EBV-encoded miRNAs target ATM-mediated response in nasopharyngeal carcinoma

Raymond W-M Lung†, Pok-Man Hau†, Ken H-O Yu†, Kevin Y Yip†, Joanna H-M Tong†, Wing-Po Chak†, Anthony W-H Chan†, Ka-Hei Lam†, Angela Kwok-Fung Lo†, Edith K-Y Tin†, Shuk-Ling Chau†, Jesse C-S Pang†, Johnny S-H Kwan†, Pierre Busson3, Lawrence S Young4, Lee-Fah Yap5, Sai-Wah Tsao6, Ka-Fai To* and Kwok-Wai Lo*†

1 Department of Anatomical and Cellular Pathology, State Key Laboratory in Oncology in South China and Li Ka Shing Institute of Health Science, The Chinese University of Hong Kong, Hong Kong
2 Department of Computer Science and Engineering, The Chinese University of Hong Kong, Hong Kong
3 UMR8126 CNRS, Université Paris-Sud, Université Paris-Saclay, Gustave Roussy, Villejuif, France
4 Warwick Medical School, University of Warwick, Coventry, UK
5 Department of Oral and Craniofacial Sciences and Oral Cancer Research and Coordinating Centre, Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia
6 School of Biomedical Sciences and Center for Cancer Research, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong

*Correspondence to: Kwok-Wai Lo, Department of Anatomical and Cellular Pathology, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong. E-mail: kwlo@cuhk.edu.hk;
Or Ka-Fai To, Department of Anatomical and Cellular Pathology, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong. E-mail: kfto@cuhk.edu.hk
†Co-first authors.

Abstract

Nasopharyngeal carcinoma (NPC) is a highly invasive epithelial malignancy that is prevalent in southern China and Southeast Asia. It is consistently associated with latent Epstein–Barr virus (EBV) infection. In NPC, miR–BARTs, the EBV-encoded miRNAs derived from BamH1-A rightward transcripts, are abundantly expressed and contribute to cancer development by targeting various cellular and viral genes. In this study, we establish a comprehensive transcriptional profile of EBV-encoded miRNAs in a panel of NPC patient-derived xenografts and an EBV-positive NPC cell line by small RNA sequencing. Among the 40 miR–BARTs, predominant expression of 22 miRNAs was consistently detected in these tumors. Among the abundantly expressed EBV-miRNAs, BART5-5p, BART7-3p, BART9-3p, and BART14-3p could negatively regulate the expression of a key DNA double-strand break (DSB) repair gene, ataxia telangiectasia mutated (ATM), by binding to multiple sites on its 3’-UTR. Notably, the expression of these four miR–BARTs represented more than 10% of all EBV-encoded miRNAs in tumor cells, while downregulation of ATM expression was commonly detected in all of our tested sequenced samples. In addition, downregulation of ATM was also observed in primary NPC tissues in both qRT-PCR (16 NP and 45 NPC cases) and immunohistochemical staining (35 NP and 46 NPC cases) analysis. Modulation of ATM expression by BART5-5p, BART7-3p, BART9-3p, and BART14-3p was demonstrated in the transient transfection assays. These findings suggest that EBV uses miRNA machinery as a key mechanism to control the ATM signaling pathway in NPC cells. By suppressing these endogenous miR–BARTs in EBV-positive NPC cells, we further demonstrated the novel function of miR–BARTs in inhibiting Zta-induced lytic reactivation. These findings imply that the four viral miRNAs work co-operatively to modulate ATM activity in response to DNA damage and to maintain viral latency, contributing to the tumorigenesis of NPC.

Keywords: Epstein–Barr virus; EBV-miRNAs; nasopharyngeal carcinoma; ATM serine/threonine kinase (ATM); transcriptome sequencing

Received 29 June 2017; Revised 9 November 2017; Accepted 5 December 2017

No conflicts of interest were declared.

Introduction

Nasopharyngeal carcinoma (NPC) is an invasive epithelial malignancy arising from the most superior part of the nasopharynx. Due to the obscure location and radio-sensitivity of cancer cells, the combination of chemotherapy and radiotherapy is currently used as the mainstay treatment of advanced disease [1]. However, the outcomes in patients with distant metastases or recurrence are still poor and survivors also frequently suffer...
from severe side effects. Hence, the development of effective treatment strategies for NPC patients is still the main focus of the field.

In NPC, clonal EBV genomes have consistently been detected in both high-grade dysplasia lesions and invasive tumors, implying the crucial role of EBV infection in NPC tumorigenesis. EBV resides in NPC cells as a type II latent infection, in which only viral EBNA1 and LMPs proteins are expressed [2,3]. The oncogenic properties of these viral proteins in epithelial malignancies have been well characterized [4,5]; LMPs always activate the host immune response [6]. Therefore, in NPC, these viral proteins are usually variable and expressed at low levels to escape from the host’s cellular defense system, thereby maintaining viral latency. In contrast, the non-immunogenic virus-encoded miRNAs in the long viral BamHI-A rightward transcripts (BARTs) region, miR-BARTs, are abundantly expressed in EBV-infected epithelial malignancies, such as NPC and lymphoepithelioma-like carcinomas [7]. The functional roles of miR-BARTs have been reported to control viral latency [8–10], host cell immunity [11–13], cell proliferation [14], apoptosis [15–17], and metastasis [18]. Increasing evidence has been reported to support the notion that these EBV-encoded miRNAs are the key molecules in augmenting NPC tumorigenesis.

Various studies have clearly demonstrated the vital roles of miRNAs in modulating cell radio-sensitivity by targeting specific DNA repair factors [19]. For example, ectopic expression of miR-101, miR-181a, and miR-421 in different cancer cell lines can suppress endogenous ataxia telangiectasia mutated (ATM) gene expression and sensitize cells to ionizing radiation (IR) treatment [19–22]. Intriguingly, it had previously been reported that ATM is consistently downregulated in EBV-positive primary NPC samples [23]. In vitro EBV re-infection in nasopharyngeal epithelial cells has been able to suppress endogenous ATM expression and, sequentially, ATM kinase activity followed by exposure to IR [23]. The indispensable role of ATM in viral replication was also demonstrated in our recent study [24]. However, the involvement of EBV in ATM regulation has remained elusive and needs further investigation.

In this study, we examined EBV-miRNA expression profiles in a panel of NPC patient-derived xenografts and an EBV-positive NPC cell line by high-throughput small RNA sequencing (RNA-seq). Although miR-BARTs are processed from the same primary transcript, several of them were predominantly expressed. Most importantly, we discovered that some highly expressed miR-BARTs could directly regulate ATM expression. Manipulating the expression of those specific miR-BARTs in the cells alters both IR sensitivity and Zta-induced EBV reactivation via the ATM signaling pathway. This is the first study to uncover the role of miR-BARTs in modulating the expression of ATM, a critical DNA double-strand break responder.

Materials and methods

Cell lines, xenografts, and patient samples
Five EBV-positive NPC xenografts (xeno-666, xeno-2117, xeno-1915, C15, and C17), a native EBV-infected NPC-derived cell line (C666-1), three immortalized nasopharyngeal epithelial cell lines (NP361, NP550, and NP69), and HeLa cells were used in the study [11,25,26]. In addition, a cohort of frozen specimens, including 16 non-cancerous nasopharyngeal epithelia (NP) and 45 primary NPC samples, were used in RT-qPCR analysis (supplementary material, Table S1). Another 35 NP and 46 NPC paraffin-embedded specimens were recruited for IHC analysis (supplementary material, Table S2). The primary specimens were prospectively collected at the Prince of Wales Hospital, Hong Kong. Ethical approval was obtained from the Joint CUHK/NTEC Clinical Research Ethics Committee, Hong Kong.

Small RNA sequencing (RNA-seq)
Total RNAs were extracted from fresh samples using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). The small RNA libraries were prepared using the Illumina TruSeq Small RNA Library Prep Kit according to the manufacturer’s instructions. Single-end 51-bp sequencing was performed on an Illumina HiSeq2000 sequencing system. Sequenced reads were aligned to human (hg19) and EBV genomes (accession number AJ507799) using Bowtie 2 [27]. The expression level of each annotated miRNA was computed based on sequencing reads that support the miRNA sequence defined in the miRBase database (http://www.mirbase.org/). Specifically, a read was considered to support a miRNA if its alignment with the miRNA sequence had at least 16 matched nucleotides. The read depth of the mature sequences was aligned using SAMtools and normalized per 10 million miRNAs sequenced [28]. The heterogeneity of the sequencing reads was examined and the results for the miR-BARTs of interest in C666-1 are listed in the supplementary material, Table S3.

Target prediction
The miRanda and RNAhybrid programs were used for miR-BART target prediction as described previously [11,17].

Reverse transcription–quantitative PCR (RT-qPCR)
Total RNA was first reverse-transcribed using the miScript Reverse Transcription Kit (Qiagen, Hilden, Germany). The ATM RT-PCR product was amplified using the SYBR Green PCR Master Mix Kit (Applied Biosystems, Foster City, CA, USA). The data were normalized with β-actin and the fold-change was calculated using the 2^(-ΔΔCt) method. The primer sequences for the
PCR are listed in the supplementary material, Table S4. The quantitative method and the oligonucleotide sequences used for EBV-miRNA expression have been described previously [7,12].

miRNA mimics, inhibitors, expression vectors, and transfection

All of the miRNA mimics, inhibitors, and their negative controls were synthesized by Ambion Inc (Austin, TX, USA) and the information is listed in the supplementary material, Table S5. The ATM-specific siRNAs were from GenePharma (Shanghai, China) and the sequences were as follows: sense, 5'-CAUACUACUAAGACAUUdTdT-3'; antisense, 5'-AAUGCUUUGAGUAGUAUGTT-3' [24]. The ATM expression vector pcDNA3.1 (+) Flag-His-ATM WT was a gift from Michael Kastan (Addgene #31985) [29]. The pcDNA3.1 (+) HA-BZLF1, C666-1-BART-Cluster 1 miRNA, and C666-1-BART-Cluster 2 miRNA expression vectors have been described previously [12,24]. In the experiment, 50 nM of siRNA, 20 nM of miRNA mimic/inhibitor, and 2.5 μg of expression vector were used to transfect the cells in a six-well plate format. All of the transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) unless otherwise specified.

Immunohistochemistry (IHC)

IHC staining was performed using the Polymer Refined Detection Kit on a Bond-Max fully automated staining system [30]. ATM-IHC was carried out with a primary antibody against ATM (1:1000 dilution, clone 11G12; Abcam, Cambridge, MA, USA) and all of the slides were counterstained with hematoxylin for analysis. The results were evaluated by a semi-quantitative approach to assign an H-score to each sample. The percentage of cells at each staining intensity level were determined. The H-score was calculated using the following formula: \[1 \times (\% \text{ cells 1+}) + 2 \times (\% \text{ cells 2+}) + 3 \times (\% \text{ cells 3+})\]. Samples with H-scores higher than 100 were considered ATM expression-positive. Statistical analysis was performed using SPSS 19.0 (IBM Corp, Armonk, NY, USA) and chi-square tests were used to analyze the significance of differences in protein expression scores in normal and tumor tissues.

Antibodies and immunoblotting

The antibody against BHRF1 was a gift from Jaap Middeldorp (VU University Medical Centre, The Netherlands). Antibodies against BZLF1 and BRLF1 were purchased from Argene (bioMérieux SA, Marcy l’Etoile, France). The rabbit monoclonal antibodies against phospho-ATM (ab81292), ATM (ab32420), and the anti-HA tag (1:5000 dilution; ab9110) rabbit polyclonal antibody were purchased from Abcam. The anti-γ-H2AX\textsuperscript{Ser139} antibody was purchased from EMD Millipore (Quincy, MA, USA). The antibodies against CHK2 (#3440) and phospho-CHK2 (#2197) were purchased from Cell Signaling Technology (Danvers, MA, USA). All of the AlexaFluor-conjugated and HRP-conjugated secondary antibodies were purchased from Molecular Probes (New York, NY, USA). The HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Western blot analysis was performed as previously described [24] and all the primary and the secondary antibodies used is 1:1000 and 1:5000 in dilutions unless otherwise specified. The signal intensity was measured by ImageJ software (http://rsb.info.nih.gov/ij/).

Luciferase reporter assay

The construction of reporter plasmids and the procedure of the dual luciferase reporter assay have been described previously [31,32]. The sequences of the oligonucleotides for plasmid construction are listed in the supplementary material, Table S6. In the experiment, transfection complex containing 80 ng of reporter plasmid, 8 ng of pRL-CMV-control plasmid, and 1 nM of miRNA mimic/inhibitor was co-transfected into 293FT cells in 24-well plates.

Ionizing radiation (IR), γ-H2AX staining, comet assay, and clonogenic survival assay

The indicated dose of IR was delivered with a cesium-137 source from an MDS Nordion Gamma-cell 1000 Elite Irradiator. The γ-H2AX staining analysis was performed as previously described [33]. The comet assays were performed using an OxiSelect Comet Assay Kit from Cell Biolabs, Inc (San Diego, CA, USA) according to the manufacturer’s instructions. At least 70 cells were analyzed using ImageJ software and the DNA repair capacity was measured by comparing the tail moment between \textit{miR-BART} transfected cells at 30 min and 6 h post-IR treatment. The standard clonogenic survival assays have been described previously [34].

Statistical analysis

The data were analyzed using Student’s \( t \)-test unless otherwise specified. The analysis of each sample was performed in triplicate. The results are expressed as the mean ± SD. To assess the similarity of \textit{miR-BART} expression patterns of the NPC samples, Spearman’s rank and Pearson’s correlations were computed for the \textit{miR-BART} reads/10 million miRNAs sequenced across the samples. The expression pattern of an miRNA is described as considerably different if Cook’s distance statistic \( D \) between any pair of sample exceeds \( 4/n \), where \( n \) is the number of \textit{miR-BART}s considered [35]. All of the analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc, San Diego, CA,
Table 1. Percentage of EBV-miRNAs found in small-RNA sequencing of EBV-associated NPCs

| Library  | No of reads | No of miRNAs | No of miRNAs | % of EBV-miRNAs |
|----------|-------------|--------------|--------------|-----------------|
|          | Total       | Mapped to human | Mapped to EBV | Human | EBV |          |
| C666-1   | 17,925,005  | 13,451,374    | 4,049,298    | 9,709,760       | 4,029,708 | 29.33    |
| X666     | 17,987,157  | 12,305,340    | 4,860,335    | 8,819,204       | 4,826,139 | 35.37    |
| X2117    | 19,820,159  | 15,573,415    | 2,533,990    | 7,037,414       | 2,500,278 | 26.22    |
| X1915    | 15,761,052  | 11,177,098    | 1,448,822    | 2,702,222       | 1,417,860 | 34.39    |
| C15      | 15,087,966  | 10,506,729    | 1,686,724    | 2,591,004       | 1,630,218 | 38.62    |
| C17      | 20,032,126  | 16,429,769    | 45,411       | 1,736,005       | 37,189    | 2.10     |

Figure 1. Expression of viral miRNAs in EBV-positive NPCs. (A) The number of EBV-miRNA reads is indicated per 10 million of the total mapped mature miRNAs to normalize the sequencing depth in each library. The libraries of C666-1, C17, and the average of four primary NPC-derived xenografts (xeno-C666, xeno-2117, xeno-1915, and C15) are shown as mean ± SD. (B) The distribution of the individual miR-BARTs to the total viral miRNAs in the libraries is shown in the pie charts.
Figure 2. Legend on next page.
USA). A value of $p < 0.05$ was taken to indicate statistical significance.

**Results**

**Expression profiles of viral miRNAs in EBV-positive NPC**

To explore the EBV-encoded miRNA expression patterns in NPC, we performed small RNA-seq on six NPC samples, including the C666-1 cell line, four NPC xenografts derived from primary tumors (xeno-666, xeno-2117, xeno-1915, and C15), and one xenograft (C17) derived from cutaneous metastasis [36–38]. In each sample, 15–20 million reads mapped to either human or EBV genomes were obtained for analysis. Curiously, almost all of the reads mapped to the EBV genome were known EBV-encoded miRNAs, indicating that miRNAs are the major components of EBV-derived small RNAs in NPC despite the existence of other small viral-encoded RNAs, such as v-snRNA-1 and evb-sisRNA-1, being reported recently [39]. As shown in Table 1, we obtained 25–40% of the EBV-encoded miRNAs from the total miRNA reads in each sample. However, a relatively smaller abundance of EBV-encoded miRNAs (2.1%) was found in C17. The low expression of miR-BARTs in C17 may be due to the selection of a metastatic subclone in a distinct microenvironment. Although microRNAs derived from the viral BHRF1 transcript (miR-BHRF1) have been suggested to restrict expression in type III latent infections, a few copy numbers of miR-BHRF1 were detected in the samples (supplementary material, Table S7). The observation aligns with our previous finding of low BHRF1 lytic transcript expression levels in NPCs [7].

The miR-BART expression pattern in C666-1 in our data was similar to the recent massive amount of sequencing data from two independent teams (supplementary material, Figure S3A). The reduction of ATM expression in the primary NPC tissues were also corroborated by immunohistochemistry (IHC) analysis in an independent cohort of 35 histologically

**Figure 2. ATM is a potential target of several EBV-encoded miRNAs.** (A) Suggested putative miR-BART recognition sites on the ATM 3'-UTRs are shown. The seed-binding regions of the miR-BARTs are underlined and the bases mutated for the luciferase reporter analysis are marked in red. (B) The expression of miR-BARTs of interest in primary NPC samples was examined using RT-qPCR ($n = 45$). The expression of miR-BARTs was normalized to EBNA1 for analysis. The low-expression miR-BARTs (BART15-5p, BART7-3p, BART9-3p, and BART14-3p) have been suggested to restrict expression in type III latent infections, a few copy numbers of miR-BHRF1 were detected in the samples (supplementary material, Table S7). The observation aligns with our previous finding of low BHRF1 lytic transcript expression levels in NPCs [7].

Although the expression levels of some highly expressed miRNAs were considerably different across several tumor lines (Cook’s distance $\geq 4/n$) (Figure 1A and supplementary material, Table S8). These highly expressed miRNAs included BART5-5p, BART6-3p, BART7-3p, BART8-5p, BART10-3p, BART19-3p, and BART22, occupying up to 60% of the total viral miRNAs in NPCs (Figure 1B). We found particularly low expression of BART20 and BART21 in all NPC samples. Similar observations have also been reported in other EBV-positive epithelial malignancies [7]. The expression of individual EBV-miRNAs in each tumor line is summarized in the supplementary material, Table S7.

**ATM is a direct target of EBV-encoded miRNAs**

We have previously demonstrated that the ATM protein is consistently downregulated in EBV-positive NPC and contributes to viral replication during EBV lytic reactivation in epithelial cells [23,24]. Based on these findings, we hypothesize that the highly expressed miR-BARTs are responsible for ATM regulation and subsequently inhibiting EBV lytic reactivation in NPC cells. We attempted to predict the putative miR-BART binding sites, specifically on the ATM transcript by using miRanda and RNAhybrid algorithms. The abun-
dantly expressed miRNAs identified in small RNA-seq were prioritized for the analysis. Using the default settings for in silico prediction, multiple putative binding sites of miR-BARTs (BART5-5p, BART7-3p, BART9-3p, and BART14-3p) on the ATM 3'-untranslated region (3'-UTR) were suggested (Figure 2A). These four miRNAs covered up to 10% and 11% of the total viral miRNAs in C666-1 and NPC xenografts, respectively (Figure 1B). Notably, the high expression levels of BART5-5p, BART7-3p, BART9-3p, and BART14-3p in primary NPC tissues were also corroborated by RT-qPCR (Figure 2B). Of note, western blot analysis revealed downregulation of ATM expression in the EBV-positive NPC samples (Figure 2C and supplementary material, Figure S3A). The reduction of ATM expression in NPC was further substantiated in the IHC analysis in an independent cohort of 35 histologically
normal nasopharyngeal epithelia (NP) and 46 primary NPC tumor cases \((p < 0.0001)\) (Figure 2D). Positive ATM expression was generally observed in the normal NP cases \((n = 32, 91.4\%)\), whereas 31 NPC cases \((67.4\%)\) scored negative (supplementary material, Table S9). Positive ATM expression was also identified in one primary EBV-negative NPC specimen, which was expected to have no miR-BART expression (supplementary material, Figure S3B). In addition, the expression level of the ATM transcript in primary NPC tumors \((n = 45)\) also showed a significant reduction compared with those in the non-cancerous NP tissues \((n = 16; \ p = 0.0003)\) in RT-qPCR analysis (Figure 2E).

The relationship between ATM and miR-BART expression was further investigated by directly comparing their mRNA expression levels in the primary NPCs. We observed a strong inverse association between ATM mRNA and BART5-5p, BART7-3p, BART9-3p, and BART14-3p expression \((-0.5306 < \text{Spearman } r < -0.4481; 0.0016 < p < 0.0475)\) (Figure 2F). To validate the direct interaction of the miR-BARTs with ATM-3’-UTR, we performed a series of luciferase reporter assays with co-transfection of different combinations of miR-BART mimics and reporter plasmids into 293FT cells. The luciferase activity of the reporter plasmids containing a predicted ATM-3’-UTR recognition site was strongly repressed by BART5-5p, BART7-3p, BART9-3p, and BART14-3p \((p < 0.01\) in all combinations). However, the suppressive effect was canceled if the corresponding miR-BART inhibitor was co-transfected simultaneously. The inhibitory effect was also not suggested when the complementarities of the seed region on the binding site were either mutated or deleted (Figure 2G and supplementary material, Figure S4).

Together, these results support the notion that multiple miR-BARTs directly modulate ATM expression via their specific binding sites on the 3’-UTR.
Figure 4. EBV-miRNAs enhance the ionizing radiation (IR) sensitivity of epithelial cells. The miR-BART transfected cells were treated with different doses of IR and the cells were harvested at 30 min post-irradiation for immunoblotting analysis. The expression of the basal ATM proteins, phospho-ATM (p-ATM), phospho-CHK2 (p-CHK2), and γ-H2AX, was analyzed. Actin was probed as the loading control. ATM knockdown cells (siATM) were included as positive controls.

Regulation of endogenous ATM expression by EBV-encoded miRNAs

To illustrate the strong regulatory effect of EBV-encoded miRNAs on ATM, we introduced an expression vector of C666-1-BART-Cluster 1, C666-1-BART-Cluster 2 or a mimic of BART5-5p, BART7-3p, BART9-3p, and BART14-3p into two EBV-negative ATM-expressing epithelial cells, NP69 and HeLa. The presence of either individual miR-BART-Cluster vector or miRNA mimic notably repressed ATM expression (Figure 3A). Interestingly, synergistic suppressive effects were observed on ATM expression in HeLa cells when the cells were simultaneously transfected with either both miR-BART-Cluster vectors or the mimics of all four miR-BARTs (Figure 3A and supplementary material, Figure S5A).

To further strengthen evidence for the importance of miR-BART in ATM expression, additional experiments with miRNA inhibitors were performed. The specificity of the miR-BART inhibitors has been confirmed by RT-qPCR. In individual miR-BART inhibitor transfected C666-1, the expression of the targeted miR-BART was dramatically repressed, whereas no obvious effect was detected in the previously reported ATM regulated miRNAs, primary BARTs, and other miR-BARTs, which are located in close proximity to the miR-BARTs of interest (supplementary material, Figures S6 and S7). In BART-Cluster 1 and BART-Cluster 2 expressing NP69 and HeLa cells, ATM expression was restored by co-transfecting with BART5-5p, BART7-3p, BART9-3p or BART14-3p inhibitors (Figure 3B, C). Likewise, ATM expression was also restored by inhibiting endogenous BART7-3p, BART9-3p, and BART14-3p activity, individually or together, in C666-1 cells (Figure 3D). The reason for the inability of the BART5-5p inhibitor to restore ATM expression in C666-1 is unclear. Endogenous BART5-5p is functionally active in C666-1 because we previously demonstrated its regulatory effect on PUMA in the same cells [17]. In addition, low expression of hsa-miR-18a/b, which shares significant seed homology with BART5-5p, and no sequence
Figure 5. Legend on next page.
EBV-miRNAs enhance the ionizing radio-sensitivity of epithelial cells

We postulated that the expression of miR-BARTs may enhance the radio-sensitivity of NPC cells through ATM suppression. Thus, we examined the contribution of miR-BARTs to the DNA damage response. NP69 and HeLa cells were transfected with either ATM-specific siRNA or miR-BART mimics and exposed to different doses of γ-irradiation afterwards. The IR-induced ATM activity, as indicated by the phosphorylation of both ATM and its downstream targets (γ-H2AX and p-CHK2), was clearly observed in the control transfected cells in a dose-dependent manner, without any apparent differences in the total ATM protein levels (Figure 4). However, the magnitude of the induced ATM activity was dramatically reduced in cells where ATM expression was knocked down by the siRNA. Similarly, the magnitude of the IR-induced responses in the cells expressing miR-BARTs, individually or together, were also obviously reduced (Figure 4). We subsequently extended our study to examine the post-IR recovery ability of the cells. In the cells exposed to 3 Gy of IR, the number of positive γ-H2AX nuclear foci was significantly reduced in all miR-BART transfected cells (p < 0.01 in all cases, Figure 5A and supplementary material, Figure S8A), suggesting that the ability of the cells to recover from the IR-induced DNA damage was impaired by the miR-BART activity. In accordance with the γ-H2AX nuclear foci staining results, the DNA repair capacity of the miR-BART transfected cells was obviously repressed in the comet assay (p < 0.05). The combined effect of these four miR-BARTs in enhancing IR-induced DNA damage could also be demonstrated (Figure 5B and supplementary material, Figure S8B). More importantly, the long-term clonogenic survival of the miR-BART transfected HeLa cells was significantly reduced after IR treatment (Figure 5C). In our experiments, approximately 50% of the untransfected cells survived after exposure to 1 Gy of IR. In contrast, the same dose of IR killed nearly 88% of the BART5-5p (p = 0.008), 80% of the BART7-3p (p = 0.009), 75% of the BART9-3p (p = 0.018), and 60% of the BART14-3p (p = 0.048) transfected cells (Figure 5C). The effects of miR-BART5-5p, miR-BART7-3p, and miR-BART9-3p on augmenting the irradiation sensitivity of the transfected cells were more obvious upon increasing the IR dose to 2 Gy in the treatment (Figure 5D). Overall, these results provide convincing evidence to support abundant EBV-encoded miRNA expression as one of the underlying mechanisms of the high radio-sensitivity of NPC.

EBV-miRNAs contribute to the maintenance of viral latency by suppressing ATM activity

Consistent with our previous finding in EBV-infected nasopharyngeal epithelial cells [24], the overexpression of EBV immediate-early lytic protein, BZLF1 (Zta), in C666-1 cells provoked the viral lytic cycle by dramatically activating the expression of other viral early lytic proteins, such as BRLF1 (Rta) and BMRF1 (EA-D). In addition, BZLF1 expression in C666-1 cells could also slightly elevate the activity of both ATM kinase (p-ATM) and its downstream target (γ-H2AX) (Figure 6A).

ATM activity is known to promote EBV DNA replication during lytic cycle reactivation [24,42]. Our new findings of the role of miR-BARTs in ATM regulation prompted us to examine whether miR-BARTs are involved in controlling viral replication. The results revealed that suppressing endogenous BART7-3p, BART9-3p or BART14-3p activity in the BZLF1-expressing C666-1 cells could activate the ATM protein and its downstream effectors (p-ATM and γ-H2AX), and these effects were accompanied by increased expression of the BMRF1 protein (Figure 6A). The expression levels of ATM and p-ATM were also greatly increased in the cells transfected with inhibitors of all four miR-BARTs (mean of ATM 1.98 and p-ATM 3.52) (supplementary material, Figure S5B) relative to the individual inhibitor (mean of ATM 1.4–1.8 and p-ATM 1.6–2.0) (Figure 6A). However, the combined effects of all four miR-BARTs inhibitors did not lead to a synergistic effect.
Figure 6. BZLF1-induced virus reactivation is suppressed by mir-BARTs. (A) The BZLF1-expressing plasmid (1.25 μg) was co-transfected with the indicated mir-BART inhibitors (10 μM) into C666-1 cells in a six-well plate. Cells were harvested for immunoblotting analysis after 48 h. The expression of ATM, ATM downstream effectors (p-ATM and γ-H2AX), and the early viral lytic proteins (BRLF1 and BMRF1) was analyzed. (B) The synergistic effects of BZLF1 and ATM on p-ATM, γ-H2AX, BRLF1, and BMRF1 expression in C666-1 cells. The cells were co-transfected with ATM and BZLF1-expressing plasmids and protein expression was examined by immunoblotting. The two isoforms of BMRF1 are indicated by arrows. The relative expression of the protein level was calculated for comparison.

to a further increase of the BMRF1 protein level (supplementary material, Figure S5B), probably due to the high level of BZLF1 which stimulates EBV reactivation through the ATM signaling pathway (supplementary material, Figure S9).

We further dissected the role of BZLF1 in the ATM-induced viral lytic cycle by introducing ATM and BZLF1 expression vectors, alone or in combination, into C666-1 cells. In the absence of BZLF1 expression, ATM alone had no effect on the expression of either early viral lytic proteins or ATM downstream effectors. Interestingly, BZLF1 and ATM synergistically increased the expression of phospho-ATM, γ-H2AX, and BMRF1 viral lytic protein (Figure 6B), indicating that ATM might enhance BZLF1 activity. Therefore, mir-BARTs may be responsible for maintaining viral latency by suppressing ATM activity, which in turn inhibits BZLF1 activity in viral lytic reactivation (supplementary material, Figure S9).

Discussion

Here, we report that the mir-BART expression patterns of six well-known EBV-positive NPC tumor lines are significantly similar (supplementary material, Figure S2). In addition, we unveil the novel suppressive effect of BART5-5p, BART7-3p, BART9-3p, and BART14-3p in modulating the ATM signaling pathway. They occupied about 11% of the total EBV-encoded miRNAs in NPCs (Figure 1B). The findings indicated that these mir-BARTs contribute to ATM suppression in this EBV-associated epithelial cancer. However, due to the low mir-BART expression in EBV-associated lymphoid malignancies [43,44], previous highly sensitive PAR-CLIP analysis only identified a number of cellular miRNAs responsible for modulating ATM expression [45,46]. Nevertheless, all of these reported cellular miRNAs, except hsa-miR-26, are rarely expressed in NPC samples, such as in C666-1 (supplementary material, Table S10). The frequent downregulation of miR-26 in NPC also suggests that the cellular miRNAs are not involved in the downregulation of ATM in this EBV-associated epithelial cancer [47].

More importantly, the expression of these four mir-BARTs significantly inhibited both IR-induced ATM kinase activity and BZLF1-induced viral lytic reactivation (Figures 4–6). Intriguingly, a recent study demonstrated that miRNAs in BART Cluster 1 can also directly target BZLF1 expression in B-cells [48].
Thus, the activity of BZLF1, a viral immediate-early protein, is tightly controlled by miR-BARTs via different mechanisms. As various genotoxic stresses constantly induce cellular DNA damage, the error-free homologous recombination (HR) pathway, which ensures accurate DNA double-strand break (DSB) repair, is a critical safeguard for maintaining genetic integrity for cell survival [49,50]. ATM is an essential molecule in the HR pathway, as it immediately responds to DNA damage and activates several downstream effectors to interrupt the cell cycle and stop DNA replication. Downstream ATM effectors then facilitate DNA repair or trigger a p53-dependent apoptotic pathway based on the severity of the damage (supplementary material, Figure S9) [51]. In the present study, we revealed that EBV employs viral-encoded miRNAs to retard the genotoxic stress-induced ATM kinase activity and eventually promote cell death. Therefore, the frequent downregulation of ATM in EBV-positive NPC may partly explain the exceptionally high radio-sensitivity of this deadly cancer.

Poly(ADP-ribose) polymerase (PARP) is an abundant nuclear zinc-finger enzyme involved in the base-excision repair of single-strand breaks (SSBs) in the earliest DNA damage response. Inhibition of PARP results in failure of the SSB repair machinery and unrepaird lesions are converted into DSBs during DNA replication [52]. HR-impaired cancer cells are therefore sensitive to PARP inhibition. Based on this mechanism, the PARP inhibitor olaparib has been evaluated in clinical trials [53,54]. Currently, two independent reports have shown that ATM-null cells exhibit selective sensitivity to olaparib treatment [55,56]. Remarkably, concurrent PARP inhibition also potentiates the cytotoxic effects of ionization radiation and platinum-based DNA-damaging agents, thus maximizing the efficacy of this deadly cancer.

In conclusion, by uncovering the interaction between miR-BARTs and the ATM signaling pathway, we report for the first time the mechanisms responsible for low ATM expression in EBV-positive NPC. It is likely that miR-BARTs work co-operatively to modulate ATM in controlling DNA damage repair and to maintain viral latency. This finding may facilitate the development of effective NPC therapies using DNA-damaging agents, such as the PARP inhibitor.

Acknowledgements

We thank the Core Utilities of Cancer Genomics and Pathology (CUHK) for providing facilities and assistance in support of this research. This study was supported by Focus Investigation Scheme-A and VC’s One-off Discretionary Fund (VCF2014017) from the Chinese University of Hong Kong, the Research Grants Council of Hong Kong-GRF (471211, 470312, 471413, 14104415 and 14138016), the Collaborative Research Fund (CUHK8/CRF/11R and C7027-16G), and the Theme-Based Research Scheme (T12-401/13R). LFY was supported by the Fundamental Research Grant Scheme (FP013-2016) from the Ministry of Higher Education Malaysia and Newton-Ungku Omar Fund (IF016-2017; MR/P013201/1) from the Academy of Sciences Malaysia and the Medical Research Council UK.

Author contributions statement

KWL, KFT, PMH, and RWML designed the study. PMH, RWML, WPC, JCS, KHL, EKYT, AKFL, and SLC carried out the experiments. KHW, SWT PB, KWL, and KFT provided the NPC tumor models, primary tumor specimens, and clinical data. KWLO, KFT, PMH, LSY, LFY, and RWML were involved in data analysis and writing the paper. All authors approved the submitted manuscript.

References

1. Chan AT, Felip E, Group EGW. Nasopharyngeal cancer: ESMO clinical recommendations for diagnosis, treatment and follow-up. Ann Oncol 2009; 20 (suppl 4): 123–125.
2. Raab-Traub N. Epstein–Barr virus in the pathogenesis of NPC. Semin Cancer Biol 2002; 12: 431–441.
3. Lo KW, Chung GT, To KF. Deciphering the molecular genetic basis of NPC through molecular, cytogenetic, and epigenetic approaches. Semin Cancer Biol 2012; 22: 79–86.
4. Lo AK, Dawson CW, Young LS, et al. Activation of the FGFR1 signalling pathway by the Epstein–Barr virus-encoded LMP1 promotes aerobic glycolysis and transformation of human nasopharyngeal epithelial cells. J Pathol 2015; 237: 238–248.
5. Raab-Traub N. Nasopharyngeal carcinoma: an evolving role for the Epstein–Barr virus. Curr Top Microbiol Immunol 2015; 390: 339–363.
6. Lee SP. Nasopharyngeal carcinoma and the EBV-specific T cell response: prospects for immunotherapy. Semin Cancer Biol 2002; 12: 463–471.
7. Lang RW, Tong JH, To KF. Emerging roles of small Epstein–Barr virus derived non-coding RNAs in epithelial malignancy. Int J Mol Sci 2013; 14: 17378–17409.
8. Jung YJ, Choi H, Kim H, et al. MicroRNA miR-BART20-5p stabilizes Epstein–Barr virus latency by directly targeting BZLF1 and BRLF1. J Virol 2014; 88: 9027–9037.
9. Qi J, Thorley-Lawson DA. EBV microRNA BART 18-5p targets MAP3K2 to facilitate persistence in vivo by inhibiting viral replication in B cells. Proc Natl Acad Sci U S A 2014; 111: 11157–11162.
10. Barth S, Pflühl T, Mamiani A, et al. Epstein–Barr virus-encoded microRNA miR-BART2 down-regulates the viral DNA polymerase BAF5. Nucleic Acids Res 2008; 36: 666–675.
11. Lang RW, Tong JH, Sung YM, et al. Modulation of LMPP2 expression by a newly identified Epstein–Barr virus-encoded microRNA miR-BART22. Neoplasia 2009; 11: 1174–1184.

© 2017 The Authors. The Journal of Pathology published by John Wiley & Sons Ltd on behalf of Pathological Society of Great Britain and Ireland. www.pathsoc.org

J Pathol 2018; 244: 394–407
www.thejournalofpathology.com
12. Lo AK, To KF, Lo KW, et al. Modulation of LMP1 protein expression by EBV-encoded microRNAs. Proc Natl Acad Sci U S A 2007; 104: 16164–16169.

13. Nachmani D, Stern-Ginossar N, Sarid R, et al. Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. Cell Host Microbe 2009; 5: 376–385.

14. Kim H, Choi H, Lee SK, Epstein–Barr virus miR-BART20-5p regulates cell proliferation and apoptosis by targeting BAD. Cancer Lett 2015; 356: 733–742.

15. Lin TC, Liu TY, Hsu SM, et al. Epstein–Barr virus-encoded miR-BART20-5p inhibits T-bet translation with secondary suppression of p53 in invasive nasal NK/T-cell lymphoma. Am J Pathol 2013; 182: 1865–1875.

16. Kang D, Skalsky RL, Cullen BR. EBV BART microRNAs target multiple pro-apoptotic cellular genes to promote epithelial cell survival. PLoS Pathog 2015; 11: e1004979.

17. Choy EY, Siu KL, Kok KH, et al. An Epstein–Barr virus-encoded microRNA targets PUMA to promote host cell survival. J Exp Med 2008; 205: 2551–2560.

18. Cai LM, Lu X, Luo WR, et al. EBV-miR-BART7-3p promotes the EMT and metastasis of nasopharyngeal carcinoma cells by suppressing the tumor suppressor PTEN. Oncogene 2015; 34: 2156–2166.

19. Methentrairut C, Slack FJ. MicroRNAs in the ionizing radiation response and in radiotherapy. Curr Opin Genet Dev 2013; 23: 12–19.

20. Yan D, Ng WL, Zhang X, et al. Targeting DNA-PKcs and ATM with miR-101 sensitizes tumors to radiation. PLoS One 2010; 5: e11397.

21. Hu H, Du L, Nagabayashi G, et al. ATM is down-regulated by N-Myec-regulated microRNA-421. Proc Natl Acad Sci U S A 2010; 107: 1506–1511.

22. Liu X, Liao W, Peng H, et al. miR-181a promotes G1/S transition and cell proliferation in pediatric acute myeloid leukemia by targeting replication. J Cancer Res Clin Oncol 2016; 142: 77–87.

23. Bose S, Yap LF, Fung M, et al. The ATM tumor suppressor gene is down-regulated in EBV-associated nasopharyngeal carcinoma. J Pathol 2009; 217: 345–352.

24. Hau PM, Deng W, Jia L, et al. Role of ATM in the formation of the replication compartment during lytic replication of Epstein–Barr virus in nasopharyngeal epithelial cells. J Virol 2015; 89: 652–668.

25. Chung GT, Lung RW, Hui AB, et al. Identification of a recurrent transforming UBR5–ZNFX423 fusion gene in EBV-associated nasopharyngeal carcinoma. J Pathol 2013; 231: 158–167.

26. Yip YL, Pang PS, Deng W, et al. Efficient immortalization of primary nasopharyngeal epithelial cells for EBV infection study. PLoS One 2013; 8: e78395.

27. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods 2012; 9: 357–359.

28. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics 2009; 25: 2078–2079.

29. Cannan CE, Lim DS, Cimprich KA, et al. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science 1998; 281: 1677–1679.

30. To KF, Tong JH, Yeung KS, et al. Detection of ALK rearrangement by immunohistochemistry in lung adenocarcinoma and the identification of a novel EML4–ALK variant. J Thorac Oncol 2013; 8: 883–891.

31. Wong QW, Lung RW, Law PT, et al. MicroRNA-223 is commonly repressed in hepatocellular carcinoma and potentiates expression of Stat3/Stat5. Gastroenterology 2008; 135: 257–269.

32. Lung RW, Wang X, Tong JH, et al. A single nucleotide polymorphism in microRNA-146a is associated with the risk for nasopharyngeal carcinoma. Mol Carcinog 2013; 52(suppl 1): E28–E38.

33. Chow JP, Man WY, Mao M, et al. PARP1 is overexpressed in nasopharyngeal carcinoma and its inhibition enhances radiotherapy. Mol Cancer Ther 2013; 12: 2517–2528.

34. Munshi A, Hobbs M, Meyn RE. Clonogenic cell survival assay. Methods Mol Cell Biol 2005; 110: 21–28.

35. Bollen KA, Jackman RW. Regression diagnostics: an expository treatment of outliers and influential cases. SocioL Methods Res 1985; 13: 510–542.

36. Cheung ST, Huang DP, Hui AB, et al. Nasopharyngeal carcinoma cell line (C666-1) consistently harbouring Epstein-Barr virus. Int J Cancer 1999; 83: 121–126.

37. Huang DP, Ho JH, Chan WK, et al. Cytogenetics of undifferentiated nasopharyngeal carcinoma xenografts from southern Chinese. Int J Cancer 1989; 43: 936–939.

38. Busson P, Ganem G, Flores P, et al. Establishment and characterization of three transplantable EBV-containing nasopharyngeal carcinomas. Int J Cancer 1988; 42: 599–606.

39. Moss WN, Lee N, Pimenta G, et al. RNA families in Epstein–Barr virus. RNA Biol 2014; 11: 10–17.

40. Marqiz AR, Mathur A, Chugh PE, et al. Expression profile of microRNAs in Epstein–Barr virus-infected AGS gastric carcinoma cells. J Virol 2014; 88: 1389–1393.

41. Hooykaas MJ, Kruse E, Wiertz EJ, et al. Comprehensive profiling of functional Epstein–Barr virus microRNA expression in human cell lines. BMC Genomics 2016; 17: 644.

42. Hagemeier SR, Barlow EA, Meng Q, et al. The cellular ataxia telangiectasia-mutated kinase promotes Epstein–Barr virus lytic reactivation in response to multiple different types of lytic reactivation-inducing stimuli. J Virol 2012; 86: 13360–13370.

43. Cai X, Schafer A, Lu S, et al. Epstein–Barr virus microRNAs are evolutionarily conserved and differentially expressed. PLoS Pathog 2006; 2: e23.

44. Qiu J, Cosmopoulos K, Pegtel M, et al. A novel persistence associated EBV miRNA expression profile is disrupted in neoplasia. PLoS Pathog 2011; 7: e1002193.

45. Gottwein E, Corcaron DL, Mukherjee N, et al. Viral microRNA targetome of KSHV-infected primary effusion lymphoma cell lines. Cell Host Microbe 2011; 10: 515–526.

46. Skalsky RL, Corcaron DL, Gottwein E, et al. The viral and cellular microRNA targetome in lymphoblastoid cell lines. PLoS Pathog 2012; 8: e1002484.

47. Lu J, He ML, Wang L, et al. MiR-26a inhibits cell growth and tumorigenesis of nasopharyngeal carcinoma through repression of EZH2. Cancer Res 2011; 71: 225–233.

48. Lin X, Tsai MH, Shumilov A, et al. The Epstein–Barr virus BART miRNA cluster of the M81 strain modulates multiple functions in primary B cells. PLoS Pathog 2015; 11: e1005344.

49. Ames BN, Gold LS. Endogenous mutagens and the causes of aging and cancer. Mutat Res 1991; 250: 3–16.

50. Ashworth A. A synthetic lethal therapeutic approach: poly(ADP) ribose polymerase inhibitors for the treatment of cancers deficient in DNA double-strand break repair. J Clin Oncol 2008; 26: 3758–3790.

51. Jasin M, Rothstein R. Repair of strand breaks by homologous recombination. Cold Spring Harb Perspect Biol 2013; 7: a012740.

52. Haber JE. DNA repair. Gatekeepers of recombination. Nature 1999; 398: 665–667.

53. Feng PC, Yap TA, Boss DS, et al. Poly(ADP-ribose)polymerase inhibition: frequent durable responses in BRCA carrier ovarian cancer correlating with platinum-free interval. J Clin Oncol 2010; 28: 2512–2519.

54. Gelmon KA, Tischkowitz M, Mackay H, et al. Olaparib in patients with recurrent high-grade serous or poorly differentiated ovarian carcinoma or triple-negative breast cancer: a phase 2, multicentre, open-label, non-randomised study. Lancet Oncol 2011; 12: 852–861.

55. Bryant HE, Helleday T. Inhibition of poly (ADP-ribose)polymerase activates ATM which is required for subsequent homologous recombination repair. Nucleic Acids Res 2006; 34: 1685–1691.
Regulation of ATM by EBV-miRNAs in NPC

56. McCabe N, Turner NC, Lord CJ, et al. Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose) polymerase inhibition. Cancer Res 2006; 66: 8109–8115.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary figure legends

Figure S1. Deep sequencing analysis shows that the miR-BART expression patterns in C666-1 are highly similar between three independent groups

Figure S2. The expression patterns of miR-BARTs are significantly similar across six tested NPC samples

Figure S3. ATM protein expression in EBV-negative NPC

Figure S4. miR-BART expression of the co-transfected cells in the dual luciferase reporter assays

Figure S5. Combination effects of BART5-5p, BART7-3p, BART9-3p, and BART14-3p on ATM signaling pathways

Figure S6. The microRNA inhibitors are specific to the intended mature miR-BARTs

Figure S7. The effect of miR-BART inhibitors on the previously reported ATM-regulated miRNAs in C666-1 cells

Figure S8. EBV-miRNAs suppress the DNA damage response

Figure S9. The role of miR-BARTs in controlling viral latency and the genotoxic stress response via the ATM signaling pathway

Table S1. Characteristics of the primary specimens recruited for quantitative RT-PCR analysis

Table S2. Characteristics of the primary specimens recruited for IHC analysis

Table S3. Heterogeneity of miR-BART5-5p, BART7-3p, BART9-3p, and BART14-3p in C666-1 cells

Table S4. The sequences of oligonucleotides used for quantitative RT-qPCR analysis

Table S5. Details of the miR-BART mimics and inhibitors

Table S6. The sequences of oligonucleotides used for the construction of luciferase reporter vectors

Table S7. Expression of EBV-miRNAs in small RNA sequencing of EBV-associated NPCs

Table S8. Cook’s distances for assessing the difference in expression among each sample

Table S9. Expression of ATM in primary NP and NPC cases

Table S10. Percentage of the reported ATM-regulatory microRNAs and four miR-BARTs of interest in small RNA sequencing of the C666-1 library

50 Years ago in the Journal of Pathology...

A comparison of the organisation and fate of autologous pulmonary emboli and of artificial plasma thrombi in the anterior chamber of the eye, in normocholesterolaeic rabbits

N. G. Ardlie and C. J. Schwartz

To view these articles, and more, please visit: www.thejournalofpathology.com

Click ‘ALL ISSUES (1892 - 2018)’, to read articles going right back to Volume 1, Issue 1.

The Journal of Pathology
Understanding Disease

© 2017 The Authors. The Journal of Pathology published by John Wiley & Sons Ltd
on behalf of Pathological Society of Great Britain and Ireland. www.pathsoc.org