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EBV-miR-BART1 is involved in regulating metabolism-associated genes in nasopharyngeal carcinoma

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ABSTRACT

EBV-miR-BART1 has been found to be highly expressed in some cancers including nasopharyngeal carcinoma (NPC), but its exact roles in the pathogenesis of NPC remain unclear. Here, we did RNA deep sequencing to compare the gene expression profile between EBV-miR-BART1-expressing CNE1 cells and the control cells to determine the possible effects of EBV-miR-BART1 in NPC. Gene expression profiling analysis unexpectedly showed a significant number of up- and down-modulated metabolism-associated genes, such as G6PD, SAT1, ASS1, PAST1, FUT1, SGPL1, DHRS3, B4GALT1, PHGDH, IDH2, PISD, UGT8, LDHB and GALNT1, in EBV-miR-BART1-expressing NPC cells, which were next confirmed by RT-qPCR. Moreover, of these metabolism-genes, PSAT1 and PHGDH expression levels were significantly upregulated and most of other genes were obviously up-expressed in NPC specimens compared with chronic nasopharyngitis (CNP) tissues. Collectively, we for the first time found the effects of EBV-miR-BART1 on the expression of mechanism-associated genes in NPC, suggesting a novel role of EBV-miR-BART1 in cancer metabolism, which remains to be fully elucidated.

1. Introduction

Cancer cells display increased metabolic autonomy compared to non-transformed cells, taking up nutrients and metabolizing them in pathways that support growth and proliferation [1]. The Warburg effect is the best characterized metabolic phenotype observed in tumor cells, which was identified in many cancers [2–3]. However, it is seldom reported in NPC.

MicroRNAs have been validated to participate in various biological processes, including cell proliferation, survival, proliferation, apoptosis, immune response and fat metabolism etc. [4]. They can participate in controlling cancer cell metabolism by regulating the expression of key genes whose protein products either directly regulate metabolic machinery or indirectly modulate the expression of metabolic enzymes [1].

Epstein–Barr virus (EBV) is the first virus found to be able to encode miRNAs and carries the largest number of encoded miRNAs among viruses encoding miRNAs so far identified [5]. Previous studies have experimentally confirmed that EBV-encoding miRNAs, contributing to viral latency, cell survival and immune escape [6–11], are highly expressed in NPC and some other malignancies. Recently EBV-miR-BART1 has been reported to be up-expressed in EBV-associated tumors, including NPC [12], gastric carcinoma (GC) [13] and lymphoblast [14], hinting its potential roles in tumor progression. However, the contribution of EBV-miR-BART1 to the NPC pathogenesis remains elusive.

Therefore, in the present study, we applied RNA deep sequencing to analyze the differential gene expression profile of EBV-miR-BART1-expressing CNE1 cells and unexpectedly found a significant number of up- or down-modulated metabolism-associated genes, such as G6PD, PHGDH, PAST1, IDH2, PISD, UGT8, LDHB, SGPL1 and DHRS3, in the presence of EBV-miR-BART1 overexpression, indicating a close relationship between EBV-miR-BART1 and metabolism regulation in NPC. Our findings shed light on a novel role of EBV-miR-BART1 in cancer metabolism.

2. Material and methods

2.1. Ethics statement

All subjects involved in this study signed an informed consent. The research was approved by the Ethics Committee of Southern Medical University, Guangzhou and Zhongshan People's Hospital, Guangdong, China.

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2.2. Cell line and cell culture

Human EBV negative NPC cell lines, CNE1 and 610B, were maintained in our lab and cultured in RPMI1640 (Invitrogen, CA) medium supplemented with 10% fetal calf serum (FCS) in a humidified incubator with 5% CO_2 at 37 °C.

2.3. Clinical specimens

Thirty primary NPC biopsies (not pretreated with radiotherapy or chemotherapy prior to surgery) and 8 CNP biopsies were obtained from patients confirmed by pathological examination in Zhongshan People's Hospital, Guangdong, China, according to the Ethics Committee of this Hospital.

2.4. Establishment of EBV-miR-BART1-overexpressing cells

Lentiviruses expressed pre-EBV-miR-BART1 (H1-miRNA-CMV-GFP-BART1) and control vector (H1-miRNA-CMV-GFP-mock) were constructed and packaged with GFP positive packaging systems by Genechemi (Shanghai, China). NPC cell lines (CNE1 and 610B) were infected with them, respectively, followed by FACS to exclude non-infected cells. The overexpression of EBV-miR-BART1 in the infected NPC cells was verified by quantitative Real-time PCR (RT-qPCR).

2.5. MiRNA transient transfection

The EBV-miR-BART1 inhibitors (EBV-miR-BART1-5p inhibitor, EBV-miR-BART1-3p 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 50 nM with lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's introduction. Total RNA isolation of the transent transfected cells were usually harvested 36 h after oligonucleotide treatment. The inhibition of EBV-miR-BART1s of the transient transfected cells was obtained with RT-qPCR.

2.6. RNA isolation, reverse transcription and RT-qPCR

For miRNA and mRNA expression analysis, total RNA from NPC cells was extracted with Trizol Reagent (Invitrogen, CA) according to the manufacturer's introduction. For miRNA analysis, total RNAs were reversely transcribed with Taqman RT reaction kit (ABI), and the expression level of EBV-miR-BART1 mature sequences (BART1-5p and BART1-3p) were determined by Taqman real-time PCR amplification performed on the Stratagene Mx3000P real-time PCR system (Agilent Technologies, Inc., USA). U6 was used for normalization. For mRNA analysis, cDNA was reversely transcribed from total RNAs with the PrimeScript RT reagent kit (TAKARA), mRNA expression of genes were determined by SYBR Green master mix (TAKARA) on the same real-time PCR system as described, GAPDH was used for normalization. The primers used for the amplification of the indicated genes were listed in Table S1. All samples were normalized to internal controls and fold changes were calculated for relative quantification ($2^{-\triangle\triangle Ct}$).

2.7. RNA deep-sequencing

RNA deep-sequencing was performed by BGI Shenzhen as previously described [15].

2.8. Statistical analysis

Data were presented as mean ± SEM unless otherwise indicated of at least three independent experiments. Statistical analysis was performed using a SPSS 16.0 package system. Statistic significance was assessed by the Student's *t*-test or one-way ANOVA analysis ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

3. Results

3.1. Establishment of EBV-miR-BART1-overexpressing NPC cells

In order to study the biological function of EBV-miR-BART1 dysregulation in the pathogenesis of NPC, Pre-EBV-miR-BART1 expression lentivirus was infected into two human NPC cells (CNE1, 6–10B) as described in Section 2. The expression Levels of these two mature EBV-miR-BART1s (EBV-miR-BART1–5p and EBV-miR-BART-3p) were evaluated by Taqman real-time PCR. As shown in Fig. 1, two mature EBV-miR-BART1s' levels in two NPC cells harboring pre-EBV-miR-BART1 transgene were significantly higher than that in their control cells treated with corresponding control vector. Notably, there were no significant differences between EBV-miR-BART1-overexpressing NPC cells and clinical NPC specimens, indicating we established EBV-miR-BART1-overexpressing NPC cells successfully.

Moreover, the expression levels of EBV-miR-BART1s in CNE1-BART1 and 6–10B-BART1 cells transfected with EBV-miR-BART1s inhibitors were significantly lower than that transfected with negative control inhibitor, indicating that EBV-miR-BART1 inhibitors efficiently down-regulated the EBV-miR-BART1s expression in CNE1-BART1 and 6–10B-BART1 cells (Fig. 1D and E).

3.2. Gene expression alteration in EBV-miR-BART1-expressing CNE1 (CNE1-BART1)

MiRNA is known to be able to regulate gene expression in post-transcription or translation level, and viruses encoding miRNAs have been shown to play their roles in diverse biological processes on similar mode of cellular miRNAs, so we reasoned that EBV-miR-BART1 over-expression should remodel gene expression patterns in NPC cells. Therefore, we applied RNA-seq analysis to compare the gene expression profile of BART1-expressing CNE1 with its corresponding control cells so as to determine the possible effects of BART1 overexpression in NPC cells. Our data showed that 555 genes were significantly up-modulated, and 995 genes were significantly down-modulated in EBV-miR-BART1-overexpressing CNE1 cells (CNE1-BART1) relative to corresponding control (CNE-mock). Unexpectedly, among 1550 significantly altered genes, we identified a large number of altered genes (Fold-change $\geqslant 2$) involved in metabolism pathways (Fig. 2A). Detailed information (gene ID, symbol, gene name, fold-change and *P*-value) of some metabolism-associated genes is summarized in Table S2.

3.3. Altered metabolism associated genes upon EBV-miR-BART1 overexpression in NPC cells

Furthermore, we used RT-qPCR to selectively verify some altered metabolism-genes identified by RNA-seq in both EBV-miR-BART1-overexpressing CNE1 and 6–10B cells (Fig. 2B and C, Table S1). As shown in Fig. 2B and C, some genes including G6PD, SAT1, ASS1, PAST1, FUT1, SGPL1, DHRS3, B4GALT1, PHGDH, IDH2, and PISD were significantly up-modulated, and some such as, UGT8, GPAM, LDHB and GALNT1 etc. appeared to be down-modulated.

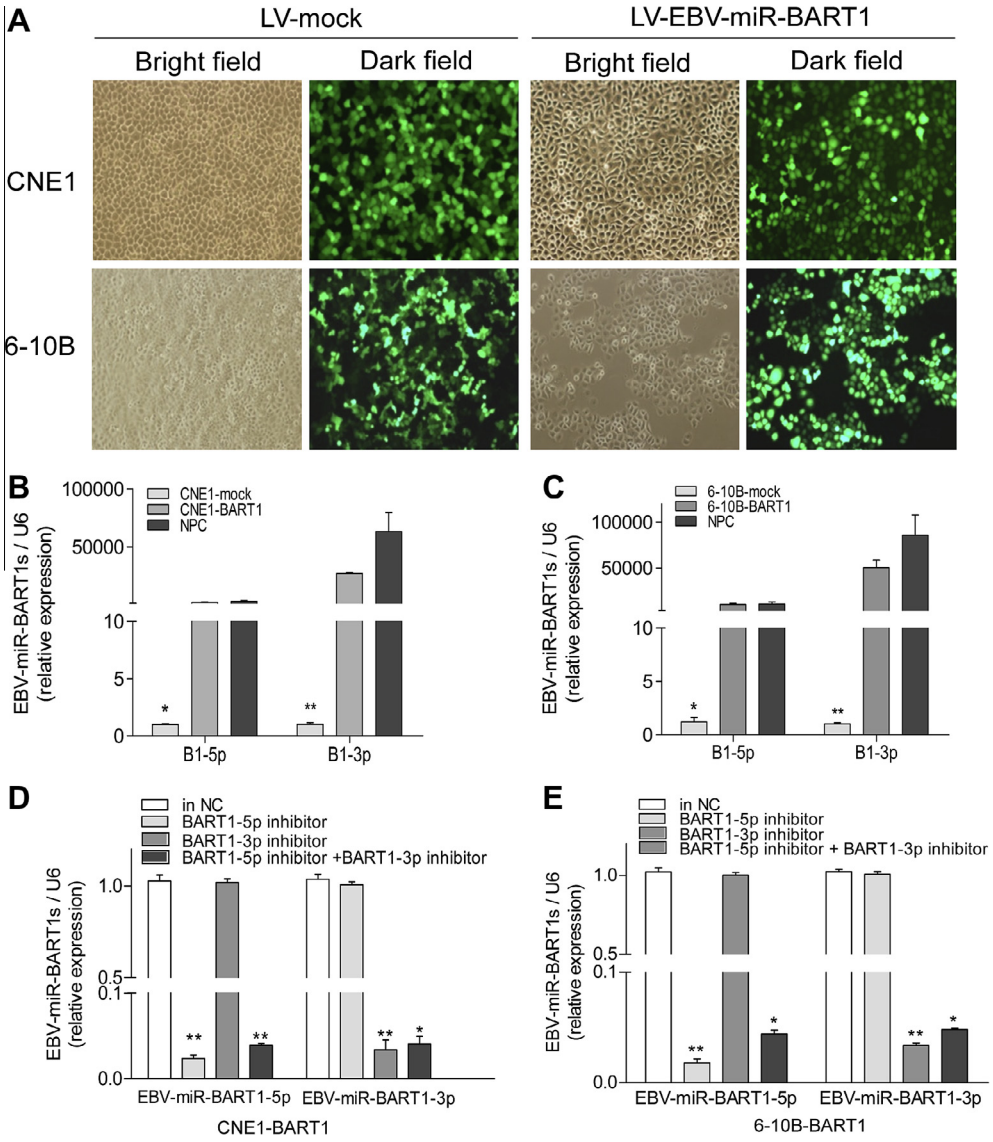

Fig. 1. EBV-miR-BART1 overexpression and down-regulation in human nasopharyngeal carcinoma (NPC) cells. (A–C) Establishment of stable NPC cell lines expressing EGFP and EBV-miR-BART1. (A) EGFP assay under inverted fluorescence microscopy; (B) expression of EBV-miR-BART1 in CNE1 cells carrying pre-EBV-miR-BART1 transgene and clinical NPC specimens by RT-qPCR; (C) expression of EBV-miR-BART1 in 6–10 B cells carrying pre-EBV-miR-BART1 transgene and clinical NPC specimens by RT-qPCR. (D–E) Detection of the expression levels of EBV-miR-BART1-5p and EBV-miR-BART1-3p after CNE1-BART1 and 6–10B-BART1 cells was transiently transfected with EBV-miR-BART11-5p, EBV-miR-BART1-3p or EBV-miR-BART1-5p and EBV-miR-BART1-3p for 36 h.

3.4. Alteration of metabolism associated genes in EBV-miR-BART1 inhibition NPC cells

In order to confirm the effects of EBV-miR-BART1 down-regulation on metabolism associated genes induced by EBV-miR-BART1 overexpression. EBV-miR-BART1 inhibitors were transiently transfected into CNE1-BART1 and 6–10B-BART1 cells, respectively. As shown in Fig. 3A and B, the results of RT-qPCR showed that anti-EBV-miR-BART1 inhibitors could significantly decrease the expression of G6PD, SAT1, ASS1, PAST1, FUT1, SGPL1, DHRS3, B4GALT1, PHGDH, IDH2 and PISD upregulated by EBV-miR-BART1 overexpression, and increase the expression of UGT8, GPAM, LDHB and GALNT1 downregulated by EBV-miR-BART1 overexpression.

3.5. EBV-miR-BART1 and metabolism associated genes altered in NPC tissues

To further validate what we found in NPC cells, we detected EBV-miR-BART1 mature sequences (EBV-miR-BART1–5p and EBV-miR-BART1–3p) in 30 NPC tissues and 8 CNP specimens with Taqman real-time PCR and found that both EBV-miR-BART1–5p and EBV-miR-BART1–3p were significantly upregulated in NPC tissues (Fig. 4A, $P < 0.01$). Compared to CNP tissues, qRT-PCR showed that consistent alterations of some metabolism associated genes, such as increased expression of G6PD, SAT1, ASS1, PAST1, FUT1, SGPL1, DHRS3, PHGDH, IDH2 and LDHB, and decreased expression of B4GALT1 and UGT8, were obviously observed in NPC specimens. Notably, PSAT1 and PHGDH were significantly upregulated in NPC tissues (Fig. 4B, $P < 0.05$), suggesting that EBV-miR-BART1 may play roles in influencing the expression of these metabolism associated genes in NPC.

4. Discussion

EBV is a herpesvirus associated with NPC, GC and other malignancies. It is the first human virus found to express miRNAs [16], most of whose functions remain largely unknown [17]. Recent evidence illustrates that EBV-encoding miR-BART1 has been found

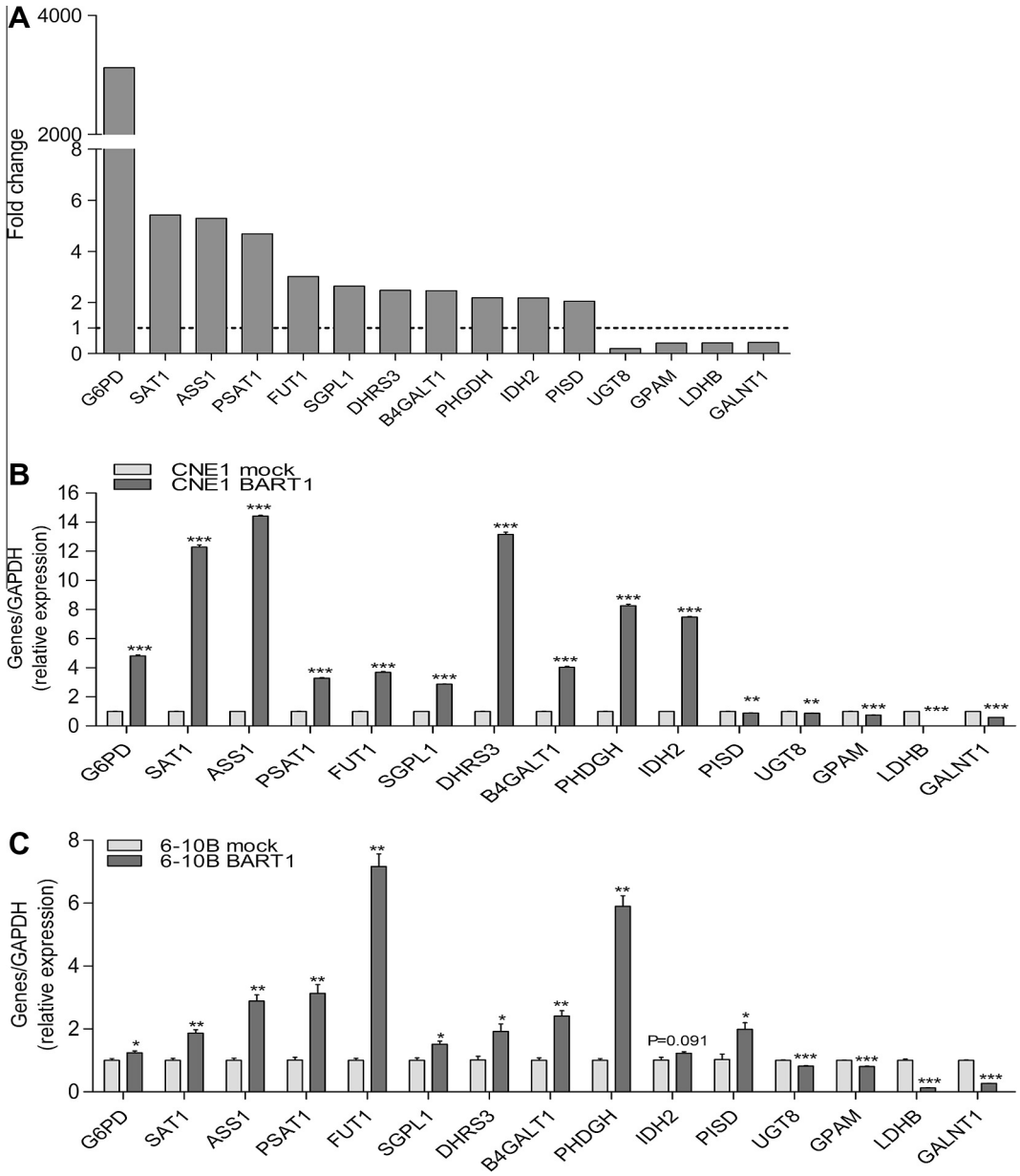

Fig. 2. EBV-miR-BART1 overexpression modulated metabolism associated genes in NPC cells. (A) The expression fold change of the representative differentially metabolism-associated genes between LV-EBV-miR-BART1 infected CNE1 cells and LV-mock infected CNE1 cells. Gene expressions of CNE1-BART1 and CNE1-mock were analyzed by RNA deep sequencing. The horizontal dashed line marks a fold change of 1 (no change). (B) RT-qPCR validation of the representative increased or decreased mRNA level of metabolism-associated genes, relative to a reference gene (GAPDH) in CNE1-BART1 cells. RT-qPCR experiments were performed in triplicate and error bars represent means ± S.E.M. from three independent experiments ($^{**}P < 0.01$, $^{***}P < 0.001$, Student's t test). (C) mRNAs expression levels of the representative metabolism-associated genes in 6–10B-BART1 cells with RT-qPCR analysis. GAPDH was used as a reference gene, RT-qPCR experiments were performed in triplicate and error bars represent means ± S.E.M. from three independent experiments ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's t test).

highly expressed in several EBV-associated malignancies, including NPC, gastric carcinoma and lymphomas [7,12,14,18]. Lo identified LMP1 as a putative target of EBV-miR-BART1–5p in NPC [19], but Skalsky figured out that LMP1 could not be directly targeted by EBV-miR-BART1–5p [14] in lymphoblastoid cells. These reports indicate that the exact role of EBV-miR-BART1 in tumor is still controversial and needed to be investigated.

Metabolic pathway alterations, including aerobic glycolysis and the Warburg effect, are known as a robust metabolic hallmark of tumor cells [20–22]. In the past decade, miRNAs have emerged as a distinct class of gene regulators involved in the biological and pathological processes, including cancer cell metabolism [23]. There are several lines of evidence that miRNAs predominantly regulate central metabolic pathways, such as amino acid biosynthesis, certain sugar and lipid metabolism, in cancer cells [24–25]. Recent studies document a role for c-Myc in stimulating glutamine catabolism partially through the repression of miRNAs miR-23a and miR-23b [26–27]. Moreover, miRNAs, such as miR-375, miR-143, miR-14, miR-29b, miR-33a/b, miR-103 and miR-107, participate in controlling various physiological and cancer cell metabolism by regulating their target genes whose protein products either directly regulate metabolic machinery or indirectly modulate the expression of metabolic enzymes, serving as master regulators, which will hopefully lead to a new therapeutic strategy for malignant cancer [1,28]. In this study, we applied RNA deep sequencing approach [15] to investigate the transcriptome and expression profile of EBV-miR-BART1 overexpressing CNE1 cells. We found that overexpression of EBV-miR-BART1 could

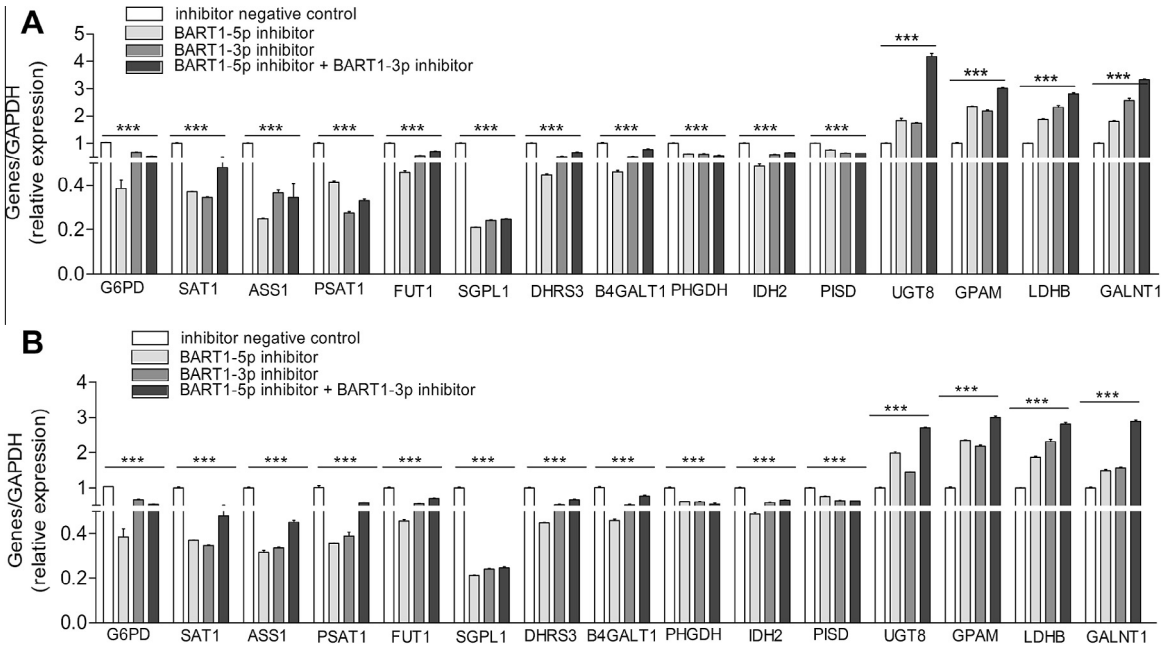

Fig. 3. EBV-miR-BART1 modulated metabolism associated gene expression in CNE1-BART1 and 6–10B-BART1 cells treated by EBV-miR-BART1 inhibitors. (A) CNE1-BART1 cells were transiently transfected with EBV-miR-BART1-5p, EBV-miR-BART1-3p, EBV-miR-BART1-5p and EBV-miR-BART-3p inhibitors or inhibitor control (50 nM) for 36 independently, followed by evaluating metabolism-associated genes expression using RT-qPCR. (B) 6–10B-BART1 cells were transiently transfected with EBV-miR-BART1-5p, EBV-miR-BART1-3p, EBV-miR-BART1-5p and EBV-miR-BART-3p inhibitors or inhibitor control (50 nM) for 36 independently, followed by evaluating metabolism-associated genes expression using RT-qPCR. GAPDH was used as a reference gene, RT-qPCR experiments were performed in triplicate and error bars represent means ± S.E.M. from three independent experiments (***$P < 0.001$, ONE-WAY ANOVA).

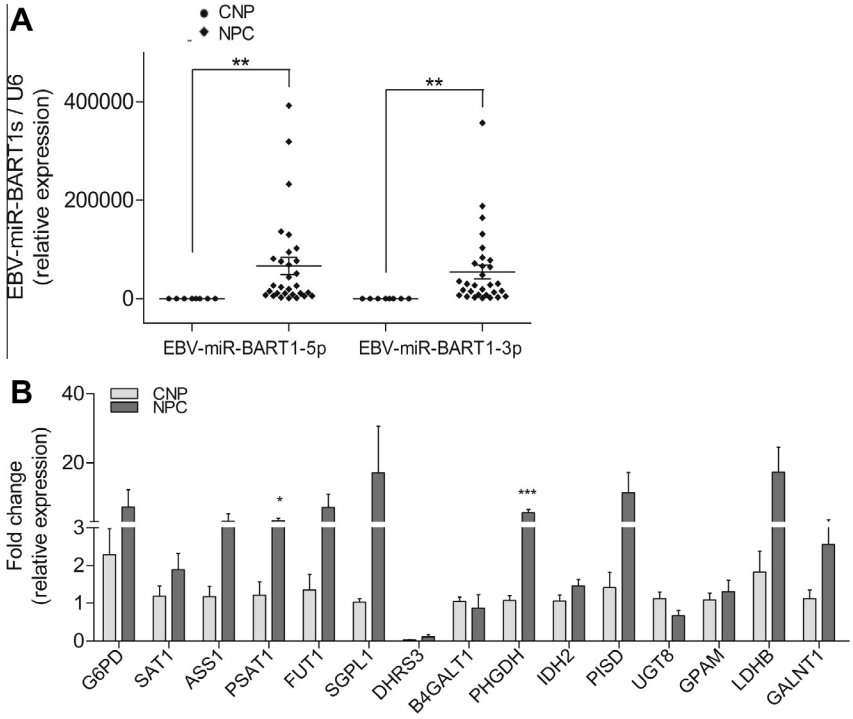

Fig. 4. EBV-miR-BART1 and EBV-miR-BART1 modulated metabolism associated genes expression in 30 NPC and 8 CNP specimens. (A) EBV-miR-BART1-5p and EBV-miR-BART1-3p expression in NPC and CNP tissues with Taq-man RT-qPCR analysis. U6 was used as a reference gene, RT-qPCR experiments were performed in triplicate and error bars represent means ± S.E.M. from three independent experiments (**$P < 0.01$, Student's t test). (B) The representative metabolism-associated genes expression in NPC and CNP tissues by SYBR green RT-qPCR analysis. GAPDH was used as a reference gene, RT-qPCR experiments were performed in triplicate and error bars represent means ± S.E.M. from three independent experiments (*$P < 0.05$, ***$P < 0.001$, Student's t test).

significantly influence the expression of metabolism associated genes including G6PD, SAT1, ASS1, PAST1, FUT1, SGPL1, DHRS3, B4GALT1, PHGDH, IDH2, PISD, UGT8, LDHB and GALNT1 etc. Moreover, inhibition of EBV-miR-BART1 in CNE1-BART1 and 6–10B-BART1 cells could significantly altered the metabolism associated genes expression induced by EBV-miR-BART1 overexpression.

Consistently, these genes were obviously altered in clinical NPC tissues as well compared with CNP specimens though the differences of some genes were less significant due to possibly insufficient sample size. These results indicated that EBV-miR-BART1, a virus-encoded miRNA might be involved in cancer metabolism in NPC. Notably, of altered metabolism-associated genes, PAST1 and PHGDH appeared to be significantly upregulated in NPC, suggesting the more important roles of these two genes in cancer metabolism in NPC. Our results were consistent with other previous studies. In an analysis of human cancers, PHGDH genomic recurrently amplification was showed to be able to divert glucose-derived carbon into a specific biosynthetic pathway and its change in metabolism can be selected for in the development of human cancer [29]. Focal copy number gain in PHGDH also suggested that its expression might be important for proliferation of cancer cells, and stable knock-down of PHGDH or the downstream enzymes PSAT could reduce cell proliferation [29]. Nadia et al. reported that overexpression of phosphoserine aminotransferase PSAT1 could stimulate cell growth and increase chemoresistance of colon cancer cells [30].

Collectively, we for the first time found that EBV-miR-BART1 could influence the expression of metabolism-associated genes (e.g. PAST1, PHGDH, DHRS3, ASS1, IDH2, PISD, UGT8, LDHB etc.) and might be involved in cancer metabolism in NPC. Therefore, a larger clinical sample-size validation and a deeper functional exploration are required to elucidate the exact contribution of EBV-miR-BART1 to the tumorigenesis of NPC by modulating genes implicated in metabolic processes in the future.

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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.2013.05.008.

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