Identification and characterisation of Epstein-Barr virus miRNA in nasopharyngeal carcinoma cells

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Key Messages

1. Two novel Epstein-Barr virus (EBV) miRNAs (miR-BART21 and miR-BART22) are preferentially expressed in nasopharyngeal carcinoma (NPC) samples.
2. Sequence polymorphisms in the primary transcript of miR-BART22 augment its biogenesis in vitro, and thus may underline the high and consistent expression of miR-BART22 in NPC cells.
3. EBV latent membrane protein 2A (LMP2A) is a putative target of miR-BART22. It is postulated that modulation of LMP2A expression by mir-BART22 may permit escape of EBV-infected cells from host immune surveillance. The large proportion of EBV-encoded miRNA compared to cellular miRNA in NPC cells underscores their significance in the establishment and/or maintenance of latent infections and pathogenesis in NPC cells.

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Introduction

Nasopharyngeal carcinoma (NPC) is prevalent in Southern China, and clonal Epstein-Barr virus (EBV) genome has been detected in both high-grade dysplastic lesions and invasive carcinoma, implicating an important aetiologic role for EBV in the NPC carcinogenesis. It is suggested that viral-encoded miRNAs play a significant role in carcinogenesis. We have identified novel EBV-encoded microRNAs from small cDNA library of native EBV-positive NPC cell line (C666-1) and xenograft (X2117) in our previous report (RFCID #06060372). These two miRNAs are highly expressed in NPC cell lines, xenografts, and primary tumour biopsies. In this study, we aimed to delineate the biological function of these novel EBV-encoded miRNAs in the nasopharyngeal epithelial cells.

Methods

This study was conducted from January 2008 to December 2008.

Proliferation rate and cell-cycle analysis

We studied the biological effect of miR-BART21 and 22 in two EBV-negative NPC cell lines (HK1 and HONE1) and an immortalised normal nasopharyngeal epithelial cell line NP69. Proliferation of cells transfected with mir-BART21 or 22 was assessed using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA). For flow cytometry analysis, cells were stained with propidium iodide and run on a FACScan flow cytometer (Becton Dickinson, CA, USA).

In vitro assay for Drosha complex digestion

An in vitro Drosha processing assay was conducted to investigate the efficacy of miRNA biogenesis in different EBV strains. The digestion substrate was prepared by in vitro transcription from T7-added miR-BART22 PCR product (~300 nt) using MAXIscript kit (Ambion). The Drosha/DGCR8 enzymatic complex was purified by Flag Tagged Protein immunoprecipitation kit (Sigma, St Louis, USA) from 293FT cells transfected with Drosha and Flag-DGCR8 expression vectors and was mixed with 100 ng RNA substrate at 37ºC for 1.5 hours. Digested products were analysed by Northern blot analysis.

In silico target prediction

We used MiRanda prediction by adjusting the energy threshold to -15 kcal/mol and the cut-off score to 90. We used the default setting for RNAhybrid program prediction. The LMP2A reference sequence for target prediction was extracted from NCBI (AB290724).

Luciferase reporter assay

293FT cells (1×10^5) grown in 24-well plates were co-transfected with miRNA and reporter construct for analysis. The cells were harvested after 2 days for luciferase activity analysis using Dual Luciferase Reporter Kit (Promega).

Western blotting and immunohistochemistry

Western Blot was preformed as previously described. The primary antibodies used were: LMP2A (MCA2467, AbD SeroTec; 1:2000 dilution), EGFP

(Clontech; 1:20,000 dilution) and β-actin (Sigma; 1:30,000 dilution). Immunohistochemical study was carried out using anti-LMP2A antibody (15F9, AbD SeroTec; 1:50) on the Ventana Nex ES automated stainer (Ventana Corporation, Tucson, AZ) using the avidin-biotin detection method.

Results

Preferentially expressed miR-BART21 and miR-BART22 in NPC cells

Greater expression of miR-BART21 and miR-BART22 was observed in NPC samples than in lymphoid cell lines (Akata, Namalwa, and Raji) using Northern blot and QRT-PCR analysis. Detailed comparison of the flanking sequences of miR-BART21 and miR-BART22 from C666-1 (EU828629.1) and Raji (AJ507799.2) revealed two nucleotide variations in miR-BART21 and four nucleotide changes in miR-BART22 (data not shown). The predicted secondary structures on miR-BART21 primary transcript (~300 nucleotides) [pri-miR-BART21] from Raji-EBV and C666-EBV strains were highly similar. However, the predicted secondary structure of miR-BART22 from Raji-EBV differed significantly from C666-EBV. The four nucleotide polymorphisms on Raji-EBV resulted in a small side-branched stem-loop adjacent to the mature miR-BART22 sequence (data not shown), which could impair miRNA maturation by concealing the DGCR8 recognition site in pri-miRNA processing. Although all four nucleotide variations in BART22 were positioned distal to the hairpin structure, changes to the two nucleotides (147144 A>T and 147146 C>A) could readily affect the stem-loop formation (data not shown). More importantly, 147144 A>T and 147146 C>A were identified in all 17 local NPC specimens examined. To elucidate whether nucleotide polymorphism of pri-miR-BART22 affected miRNA maturation, we examined the digestion efficiency of the Drosha/DGCR8 enzymatic complex by incubating immunoprecipitated flag-tagged Drosha/DGCR8 with in vitro transcribed EBV RNA substrates (AJ507799.2; 147137-147456) from the C666-1 and Namalwa EBV genomes, which shared the same polymorphism as Raji-EBV. In vitro digestion of pri-miR-BART22 in C666-1 was much facilitated in Drosha/DGCR8 processing, compared to the Namalwa strain (data not shown). Therefore, we hypothesised that nucleotide polymorphisms within the primary miR-BART22 transcript could augment its maturation in NPC cells, and at least partly explain the varying transcript levels in different EBV strains.

Functional effect of miR-BART21 and miR-BART22

The functional effect of miR-BART21 and miR-BART22 on cell proliferation and the cell cycle was investigated in three EBV-negative cell lines (NP69, HK1, and HONE1). In all three cell lines, transfection of miR-BART21 or miR-BART22 precursor did not show morphologic changes as in mock experiments; nor did they alter the proliferation and cell cycle.

LMP2A is a potential target of miR-BART22

In silico prediction suggested that LMP2A might be a putative target of miR-BART22. Using the luciferase reporter assay, we demonstrated that miR-BART22 exerted a strong inhibitory effect on LMP2A-3'UTR (42%, $P<0.001$). The repression was eliminated when the complementarities of the seed region were either deleted or mutated (data not shown). This suggests that the repressive property of BART22 on LMP2A 3'UTR was both functional and specific.

To confirm the importance of seed sequence complementarity in the BART22-LMP2A interaction, we performed additional luciferase assays using LMP2A-M1-M3 reporter plasmids with miR-BART22 and two miRNA mimics (miR-M2-BART22 and miR-M3-BART22) designed to compensate for the mutated seed regions of the LMP2A-M2 and M3 reporters (data not shown). The miRNA mimics exerted differing levels of suppression with the BART22 3'UTR, indicating that the miRNA mimics were functionally active. However in co-transfection with LMP2A-WT 3'UTR reporter, only miR-BART22 was able to suppress translation. Although seed binding may be critical for initiating repression, mutant miR-M3-BART22 with restored complementarity to LMP2A-M3 failed to exert an inhibitory effect on its corresponding mutated reporter. While miR-M2-BART22 significantly inhibited translation of the LMP2A-M2 mutated reporter, it expressed only slightly below the control level and clearly did not exhibit profound repression (data not shown). This suggests that the seed interaction between miR-BART22 and LMP2A-3'UTR was unique. Replacing seed pairing with other complementary sequences yielded negligible or only partial suppressive effects.

Differential expression of LMP2A in NPC cells

To investigate whether LMP2A is commonly expressed in NPC cells, we performed both RT-PCR and Western blot on a panel of NPC samples from South China. Although LMP2A RNA transcript could be detected in C666-1 cells, and NPC xenografts X666 and X2117, none of them showed detectable LMP2A protein level in Western blot (data not shown). Nevertheless, we were able to detect weak focally expressed LMP2A in X2117 by immunohistochemical staining. In addition, we detected weak LMP2A expression in six out of 26 (23%) primary NPC tumours (data not shown). The expression levels of miR-BART22 and LMP2A mRNA were also determined in 11 of these tumours. Interestingly, the LMP2A mRNA expression level did not correlate directly with protein expression. This finding supports the possible regulatory role of miR-BART22 on LMP2A expression.

Suppression of LMP2A protein expression by miR-BART22

To establish the strong interaction between miR-BART22 and LMP2A-3'UTR, two supportive experiments were designed. First, the dose effect of miR-BART22 on

LMP2A expression was studied by co-transfection of different amounts of miR-BART22 with the LMP2A expression vector with the complete 3'UTR. MiR-BART22 suppressed the LMP2A protein level in a dose-dependent manner without an apparent effect on LMP2A mRNA level (data not shown). MiR-BART22 expressions also had no obvious effect on the EGFP control protein. Second, transfection of miR-BART22 into HEK293 that had been stably transfected with pcDNA3.1-LMP2A (data not shown) readily suppressed the LMP2A protein. The transfection again had no significant effect on the LMP2A miRNA level. These results strongly suggest that LMP2A is a direct target of miR-BART22, which specifically represses LMP2A expression at the post-transcriptional level.

Discussion

Transcriptional regulation of LMP2A in EBV-infected cells by epigenetic and viral latent protein mechanisms has been reported. In the current study, LMP2A could also be regulated at the translational level by miR-BART22. LMP2A protein was expressed in NPC biopsies, which tended to have relatively low expressions of miR-BART22. However, not all biopsies showing low miR-BART22 expression had detectable LMP2A. This indicates that LMP2A expression might also be regulated by other pathways. Such multiple regulatory mechanisms have also been implicated in LMP1 modulation.[1]

There are many potential benefits when LMP2A expression is suppressed during NPC development. LMP2A in particular has a stronger immunogenicity than two other EBV latent gene products (EBNA1 and LMP1). In this regard, limiting LMP2A protein expression has potential advantages for NPC cells to escape host immune surveillance, and thus LMP2A expression in NPC cells is predictably low. In fact, immunomodulatory effects of other EBV-miRNAs have recently been demonstrated. For example, down-regulation of LMP1 by mir-BARTs may favour immune escape by decreasing the antigen processing function of NPC cells.[1] In primary effusion lymphoma, EBV-miR-BHRF1-3 can target CXCL-11/I-TAC, an IFN-inducible T-cell attracting chemokines.[2] Apart from immunogenicity, LMP2A (as oppose to LMP1) could suppress NF-κB level resulting in an anti-proliferative effect.[3] Moreover, LMP2A expressing epithelial cells could also inhibit telomerase reverse transcriptase activity, an enzyme important for cell immortalisation and transformation.[4] Since LMP2A has diverse functional roles in epithelial cells, its expression is therefore needed to be tightly regulated during the development of NPC.

We have reported that miR-BARTs regulates LMP1 expression,[1] and miR-BART5 affects the expression of the cellular target gene PUMA.[5] In this study, we further identified miR-BART22 as a modulator of an important oncogenic and immunogenic viral gene—LMP2A. This provides evidence for the vital roles of EBV-encoded miRNAs in regulating oncogenic and immunogenic latent viral protein expression, which may be important for the progression and survival of EBV-infected NPC cells.

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