Epstein–Barr virus-encoded microRNA BART1 induces tumour metastasis by regulating PTEN-dependent pathways in nasopharyngeal carcinoma

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Epstein–Barr virus (EBV), aetiologically linked to nasopharyngeal carcinoma (NPC), is the first human virus found to encode many miRNAs. However, how these viral miRNAs precisely regulate the tumour metastasis in NPC remains obscure. Here we report that EBV-miR-BART1 is highly expressed in NPC and closely associated with pathological and advanced clinical stages of NPC. Alteration of EBV-miR-BART1 expression results in an increase in migration and invasion of NPC cells in vitro and causes tumour metastasis in vivo. Mechanistically, EBV-miR-BART1 directly targets the cellular tumour suppressor PTEN. Reduction of PTEN dosage by EBV-miR-BART1 activates PTEN-dependent pathways including PI3K-Akt, FAK-p130Cas and Shc-MAPK/ERK1/2 signalling, drives EMT, and consequently increases migration, invasion and metastasis of NPC cells. Reconstitution of PTEN rescues all phenotypes generated by EBV-miR-BART1, highlighting the role of PTEN in EBV-miR-BART1-driven metastasis in NPC. Our findings provide new insights into the metastasis of NPC regulated by EBV and advocate for developing clinical intervention strategies against NPC.

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Nasopharyngeal carcinoma (NPC) represents a serious health problem in Southern China and Southeast Asia and has a high metastatic potential. Most patients with the disease are diagnosed only when the tumour has reached an advanced stage (stages III and IV). Interactions among environmental carcinogens, genetic alternations and viral infections are believed to play a major role in the pathogenesis of NPC. However, the molecular mechanisms for the metastases of NPC remain obscure.

The Epstein–Barr virus (EBV), latently infected >90% of the global population, is etiologically linked to several human lymphocytic and epithelial malignancies including Burkitt’s lymphoma, Hodgkin’s disease, post-transplant lymphoma, gastric cancer (GC) and NPC. To date, most studies have focused on EBV-encoded proteins such as the latent nuclear antigen EBNA1 (ref. 4) and the latent membrane proteins LMP1, LMP2A and LMP2B, which have the abilities to transform cells, to alter cellular gene expression and to increase the growth, survival and invasion of transformed cells. EBV-associated tumours usually express a restricted repertoire of viral proteins in a histological specific fashion, suggesting that the pathogenesis and progression of these tumours are not only regulated by viral proteins but also by other important factors such as EBV-encoded microRNAs (EBV-miRs).

MicroRNAs (miRNAs), a class of 17–23 nucleotide non-coding RNAs, are capable of inhibiting the expression of multiple target genes by forming imperfect complementary duplexes with their target miRNAs in the 3′- untranslated region (3′-UTR), leading to mRNA degradation or translational inhibition, and participate in diverse biological processes including tumorigenesis and metastasis. EBV is the first human virus found to encode miRNAs. To date, a total of 25 EBV-miR precursors containing 48 mature miRNAs have been identified within two regions of the EBV genome. The BamHI fragment H rightward reading frame 1 (BHRF1) gene encodes 3 miRNA precursors (that is, EBV-miRBART1-1–3) generating four mature miRNAs, while the BamHI fragment A rightward transcript (BART) region contains 22 miRNA precursors (that is, EBV-miR-BART1-22) producing 44 mature miRNAs. The abundance of individual miRNAs within cell lines and tumour tissues varies widely and appears to be cell-type and tumour-type specific. The expression of EBV-miR-BHRF1 was highly restricted to lytic and latency III-infected cell lines such as B lymphoma cells, whereas EBV BART miRNAs were detected in all types of EBV-associated latency.

Interestingly, the expression of BART miRNAs was shown to be relative low in EBV-infected B lymphocytes but extremely high in infected epithelial tissues, suggesting a pathogenic role in the development of epithelial malignancies.

Many EBV BART miRNAs were found highly expressed in NPC and GC, contributing to viral latency and immune escape. However, their functions in tumorigenesis and tumour progression of NPC remain elusive. We are interested in the viral and cellular miRNAs in the tumour biology and clinical significance in NPC. In this study, we report that EBV-miR-BART1 is highly expressed in NPC and closely associated with pathological features of NPC. Alterations of the expression of EBV-miR-BART1 demonstrated that it induces the migration and invasion of NPC cells in vitro and the tumour metastasis in vivo. The underlying molecular mechanisms by which BART1 causes tumour metastasis are revealed to directly target the major tumour suppressor, phosphatase and tensin homologue (PTEN) and consequently to activate PTEN-dependent pathways and induce epithelial–mesenchymal transition (EMT). Our findings provide new insights into the metastasis of NPC regulated by EBV and advocate for developing clinical intervention strategies for NPC.

Results

EBV-miR-BART1 is associated with pathological features. We performed miRNA expression profile microarray analysis of 20 NPC versus 20 non-cancerous nasopharyngeal (NP) tissues (Supplementary Table 1) and identified 69 miRNAs that were differentially expressed between NPC and NP samples (Fig. 1a). Of them, 24 miRNAs were upregulated and 45 miRNAs were downregulated in NPC specimens. Intriguingly, among all upregulated miRNAs, 62.5% (15/24) were EBV BART miRNAs, while only 29.2% (7/24) were human cellular miRNAs (Supplementary Table 2). In contrast, all downregulated miRNAs were host derived, of which a large proportion (55.6%, 25/45) were tumour suppressive miRNAs such as the let-7 family (hsa-let-7c, d, g and i), miR-34 family (miR-34c and d), miR-29 family (miR-29a, b and c), miR-30 family (miR-30a-5p, 3a-3p, b, d and e), miR-26 family (miR-26a and b), miR-143/145 cluster, miR-16 (ref. 36), miR-31 (ref. 37), miR-101 (ref. 38), miR-146a (ref. 40), miR-150 (ref. 40), miR-195 (ref. 41) and miR-199 (ref. 42). Notably, BART1, BART7, BART3, BART10, BART8, BART9 and BART5 were in the top seven and clustered together, suggesting that EBV BART miRNAs may play a critical role in NPC.

To investigate if the EBV-miR-BART1 expression is associated with pathological features of NPC, we determined the expression of EBV-miR-BART1 in another cohort of 82 NPC samples that contain the TNM-stage information (Supplementary Table 3). The expression of either BART1-5p (3.8-fold) or BART1-3p (2.9-fold) was dramatically increased in N2–3 stages as compared with N0–1 stages (Fig. 1b). Similarly, a substantial higher level of BART1-5p (3.4-fold) or BART1-3p (3.0-fold) was observed in advanced clinical stage III–IV than in early clinical stage I–II (Fig. 1c). Thus, the results suggest that EBV-miR-BART1 might contribute to the metastasis of NPC.

EBV-miR-BART1 impels NPC metastasis in vitro and in vivo. We upregulated the expression of BART1 in two EBV-negative NPC cell lines (CNE1 and 5-8F) by lentivirus-mediated transduction (Supplementary Fig. 1). Transwell migration assays (Fig. 2a) and wound-healing assays (Supplementary Fig. 2) demonstrated that upregulation of BART1 dramatically increased the cell migration of either CNE1-BART1 or 5-8F-BART1 compared with the relative control. Boyden chamber invasion assays (Fig. 2b) revealed that the upregulation of BART1 significantly increased the cell invasion of both CNE1-BART1 and 5-8F-BART1 compared with the relative control. To verify the results, we downregulated the expression of BART1 in CNE1-BART1 and 5-8F-BART1 by transfection of BART1-inhibitory oligonucleotides (Supplementary Fig. 3). Consistent with the upregulation results, downregulation of BART1 by either in-b1-5p or in-b1-3p dramatically decreased the migration (Fig. 2c) and invasion (Fig. 2d) of either CNE1-BART1 or 5-8F-BART1 cells compared with the relative in-NC control. An additive effect on cell migration or invasion was observed when combined the two BART1 inhibitors (Fig. 2c,d). Together these results suggest that EBV-miR-BART1 propels the migration and invasion of NPC cells in vitro.

We xenografted 5-8F-BART1 cells into nude mice. Upregulation of BART1 significantly increased the tumour growth of 5-8F-BART1 compared with the 5-8F-mock control (Fig. 2e,f). Transplantation of 5-8F cells underneath the liver envelop demonstrated that the upregulation of BART1 promoted the tumour metastasis of 5-8F-BART1 compared with the 5-8F-mock control (Fig. 2g). Three weeks after transplantation, 86% of mice (6/7) in the 5-8F-BART1 group showed both liver and lymph node metastases compared with
14% of mice (1/7) in the 5-8F-mock group (Chi-square test \(P<0.05\)); 43% of mice (3/7) in the 5-8F-BART1 group displayed lung metastasis compared with 14% of mice (1/7) in the control group (Fig. 2h). In the metastasized lymph nodes, 45 tumour nodules in the 5-8F-BART1 tumours were observed compared with five nodules in the control tumours (6.43 ± 2.13 versus 0.71 ± 0.71, Student’s \(t\)-test \(P<0.05\); see Fig. 2h). Re-examination of EBV-miR-BART1 expression in liver-metastasized tumours by quantitative reverse transcription PCR (qRT–PCR) demonstrated that the metastasized tumour cells stably expressed BART1-5p and BART1-3p at the levels close to that of NPC clinical samples (Supplementary Fig. 4). Thus, the results indicate that EBV-miR-BART1 impels the growth and metastasis of NPC cells in vivo.

**EBV-miR-BART1 directly targets cellular PTEN in NPC cells.**

We investigated BART1-targeted genes that are associated with tumour metastasis by developing an integrated strategy (Fig. 3a). RNA-deep sequencing analysis of the gene expression profiling of CNE1-BART1 cells versus CNE1-mock cells identified 995 downregulated genes, from which many metastasis-associated candidates can be retrieved (Supplementary Table 4 and Supplementary Fig. 5). Pathway enrichment analysis for these downregulated genes revealed that BART1 regulated many important signalling pathways such as p53 signalling pathway, pathway in cancer, MAPK pathway, regulation of actin cytoskeleton, Jak-STAT signalling pathway and focal adhesion pathway. At least 10 genes were significantly altered in each of these pathways by BART1. PTEN was identified as one of the top genes regulated by BART1 and involved in five tumour metastasis- or cell invasion-associated pathways.

Bioinformatics analysis showed that PTEN 3’-UTR region contains two sites complementary with the seed sequences of BART1-5p and BART1-3p from the nucleotide position 425 and 921, respectively (Fig. 3b). To clarify if PTEN is a direct cellular target for BART1, we performed luciferase reporter assays by co-transfection of a wild-type (wt) or mutant (mut 1 or mut 2) PTEN 3’-UTR-containing luciferase reporter vector with a

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**Figure 1** | **EBV-miR-BART1 is highly expressed in NPC and associated with clinicopathological features.** (a) MiRNA expression profile microarray screening. Supervised hierarchical cluster analysis of 69 miRNAs that were differentially expressed between 20 NPC and 20 NP biopsies (universal test \(P<0.005\), fold-change >1.5, false discovery rate <0.05). Left: heatmap of the 69 miRNAs differentially expressed between NPC and NP samples. Red represents upregulated miRNAs and blue for downregulated miRNAs. Right-up: the top seven upregulated EBV BART miRNAs clustered together. Right-down: a volcano plot for the visualization of differentially expressed miRNAs with significance cut off \(P<0.005\) and fold-change ≥1.5 symmetrically in NPC compared with NP. The top 11 upregulated EBV BART miRNAs in NPC were highlighted by a red dot-line square. (b) EBV-miR-BART1 was highly expressed in late pathological stages of NPC. The expression of EBV-miR-BART1 in a separated cohort of 82 NPC samples was determined by qRT-PCR. RPMU6B was used for normalizing the expression of BART1-5p or BART1-3p. Student’s \(t\)-test, mean ± s.e.m., *\(P<0.05\), **\(P<0.01\). (c) EBV-miR-BART1 was highly expressed in advanced clinical stages of NPC. Both BART1-5p and BART1-3p were highly expressed in advanced clinical stages (III-IV) compared with that of early clinical stages (I-II). Student’s \(t\)-test, mean ± s.e.m., *\(P<0.05\), **\(P<0.01\).
Figure 3 | EBV-miR-BART1 targets tumour suppressor PTEN in NPC. (a) Development of the integrated strategy for discovering targeted genes of EBV-miR-BART1. (b) Bioinformatic predictions of two binding sites by BART1-5p and BART1-3p in PTEN 3′-UTR region. Wild-type (wt) and two mutant sequences are indicated. (c) Luciferase reporter assays. HEK293T cells were co-transfected with wt or mutated PTEN 3′-UTR and the BART1 mimic (either b1-5p for BART1-5p or b1-3p for BART1-3p) or nonspecific mimic control (NC). Relative repression of firefly luciferase expression was standardized to a transfection control. One-way ANOVA and Dunnett’s multiple comparison test. (d) The expression of PTEN in two cell lines and tumour sections of 5-8F xenografts and metastasized organs. Up panels are representatives of IF staining for PTEN (red) in both CNE1-BART1 and 5-8F-BART1 cells and relative mock control. Nuclei were stained with DAPI (blue). Low panels are the representatives of IHC staining for PTEN in metastasized tumours (liver and lymph node) from 5-8F tumours. Original magnification, ×400; scale bar, top panel 10 μm, bottom panel 25 μm. (e) The expression of PTEN at mRNA level in 32 NP and 82 NPC specimens determined by qRT-PCR. (((b)–(d))) Luciferase activity

Figure 2 | EBV-miR-BART1 propels cell migration and invasion in vitro and tumour metastasis of NPC in vivo. (a) Transwell migration assays. Upregulation of BART1 miRNA increased the cell migration of both CNE1-BART1 and 5-8F-BART1 compared with relative mock cells. Student’s t-test. (b) Boyden chamber invasion assays. Upregulation of BART1 miRNA increased the cell invasion of both CNE1-BART1 and 5-8F-BART1 compared with relative mock cells. Student’s t-test. (c) Downregulation of BART1 miRNA by the BART1 inhibitor decreased the cell migration of both CNE1-BART1 and 5-8F-BART1. One-way ANOVA and Dunnett’s multiple comparison test. (d) Downregulation of BART1 miRNA by the BART1 inhibitor decreased the cell invasion of both CNE1-BART1 and 5-8F-BART1. One-way ANOVA and Dunnett’s multiple comparison test. (e) Xenograft tumours collected on day 21 post subcutaneous implantation of 5-8F cells. Upregulation of BART1 increased the tumour growth in nude mice (5-8F-BART1 versus 5-8F-mock). (f) Tumour growth curves. Upregulation of BART1 increased the tumour growth in nude mice (5-8F-BART1 versus 5-8F-mock). N = 7, parametric generalized linear model with random effects. (g) Representative GFP images of tumour metastasis. Upregulation of BART1 increased the tumour metastasis in lung, liver and lymph nodes. N = 7, two-tailed Spearman’s correlation analysis. One-way ANOVA and Dunnett’s multiple comparison test. Mean ± s.e.m., **P < 0.01, ***P < 0.001.
BART1 mimic. The luciferase activity of the wt PTEN 3'-UTR but not the mutant 3'-UTR (either mut 1 or mut 2) was significantly reduced by b1-5p mimic alone or b1-3p mimic alone but not by the control mimic (NC). The combination of b1-5p with b1-3p produced an additive effect on the luciferase activity (Fig. 3c).

The effects of EBV-miR-BART1 on PTEN mRNA and protein expression were examined in NPC cell lines, xenografted tumours and clinical samples. Upregulation of BART1 significantly reduced the expression of PTEN in both CNE1-BART1 and 5-8F-BART1 cell lines compared with the relative control as revealed by western blotting and immunofluorescent (IF) staining (Fig. 3d, upper panels). Immunohistochemical (IHC) staining on tumour sections of xenografts and metastasized organs demonstrated that the upregulation of BART1 significantly reduced the expression of PTEN in 5-8F-BART1 tumours compared with the 5-8F-control tumours (Fig. 3d, lower panels). Detection of the PTEN mRNA by qRT–PCR revealed that the expression of PTEN in clinical tumour samples was significantly decreased in N2–3 stages as compared with either N0–1 stages (0.1673 ± 0.0295 versus 0.3927 ± 0.0435, P < 0.0001) or NP (0.1673 ± 0.0295 versus 1.2100 ± 0.1247, P < 0.0001; Fig. 3e, left panel). Similarly, the expression of PTEN was significantly lower in advanced clinical stages III–IV than in either early clinical stages I–II (0.1683 ± 0.0210 versus 0.4088 ± 0.0331, P < 0.0001) or NP (0.1683 ± 0.0210 versus 1.2100 ± 0.1247, P < 0.0001; Fig. 3e, right panel). Strikingly, the expression of PTEN in NPC was highly inversely correlated with the expression of either BART1-5p (r = -0.5205, P < 0.0001; Fig. 3f, right panel) or BART1-3p (r = -0.3687, P < 0.0007; Fig. 3f, left panel). Taken together, these data indicate that PTEN is a direct cellular target of EBV-miR-BART1 in NPC.

**EBV-miR-BART1 activates PTEN-dependent signalling pathways.** We examined the expression alterations of key components of PTEN-dependent pathways in NPC. Western blotting showed

![Figure 4](image-url)
that the upregulation of BART1 significantly reduced the protein level of PTEN but increased the phosphorylation level of p-Akt, p-FAK, p130Cas, p-Shc or p-ERK1/2 in either CNE1-BART1 or 5-8F-BART1 cells when compared with the relative control (Fig. 4a). On the contrary, downregulation of BART1 by in-b1-5p alone, in-b1-3p alone or both inhibitors increased the PTEN level but decreased the phosphorylation of p-Akt, p-FAK, p130Cas, p-Shc or p-ERK1/2 in both CNE1-BART1 and 5-8F-BART1 cells (Fig. 4b). IHC staining on tumour sections of xenografts and metastasized organs demonstrated that the upregulation of BART1 significantly reduced the expression of PTEN (see Fig. 3d) but clearly increased the phosphorylation levels of p-Akt, p130Cas, p-Shc and p-ERK1/2 in 5-8F-BART1 tumours compared with the 5-8F-mock tumours (Fig. 4c). Collectively, these results indicate that EBV-miR-BART1 activates PTEN-dependent signalling pathways by decreased PTEN in NPC.

EBV-miR-BART1 compels EMT. We investigated the expression of E-cadherin, N-cadherin and vimentin, as well as the rearrangement of actin cytoskeleton, a characteristic event of EMT. Western blotting demonstrated that the upregulation of BART1 strikingly decreased the expression of E-cadherin but increased the expressions of N-cadherin and vimentin in both CNE1-BART1 and 5-8F-BART1 cells compared with the relative control cells (Fig. 5a). In agreement with the upregulation results, downregulation of BART1 by in-b1-5p alone, in-b1-3p alone or both inhibitors increased the E-cadherin expression but decreased the expression of N-cadherin and vimentin in both CNE1-BART1 and 5-8F-BART1 cells as compared with the in-NC control (Fig. 5b). IF staining on tumour sections of xenografts and metastasized organs demonstrated that the upregulation of BART1 overtly reduced the E-cadherin expression but increased the expression of vimentin in 5-8F-BART1 tumours compared with the 5-8F-mock control tumours (Fig. 5c).

We then performed IF staining for F-actin with Rhodamine-phalloidin. Confocal microscopy examinations showed that the upregulation of BART1 modestly increased the expression density of F-actin and triggered actin cytoskeleton rearrangement in both CNE1-BART1 and 6-8F-BART1 cells as compared with that of relative mock control cells. The organization of the actin bundles

**Figure 5 |** EBV-miR-BART1 compels EMT. (a) Upregulation of EBV-miR-BART1 expression by retrovirus transductions decreased the protein level of E-cadherin but increased the expression level of N-cadherin and vimentin in both CNE1-BART1 and 5-8F-BART1 cells. (b) Downregulation of EBV-miR-BART1 expression by the BART1 miRNA inhibitor increased the protein level of E-cadherin but decreased the expression level of N-cadherin and vimentin in both CNE1-BART1 and 5-8F-BART1 cells. (c) Upregulation of EBV-miR-BART1 decreased the expression of E-cadherin but increased the expression of vimentin in tumour sections of 5-8F metastasized organs revealed by IHC staining (5-8F-BART1 versus 5-8F-mock). Original magnification, ×400; scale bar, 25 μm. One-way ANOVA and Dunnett’s multiple comparison test. Mean ± s.e.m., N = 5, *P < 0.05, **P < 0.01. (d) EBV-miR-BART1 enhanced the rearrangement of actin cytoskeleton. Stress fibres and actin filaments were visualized by Rhodamine-phalloidin staining (red) in NPC cells transduced with lentivirus (green for GFP). Nuclei were stained with DAPI (blue). Upregulation of BART1 modestly increased F-actin expression (red) and actin stress fibres throughout the BART1-expressing cells compared with the control cells in which the actin bundles were predominantly localized underneath cell membranes. Original magnification, ×1,200; scale bar, 5 μm.
and associated proteins underneath the cell membranes (that is, cortical actin cytoskeleton) was clearly observed in either CNE1-mock or 5-8F-mock cells. In sharply contrast, dynamic structures of actin filaments and associated proteins (that is, actin stress fibre) throughout the cells, characteristic of migrating mesenchymal cells, were explicitly visualized in both CNE1-BART1 and 5-8F-BART1 cells (Fig. 5d). Taken together, the results suggest that EBV-miR-BART1 promotes cell motility and invasiveness by driving EMT and increasing actin stress fibres in NPC cells.

Reconstituted PTEN rescued the phenotypes produced by BART1. We transfected the PTEN expression vector into CNE1-BART1 and 5-8F-BART1 cells. Restitution of PTEN expression (Supplementary Fig 6) significantly reduced the migration (Fig. 6a) and invasion (Fig. 6b) of both CNE1-BART1 and 5-8F-BART1 cells compared with the relative PTEN-negative plasmid control (NC). Detection of the key components of PTEN-dependent pathways by western blotting demonstrated that the restoration of PTEN protein decreased phosphorylation levels of p-Akt, p-FAK, p130Cas, p-Shc and p-ERK1/2 in both CNE1-BART1 and 5-8F-BART1 cells (PTEN versus NC). (d) Restoration of PTEN dosage increased the expression of E-cadherin but decreased the expression of either N-cadherin or vimentin in both CNE1-BART1 and 5-8F-BART1 cells (PTEN versus NC). Original magnification, × 200; scale bar, 25 μm. Student’s t-test, mean ± s.e.m., N = 3. ***P < 0.001.

Reproducibility of the phenotypes in EBV-positive NPC cells. We quantified the expression of EBV-miR-BART1 in three EBV-positive NPC cell lines (that is, C666-1-EBV, HONE1-EBV and HK1-EBV) by qRT–PCR. The expression levels of both BART1-5p and BART1-3p were found comparable among three EBV-positive cell lines although lower in the cell lines than in NPC clinical samples (Supplementary Fig. 7a). We used HONE1-EBV cell line as a representative to validate the function of BART1 in NPC metastasis and underlying molecular mechanisms.
Transwell migration and Boyden chamber invasion assays demonstrated that the downregulation of endogenous BART1 by either in-b1-5p or in-b1-3b (Supplementary Fig. 7b) significantly decreased the migration (Fig. 7a) and invasion (Fig. 7b) of HONE1-EBV-positive cells compared with the in-NC control. An additive effect was obtained when combined the two BART1 inhibitors (see Fig. 7a,b). Examination of the key components of PTEN-dependent pathways by western blotting showed that the downregulation of endogenous BART1 increased PTEN expression but decreased the phosphorylation of p-Akt, p-FAK and p-ERK1/2 in HONE1-EBV-positive cells compared with the in-NC control (Fig. 7c). In agreement with the results obtained from EBV-negative NPC cell lines (see Fig. 5b), downregulation of endogenous BART1 increased E-cadherin expression but decreased the expression of N-cadherin and vimentin in HONE1-EBV-positive cells. Thus, the results from the EBV-positive cell line substantiate that EBV-miR-BART1 promotes cell motility and invasiveness by activating PTEN-dependent signalling pathway and driving EMT.

**Discussion**

In this study, we have demonstrated that EBV-miR-BARTs are highly upregulated while cellular miRNAs are largely...
In NPC specimens, EBV-miR-BART1, 7, 3, 10, 8, 9, 5, 6, 2, 14 and 16 were all upregulated (Supplementary Table 2), reminiscent of the pattern of abundance of individual BART miRNAs in C666-1-EBV-positive cell line, C15 xenograft tumour and clinical samples of NPC10,15,16,20,24,46, highlighting the association of latent EBV infection with NPC and the importance of EBV BART miRNAs in NPC. Surprisingly, more than half (55.6%) of the downregulated miRNAs are tumour suppressor miRNAs, including the let-7 family, miR-34 family, miR-29 family, miR-30 family, miR-26 family, miR-143/145 cluster, miR-16, miR-31, miR-101, miR-146a, miR-150, miR-152 and miR-195, suggesting that effects of EBV infection on NPC may be partially through regulating the abundance of cellular tumour suppressive miRNAs. Consistent with our findings, EBV-infected AGS cells were shown to decrease the overall abundance of human miRNAs with specific downregulation of three types of tumour suppressive miRNAs, let-7 family, miR-200 family and miR-143 (refs 5,16).

Several of these upregulated EBV BART miRNAs have been partially defined in epithelial carcinomas such as BART5 that inhibited cell apoptosis through targeting PUMA18, BART* that promoted cellular growth by targeting DICE1 (ref. 47), BART5, 1-5p, 1-17p that have been linked to viral latency by targeting LMP1 (refs 23,48), BART9 that increased tumour metastasis by targeting E-cadherin19, and BART7-3p that promoted tumour metastasis by regulation of the PTEN-P13K/Akt-EMT pathway50. Here we demonstrated that EBV-miR-BART1 is closely associated with pathological and clinical stages, suggesting that BART1 may contribute to NPC metastasis, underscoring the clinical significance. To test this hypothesis, we modulated the expression of BART1 in both EBV-negative and EBV-positive NPC cells and revealed that BART1 significantly propels the migration and invasion of NPC cells in vitro and the tumour metastasis in vivo animal models. Thus, our findings shed new lights on the association of EBV BART miRNAs with NPC metastasis.

To explore underlying mechanisms of EBV-miR-BART1 in NPC invasion and metastasis, we exploited the integrated strategy consisting of RNA-deep sequencing, literature retrieval and pathway enrichment analysis, bioinformatics prediction, luciferase reporter assay and expression validation of targeted gene in cell lines, xenograft tumours and clinical samples to investigate targeted genes and related signalling pathways. We have identified 995 downregulated genes and many important pathways regulated by BART1. We found that PTEN is the major cellular target of BART1 in NPC. PTEN, one of the most frequently mutated and deleted tumour suppressor in the multitude of tumour types34, has frequently been reported downregulated in NPC52,53 while the mutation or deletion of PTEN has rarely been detected in oral squamous cell carcinoma34, suggesting other mechanisms in NPC are likely responsible for the downregulation of PTEN. Consistently, we found that the expression of PTEN is extremely lower (4.4-fold) in NPC than that of NP and highly inversely correlated with the expression of BART1-5p or BART1-3p in NPC. More strikingly, the expression PTEN is decreased by 2.4-fold in advanced stages III–IV compared with that of early clinical stages I–II, concomitant with the higher expression of BART1 (3.4-fold for BART1-5p, 3.0-fold for BART1-3p) in advanced stages III–IV than that of early stages I–II. Recently, many human miRNAs including but not limited to miR-26a, 29b, 141, 144, 205 and 221 were reported to target PTEN in various tumours35,55–57. However, we found that several of these miRNAs including miR-26a, 29b, 144 and 221 are significantly downregulated in NPC (see Supplementary Table 2), implying that EBV BART miRNAs in NPC may be more important than cellular miRNAs in the PTEN dosage regulation. Indeed, BART1 (both BART1-5p and BART1-3p) and BART7-3p target PTEN in NPC. It was noted that, based on bioinformatics analysis or PAR-CLIP predictions, several BART miRNAs (that is, BART9 and BART18-5p in NPC cells; BART2-3p, BART5, BART14* and BART21-3p in BC1 or AGS cell) would be possible to regulate PTEN54,56. However, their authenticity in epithelial carcinomas remains to be determined.

PTEN functions both as a dual specificity protein phosphatase and a lipid phosphatase59. The principal catalytic function of PTEN is to dephosphorylate phosphatidylinositol 3,4,5-trisphosphate (PIP3), a secondary messenger for Akt, to phosphatidylinositol 4,5-bisphosphate, thereby suppressing PI3K-Akt signalling by preventing PI3P-dependent processes such as the membrane recruitment and the activation of Akt and PKC. PTEN is also able to dephosphorylate protein substrates such as FAK and Shc, thus inhibiting the FAK-p130Cas pathway60. Moreover, PTEN is capable of regulating MAPK-ERK1/2 signalling either by dephosphorylating Shc (resulting in the inactivation of Ras, Raf and ultimately ERK1/2) or by reducing PIP3-mediated activation of PKC and Akt (leading to the suppression of Raf, MEK and ERK1/2)61. In NPC, we found that BART1 significantly increased the phosphorylation levels of p-Akt, p-FAK, p130Cas, p-Shc and p-ERK1/2 in NPC cell lines. Importantly, such expression alterations also occurred in vivo, as revealed by IHC staining on tumour sections of xenografts and metastasized organs. Therefore, we concluded that EBV-miR-BART1 impels the migration and metastasis by targeting cellular PTEN and consequently activating PI3K-Akt, FAK-p130Cas and MAPK-ERK1/2 pathways in NPC.

EMT, a fundamental biological process that enables polarized epithelial cells to convert mesenchymal-like cells, which includes the rearrangement of actin cytoskeleton and enhanced migratory and invasive capacity, is considered as the initial step of tumour metastasis62. FAK/p130Cas, PI3K/Akt and MAPK/ERK pathways were known to regulate EMT and promote cell migration45,62. In this study, we demonstrated that EBV-miR-BART1 in NPC cells significantly reduced the expression of E-cadherin, an important caretaker for the epithelial phenotype, but increased the expression of N-cadherin and vimentin, two goalkeeper for the mesenchymal phenotype. Consistently, downregulation of EBV-miR-BART1 increased the expression of E-cadherin but decreased the expression of N-cadherin and vimentin in NPC cell lines. IHC staining on tumour sections of xenografts and metastasized organs confirmed that BART1 decreased the expression of E-cadherin but increased the expression of vimentin in vivo. In clinical tumour samples, the expression of E-cadherin was reported downregulated and inversely correlated with lymph node metastasis of NPC63. Interestingly, we also found that BART1 modestly increased F-actin level and triggered actin cytoskeleton rearrangement in NPC cells apparently by activating FAK-p130Cas and Shc-MEK-ERK1/2 pathways59–61. Collectively, our findings indicated that EBV-miR-BART1 increases cell motility and invasiveness by impelling EMT and increasing actin stress fibres in NPC cells.

In conclusion, we have demonstrated that EBV-miR-BART1 is highly expressed in NPC and closely associated with advanced stages of the patients. BART1 increased the cell migration and invasion of NPC in vitro and impelled the tumour metastasis in vivo by directly targeting PTEN. Reduction of PTEN dosage by BART1 activated PTEN-dependent pathways, compelled EMT and consequently increased the migration, invasion and metastasis of NPC (Fig. 7d). Reconstitution of PTEN expression rescued all phenotypes generated by BART1, highlighting the dominant role of PTEN in EBV-miR-BART1-driven metastasis in NPC. Our findings provide new insights into the invasiveness and
metastasis of NPC regulated by EBV and rational for the development of clinical intervention strategies for NPC.

Methods

Cell culture. Two EBV-negative NPC cell lines (CNE1 and 5-8F) and HEK293T cells were obtained from Cancer Research Institute Southern Medical University. Three EBV-positive NPC cell lines (HONE1-EBV, HK1-EBV and C661-EBV) were kindly provided by Professor W.-S. Tsao, University of Hong Kong. The NPC cell lines were cultured in RPMI-1640 (Invitrogen) supplemented with 10% newborn cow serum (NCS; Hyclone, Invitrogen), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin, while HEK293T cells grew in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% NCS, 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin. All cells were maintained at 37°C with 5% CO₂.

Patients and clinical tissue specimens. Primary NPC (no treatment before biopsy taking) and NP biopsies were collected from patients in Zhongshan People’s Hospital, Guangdong, China. Informed written consent was obtained from all subjects and approval from the Ethics Committee of Zhongshan People’s Hospital was obtained for research purposes in use of clinical materials. Twenty NPC and 20 NP specimens were used for microarray analysis and qRT-PCR validation (Supplementary Table 1) and another cohort containing 82 NPC specimens with TNM staging and 32 NP specimens were collected for the association analysis of EBV-miR-BART1 expression with pathological and clinical data (Supplementary Table 3).

Microarray analysis. miRNA and mRNA, respectively. The fold changes were calculated by using the expression of All-in-One miRNA qRT–PCR Detection Kit following the manufacturer's protocol (GeneCopoeia). RPU6B and GAPDH were used for normalizing the expression of complementary DNA (cDNA) was synthesized with the PrimeScript RT reagent Kit (Takara, Dalian, China). qRT-PCR was performed in triplicate with SYBR Premix Ex Taq (Takara, Dalian, China). The primers used for amplification of the targeted genes were listed in Supplementary Table 5. Quantification of EBV-miR-BART1 was conducted with TaqMan microRNA assays (Applied Biosystems). Mature miRNAs were reverse transcribed, and qRT-PCR was performed using All-in-One miRNA qRT–PCR Detection Kit following the manufacturer’s protocol (GeneCopoeia). EUPR and GAPDH were used for normalizing the expression of miRNA and mRNA, respectively. The fold changes were calculated by using the formula 2⁻ΔΔCt method.

MiRNA microarray analysis. MiRNA microarrays (CCDDTM-miRNA850-V4p1.4) were kindly provided by Infectious Disease and Immunogenetics section, Department of Transfusion Medicine, Clinical Center, National Institutes of Health, USA. Total RNA was labelled by miRCURY LNA miRNA Power Labeling Kit (Exiqon Life Sciences, Vedbaek, Denmark) according to the manufacturer’s instructions. The test sample and the reference were labelled with Hy5 and Hy3, respectively, and co-hybridized to the miRNA arrays. The hybridized arrays were scanned by LuxScanTM 10 K microarray Scanner (Capital Bio, Corporation, Beijing, China). The resulting data files were uploaded to the miRdb database. Any gene containing one of the follows was excluded: (1) <20% of expression data had at least a 1.5-fold change in either direction from gene’s median value; (2) per cent data missing of filtered out exceeds 50%. Further analysis was done by using the BRB-Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.html). Expression profile clustering and visualization were performed with Cluster and Treeview software (Ernest Orlando Lawrence Berkeley National Laboratory, Berkeley, CA, USA). The raw data were deposited in GEO database (Superseries Accession Number: GSE42945).

Lentivirus transductions. Lentiviral particles containing the GV209 expression vector encoding 282-nt pri-EBV-miR-BART1 precursor that produces both EBV-miR-BART1-3p and associated nonspecific miRNAs were generated by the Lentiviral expression vector (Sino Biological Inc, Beijing, China). The resulting data files were uploaded to the mAdb database. Any gene containing one of the follows was excluded: (1) at least a 1.5-fold-change in either direction from gene’s median value; (2) per cent data missing of filtered out exceeds 50%. Further analysis was done by using the BRB-Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.html). Expression profile clustering and visualization were performed with Cluster and Treeview software (Ernest Orlando Lawrence Berkeley National Laboratory, Berkeley, CA, USA).

Plasmid preparation and cell transfection. The expression vector GV230 (http://www.genchem.com.cn) containing whole coding sequence of PTEN and the control vector GV170 were purchased from GeneChem (Shanghai, China). Plasmid DNAs were extracted using a TIANprep Mini Plasmid Kit (TIANGEN, China). Both CNE1-BART1 and 5-8F-BART1 cells were transfected with 200 ng plasmid DNA using Lipofectamine 2000 reagent (Invitrogen). Forty-eight hours post transfection, the cells were harvested for qRT-PCR and western blotting analyses. miRNA mimics or inhibitors (miRNA antisense oligonucleotides) were transfected at 50 nM (Sino Biological Inc, Beijing, China), CNE1-BART1 and 5-8F-BART1 cells were transfected with 200 ng plasmid DNA using Lipofectamine 2000 reagent (Invitrogen). Forty-eight hours post transfection, the cells were harvested for qRT-PCR and western blotting analyses. miRNA mimics or inhibitors (miRNA antisense oligonucleotides) were transfected at 50 nM (Sino Biological Inc, Beijing, China).

Western blotting. Western blotting analyses were performed by using standard methods. In brief, cells were harvested and lysed in the RIPA buffer containing protease inhibitors (Sigma-Aldrich) and phosphatase inhibitors (Keygen, China). Protein samples were separated by gel and electrophoresis gels, and transferred onto PVDF membrane (Millipore). The membrane was probed with the first antibody listed in Supplementary Table 6 and then with the peroxidase-conjugated secondary antibody. GAPDH was used as a protein loading control. Western blotting bands were visualized by eCL2 Western Blot Kit (CWBIO Technology) and captured with ChemiDocTM CB+- Molecular Imaging System (BioRad). All blots in figures were compared by the location of one molecular weight/size markers. Original blotting images for the key components of PTEN-dependent pathways and EMT were shown in Supplementary Fig. 9.

Tumour xenografts in nude mice. Animal experiments were approved by the Ethical Committee for Animal Research of Southern Medical University (protocol number: 2011-020) and conducted based on the state guidelines from the Ministry of Science and Technology of China. All nude mice (6–8 weeks old, female) were purchased from the Central Animal Facility of Southern Medical University, Wuhan, China. To assess tumour growth, 200 μl of 5-8F-BART1 cells or mock control cells (10⁶) was subcutaneously injected into the left or right side on the back of each mouse (seven mice per group). The tumour sizes were measured regularly and calculated using the formula W²/2L where L and W are the long and short diameter of the tumour, respectively.

We chose the well-established mouse model to evaluate tumour metastasis. Under the anaesthesia by peritoneal injection of 1% pentobarbital sodium, a small cut on the abdominal region was made. The mouse liver was gently diluted into original abdominal cavity and 50 μl of 5-8F cells (2 × 10⁶) was then injected underneath the liver envelope. The liver was then pushed back into abdominal cavity after lightly pressed the pinhole with alcohol cotton balls for two minutes. All mice (seven per group) were killed on day 21 after transplantation, or earlier if the needle reached the ethical endpoint. Whole bodies, freshly dissected tumours and internal organs including lungs, livers and lymph nodes were collected for fluorescence imaging with FL-3-9M-ACTIMSYSPLUS whole-body imaging system (Encinitas, CA). To assess tumour growth, 200 μl of 5-8F-BART1 cells or mock control cells (10⁶) was then injected underneath the left or right side on the back of each mouse (seven mice per group). The tumour sizes were measured regularly and calculated using the formula W²/2L where L and W are the long and short diameter of the tumour, respectively.

F-actin staining and confocal microscope examinations. F-actin staining was monitored by Rhodamine-phalloidin (5 μg ml⁻¹) and 4′,6-diamidino-2-phenylindole (DAPI) (0.1 μg ml⁻¹) staining according to the manufacturer’s instructions. In brief, the cells were seeded on sterile coverslips with culture on the day before staining. Cells were then washed with prewarmed PBS twice, fixed with 4% paraformaldehyde for 48 h and transferred to gradient ethanol. Tumours were immersed in paraffin, sectioned with Leica RM2235 and processed for histological examinations.

IF assays. Cells were cultured on coverslips overnight, fixed with 4% formaldehyde in PBS for 15 min at 4°C and then permeabilized with 0.3% Triton-X-100 in PBS for 30 min. Subsequently, cells were blocked for nonspecific binding with 5% milk in TBS for 30 min, incubated with PTEN-specific antibody (1:100, Bioworld) at 4°C overnight. The cells were then washed with prewarmed PBS twice, fixed with 4% paraformaldehyde in PBS for 10 min and washed with PBS three times, each for 5 min. Cells were then permeabilized with cold 100% acetone for 5 min, washed and treated with 1% bovine serum albumin/PBS/0.1% azide for 30 min for blocking nonspecific binding. Subsequently, cells were stained with Rhodamine-phalloidin at RT for 30 min, washed with PBS twice, stained for nuclei with DAPI for 1 min. The coverslips were mounted on slides using anti-fade mounting medium. Confocal images were acquired on the OLYMPUS confocal microscope system, and analysed with FV10-ASW1.7 viewer software (Olympus).

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coverslips were mounted on slides using anti-fade mounting medium with DAPI. IF images were acquired on Nikon ECLIPSE 80i microscope (Nikon).

**IHC staining.** Paraffin sections prepared from *in vivo* experiments were applied to IHC staining for the detection of protein levels of PTEN and some key components of PTEN-pathways. The indirect streptavidin-peroxidase method was used29. All antibodies used for IHC were listed in Supplementary Table 6. The stained results were reviewed and scored by two pathologists independently 30. In brief, the intensity of immunostaining was scored as negative (0), weak (1), medium (2) and strong (3). The extent of staining, defined as the per cent of positive staining cells, was scored as 1 (≤10%), 2 (11–50%), 3 (51–75%) and 4 (>75%). An overall expression score, ranging from 0 to 12, was obtained by multiplying the score of intensity and that of extent. The final staining score was presented as negative (overall score of 0), 1 (overall score of 1–3), 2 (overall score of 4) or 3 (overall score of ≥ 5).

**RNA-deep sequencing.** RNA-deep sequencing was performed and analysed in BGI-Shenzhen of China. In brief, miRNAs were isolated from the DNase-treated total RNAs with the Dynabeads mRNA Purification Kit (Life Technologies). According to the manufacturer’s instructions, the miRNAs were fragmented with divalent cations and converted to single-strand cDNA with random hexamer primers and Superscript II reverse transcriptase (Life Technologies). The second strand of cDNA was generated by RNase H (Enzymatics) and DNA polymerase. cDNA products were purified by Ampure beads XP (Beckman). After converting the overhangs into blunt ends using T4 DNA polymerase and Klenow DNA polymerase, extra ‘A’ base was added to the 3’-end of cDNA by Klenow enzyme. Sequencing adapters were then ligated to the end of cDNA by T4 DNA Ligase (Enzymatics). The fragments of 200 bp were selected by Ampure beads XP (Beckman) and enriched by 12 cycles of PCR. The PCR products were loaded into flowcell to generate clusters and then sequenced by HiSeq 2000 (Illumina). The resulting data were deposited in GEO database (Accession Number: GSE42945). Selected results were shown in Supplementary Table 4.

**MiRNA target predictions.** EBV-miR-BART1 candidate targets were initially obtained from the RNA-deep sequencing in BGI-Shenzhen of China (Supplementary Table 4) and then enriched with literature retrieval. The RNA-hybrid programme (http://bibiserv.techfak.uni-bielefeld.de/mahybrid/submission.html) was used to predict duplex complementation between human PTEN 3’-UTR and EBV-miR-BART1-S or EBV-miR-BART1-3p.

**Luciferase reporter assays.** The MiTarget microRNA 3’-UTR target vector (pEZX-MT01) containing full-length 3’-UTR of PTEN with two binding sites for EBV-miR-BART1-5p and EBV-miR-BART1-3p (wild-type 3’-UTR) was produced by Genecopoeia. Two mutant sites (mut1 and mut2) in PTEN 3’-UTR were then generated by site-directed mutagenesis with KOD-Plus-Mutagenesis Kit (SMK-101, Toyobo C. Ltd). For luciferase reporter assays, wild-type or mutant 3’-UTR vector was co-transfected with EBV-miR-BART1 mimic or nonspecific mimic control (Genecopoeia) into HEK 293 T cells, respectively. Luciferase activity was measured 48 h after transfection using Luc-Pair miR Luciferase Assay Kit (GeneCopoeia) on Panomics Luminometer.

**Statistical analysis.** All experiments were performed in triplicate. Data shown are mean ± s.e.m. (unless otherwise specified) from at least three independent experiments. SPSS 16.0 software was used for statistical analysis. Differences were considered to be statistically significant at values of *P* < 0.05 by Student’s *t*-test for two groups, one-way ANOVA (analysis of variance) for multiple groups and parametric generalized linear model with random effects for tumour growth. Correlation was analysed with two-tailed Spearman’s correlation analysis. Single, double and triple asterisks indicate statistical significance—*P* < 0.05, **P** < 0.01 and ***P*** < 0.001.

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