Short Communication

Perturbation of biogenesis and targeting of Epstein–Barr virus-encoded miR-BART3 microRNA by adenosine-to-inosine editing

Ting Lei,1,2 Kit-San Yuen,2 Sai Wah Tsao,3 Honglin Chen,4 Kin-Hang Kok2 and Dong-Yan Jin2

1Department of Pathology, School of Medicine, Xi'an Jiaotong University, Xi'an, PR China
2Department of Biochemistry, University of Hong Kong, Pokfulam, Hong Kong SAR, PR China
3Department of Anatomy, University of Hong Kong, Pokfulam, Hong Kong SAR, PR China
4Department of Microbiology, University of Hong Kong, Pokfulam, Hong Kong SAR, PR China

Correspondence
Ting Lei
leiting@mail.xjtu.edu.cn
Dong-Yan Jin
dyjin@hku.hk

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Adenosine-to-inosine (A-to-I) RNA editing catalysed by adenosine deaminases that act on RNA (ADARs) is the most common type of editing in metazoans. As I preferentially base pairs with cytidine (C), this change is read as guanosine (G) by the splicing and translation machineries, leading to the change of RNA splicing patterns and translation results (Bass, 2002). ADARs are found in all animals and are expressed most abundantly in the nervous system. Three separate ADAR gene family members (ADAR1–3) are known in mammals. Both ADAR1 and ADAR2 are detected in many tissues and localized to the nucleus. ADAR3 is expressed only in brain and does not have adenosine deaminase activity (Bass, 2002). Interestingly, ADAR1 and ADAR2 exhibit both proviral and antiviral properties. Whereas ADAR2 is expressed constitutively, ADAR1 can be induced by interferons during the course of viral infection (Samuel, 2011).

Many A-to-I RNA editing sites have been revealed through systematic computational or experimental analysis, and most reside in dsRNA region (Bahn et al., 2012; Eggington et al., 2011; Levanon et al., 2004; Peng et al., 2012). Mature microRNAs (miRNAs) are produced from primary miRNAs (pri-miRNAs) in the nucleus and precursor miRNAs (pre-miRNAs) in the cytoplasm. Because pri-miRNAs also contain an imperfect short dsRNA duplex, they were predicted to be substrates for nuclear ADARs (Bass, 2000). The first mammalian pri-miRNA validated to be A-to-I edited was pri-miR-22 (Luciano et al., 2004). Subsequently, other human and mouse pri-miRNAs were confirmed to be A-to-I edited in various tissues (Blow et al., 2006; Choudhury et al., 2012; Garcia-Lopez et al., 2013; Pfeffer et al., 2005; Yang et al., 2006). A systematic survey of human pri-miRNA sequences identified A-to-I editing sites in ~6% of all pri-miRNAs examined (Blow et al., 2006). A comprehensive analysis of RNA-sequencing data also revealed 44 editing sites in human miRNAs (Peng et al., 2012). However, in vitro editing studies of randomly selected pri-miRNAs have suggested that as many as 50% of all pri-miRNAs might have specific A-to-I editing sites (Yang et al., 2006). A-to-I editing can affect miRNA biogenesis and reprogramme miRNA targeting (Gommans, 2012).

Epstein–Barr virus (EBV) successfully infects more than 90% of the world’s adult population. It establishes a lifelong latent infection and causes no disease in the vast majority of healthy carriers. However, EBV-associated lymphoid and epithelial malignancies, including nasopharyngeal carcinoma (NPC) and gastric cancer, occur in a small subset of individuals usually decades after initial infection (Raab-Traub, 2012; Tao et al., 2006). Although the mechanisms of EBV-associated carcinogenesis remain elusive (Gourzones et al., 2012), EBV-encoded miRNAs...
have emerged recently as important regulators of the virus life cycle, virus–host interactions and cancer development (Lo et al., 2012; Umbach & Cullen, 2009).

EBV encodes at least 44 mature miRNAs from two distinct clusters: BHRF1 and BART. These miRNAs are expressed differentially in different tissues and during different phases of latent infection. BHRF1 miRNAs are abundant in B-cells of latency III, but undetectable in B-cells or epithelial cells of latency I or II (Cai et al., 2006; Xing & Kieff, 2007). In contrast, whereas BART miRNAs are expressed sparsely in infected B-cells, their expression levels are high in epithelial cells of latency II (Cai et al., 2006; Choy et al., 2008b; Kim et al., 2007; Lo et al., 2007).

Interestingly, even in epithelial carcinoma tissues and cell lines, the BART miRNAs derived from the same primary transcript are not present at the same level. The copy numbers of BART miRNAs in the same biopsy sample were found to range from $10^2$ (miR-BART2-3p) to $10^6$ (miR-BART17-3p) (Cosmopoulos et al., 2009). Likewise, steady-state levels of different BART miRNAs were also found to vary by up to 50-fold within a cell line (Amoroso et al., 2011). In addition, the unique expression pattern of BART miRNAs in persistently infected cells was found to be disrupted in EBV-associated tumours (Qiu et al., 2011). Although insights into the targets and regulatory function of EBV miRNAs are beginning to emerge (Lo et al., 2012; Riley et al., 2012; Skalsky et al., 2012), much less is known about the regulation of their expression and activity.

BART miRNAs are processed from a large intron in the BART transcript prior to splicing by the same Drosha- and Dicer-dependent pathways used by the majority of cellular miRNAs (Edwards et al., 2008). Plausibly, A-to-I editing may serve as one mechanism to modulate the expression and activity of BART miRNAs, leading to repertoire diversity. Indeed, several EBV-encoded miRNAs, including pri-miR-BART6, pri-miR-BART8 and pri-miR-BART11, have been found to be edited at specific sites. Moreover, A-to-I editing of pri-miR-BART6 suppresses its processing and Dicer targeting (Iizasa et al., 2010). Sequence divergence from the reference EBV strain, which is compatible with A-to-I editing of BART miRNAs including miR-BART3 in NPC cells but unrecognized as such, has also been detected by deep sequencing (Chen et al., 2010). However, the editing of additional EBV miRNAs and the fate of these edited miRNAs remain to be identified and characterized.

![Fig. 1. A-to-I editing of EBV miR-BART3.](image)
Activation of type I interferon production is integral to the EBV latency programme (Xu et al., 2006). As a result, expression of the interferon-inducible ADAR1 editing enzyme might also be elevated in EBV-infected epithelial carcinoma cells. To verify this, we examined ADAR1 expression in three EBV + cell lines by Western blotting. HK1 and HK1/EBV are a pair of NPC cell lines. Whereas HK1 does not harbour EBV (Huang et al., 1980), introduction of EBV into HK1 through co-culture with EBV + B-cells yielded HK1/EBV cells. C666-1 is an NPC cell line constitutively carrying EBV (Cheung et al., 1999) and highly representative of primary NPC tumours (Cosmopoulos et al., 2009). AGS/BX1 is another gastric cancer cell line carrying EBV (Kim et al., 2007). Although ADAR1 was detected in all cell lines tested, the expression was more pronounced in EBV + cell lines HK1/EBV, C666-1 and AGS/BX1 versus the EBV − cell lines HK1 and HEK293 (human embryonic kidney) (Fig. 1a). Thus, ADAR1-dependent A-to-I editing might also be activated in EBV-infected cells.

To test the possibility of A-to-I editing of BART miRNAs, we amplified pri-miR-BARTs from C666-1 cells by reverse transcription-PCR (RT-PCR) and directly sequenced the products. If A-to-I editing occurs, an A-to-G change will be observed in the sequencing result. The co-appearance of A and G peaks in the sequencing chromatogram indicates incomplete editing. We first verified the A-to-G changes at the previously reported A-to-I editing sites of pri-miR-BART6 (Iizasa et al., 2010). In addition, we found the same A-to-G changes at four sites in pri-miR-BART3 suggestive of extensive A-to-I editing. In our experimental setting, these were the most prominent changes found in pri-miR-BARTs. We then cloned the PCR product of pri-miR-BART3 and sequenced 17 clones. Among them 13 had A-to-G changes indicating A-to-I editing. In most of the edited clones, all four sites were changed (Fig. 1b). Similar results were also obtained when we amplified and cloned pri-miR-BART3 from three EBV + NPC tumour samples. To exclude the possibility that the sequence changes were due to heterogeneity of the EBV genome, we determined the DNA sequence of the relevant BART region of EBV genome recovered from C666-1 cells and the three NPC samples. The genome sequence was unchanged and no heterogeneity was found. In addition, we verified the editing of the same four sites in pri-miR-BART3 in HK1/EBV and AGS/BX1 cells as well as in HEK293 cells ectopically expressing pri-miR-BART3 and ADAR1. Thus, we identified A-to-I editing of pri-miR-BART3 at four sites in EBV-infected cells.
of interest to see whether A-to-I editing perturbs Drosha processing of pri-miR-BART3 or silencing of target mRNAs. We recently demonstrated the DICE1 tumour suppressor to be a cellular target of EBV miR-BART3-5p (Lei et al., 2013). Hence, we sought to determine how A-to-I editing might impact on DICE1 targeting. A reporter construct pGL3-DICE1.UTR, in which luciferase expression is controlled by the full-length 3′ untranslated region (UTR) of DICE1, was co-transfected into HEK293 cells together with the pSuper-BART3 plasmid expressing pri-miR-BART3. The luciferase activity was diminished in co-transfected cells, indicating the targeting activity of

Fig. 3. A-to-I editing affects miR-BART3 biogenesis. The indicated pri-miRNAs were expressed in HEK293 cells. Total RNA was extracted using Trizol reagent (Invitrogen). Northern blotting was performed as described previously (Choy et al., 2008a). Mixed probes were labelled with 32P and hybridized perfectly to miR-BART3-5p and miR-BART3-5p-2 (a) or to miR-BART3-3p, miR-BART3-3p-3, miR-BART3-3p-4 and miR-BART3-3p-34 (b). Chemical cross-linking with 1-ethyl-3-(3-dimethylaminopropyl) carbodimide was used to enhance the sensitivity of small-RNA detection (Pall & Hamilton, 2008). Relative ratios of miRNA levels were quantified and are indicated below the lane numbers. Three independent experiments were carried out and the means ± SD are presented in the bar plots. An asterisk indicates that the differences between the indicated groups were statistically highly significant (P<0.001 by Student's t-test).
miR-BART3-5p. Interestingly, the suppressive activity was reversed completely when the second editing site was mutated to G, and almost completely when all four sites were changed. In addition, the suppression was also alleviated when the third editing site was mutated to G (Fig. 2a). As a positive control, four copies of perfectly matched miR-BART3-5p target sequence were cloned into the 3′ UTR of the luciferase gene, giving rise to reporter plasmid pGL3-miBART3.UTR. Expression of pSuper-BART3 and its editing site mutants dampened the luciferase activity recovered from pGL3-miBART3.UTR to the same extent. This indicated that pri-miR-BART3 and its mutants were expressed and functional. Consistent with the results of the luciferase assays, expression of ADAR1 in HEK293 cells alleviated the suppression of DICE1 protein expression by miR-BART3-5p (Fig. 2b, compare lane 1 with lanes 2 and 3). This was in agreement with the notion that ADAR1-mediated A-to-I editing perturbs DICE1 targeting by miR-BART3-5p. Collectively, these data support the suggestion that A-to-I editing of the second site within the seed region of miR-BART3-5p compromised DICE1 targeting.

miRNA processing by Drosha and Dicer might also be affected by A-to-I editing at some sites (Bass, 2002; Gommans, 2012). To investigate whether A-to-I editing at the four sites might impede biogenesis of miR-BART3, we compared the steady-state amounts of different forms of pre-miR-BART3 as well as mature miR-BART3-5p and miR-BART3-3p in HEK293 cells by Northern blotting. Notably, the relative levels of mature products of miR-BART3 mutated either at the third editing site or at all four editing sites were substantially reduced (Fig. 3, compare lane 1 with lanes 4 and 6). Thus, A-to-I editing of pri-miR-BART3 affected not only target selection but also biogenesis.

In this work, we identified and characterized A-to-I editing of EBV-encoded miR-BART3. Four novel editing sites were identified and the functional impact of editing on DICE1 targeting and biogenesis was characterized. A-to-G mutation at the third editing site affected miRNA biogenesis (Fig. 3); this might contribute to the partial reversal of suppressive effect on DICE1 transcript (Fig. 2a). In contrast, A-to-G mutation at the second editing site had no influence on the production of mature miR-BART3-5p and miR-BART3-3p (Fig. 3), but eliminated the ability to silence DICE1 expression (Fig. 2a). Thus, A-to-I editing of the second site suppressed DICE1 targeting plausibly through alteration of base pairing of miR-BART3-5p with the target sequence in 3′ UTR of DICE (Lei et al., 2013). A-to-I editing was not influential on the targeting of a perfectly matched miR-BART3-5p target (Fig. 2a), suggesting that the silencing of such a target could occur efficiently even when mature miR-BART3-5p was limiting due to impaired processing and when base pairing with the target was affected by the change of one nucleotide. Plausibly, due to a high degree of complementarity between miR-BART3-5p and such a target, only a small amount of miR-BART3-5p would be sufficient for silencing. It is not too surprising that one nucleotide change was well tolerated. This might only affect the mechanism but not the functional outcome of silencing. In other words, such a target was effectively silenced by miR-BART3-5p through translational repression but not specific RNA cleavage. Nevertheless, our findings suggest that A-to-I editing of pri-miR-BART3 could prevent the suppression of DICE1. It will be of great interest to determine the biological significance of this effect in EBV infection and NPC development.

Other herpesvirus-encoded miRNAs are also known to be A-to-I edited. Kaposi’s sarcoma-associated herpesvirus-encoded miR-K12-10 was the first viral miRNA validated to be A-to-I edited (Gandy et al., 2007; Pfeffer et al., 2005). A subsequent study characterized A-to-I editing of EBV-encoded miR-BART6 (Iizasa et al., 2010). Consistent with these reports, our findings supported the notion that A-to-I editing of herpesvirus-encoded pri-miRNAs might redirect targets and modulate the steady-state levels of mature miRNAs in infected cells. Although DICE1 targeting was compromised by A-to-I editing, the edited miR-BART3 could also gain new specificity in targeting. It remains to be seen what new viral and cellular RNAs might be targeted more efficiently by the edited miR-BART3-5p and miR-BART3-3p. As it impacts on miRNA biogenesis, A-to-I editing might also provide another level of regulation for miRNA expression and function. Plausibly, A-to-I editing of particular BART miRNAs including miR-BART3 and miR-BART6 could contribute to the previously documented wide range of miRNA expression levels in infected cells (Amoroso et al., 2011; Cosmopoulos et al., 2009). In this regard, further investigations are required to clarify how A-to-I editing might affect the levels of mature miR-BART3 and other EBV-encoded miRNAs in NPC cells.

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References


