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RAPID COMMUNICATION

EBV-Mir-BART5-5p targets p53 independent pathway in cytoplasm: Potential role in EBV lymphomagenesis

EBV-positive diffuse large B-cell lymphoma (DLBCL), which has a clonal EBV carrying proliferation of B-cells, defines a new DLBCL subtype and predicts a poor prognosis. Further studies are needed to explore the underlying mechanisms in EBV lymphomagenesis. EBV encoded microRNAs (miRNAs) have been proven to contribute to the pathogenesis and oncogenesis of EBV-associated malignancies.[1] In this study, research has focused on the pathological roles of miRNAs in EBV-positive DLBCL progression and survival, which might provide ideas for therapeutic decision to improve the prognosis.

We identified that high expression of miR-BART5-5p correlates with low p53/PUMA expression in DLBCL cell lines (Fig. 1A, B). Based on the computational predictions of miRNA target sites, PUMA was identified as a potential target of miR-BART5-5p by the bioinformatics analysis shown in Figure 1C. Dual-luciferase reporter assay results showed that miR-BART5-5p had decreased the luciferase activity of PUMA wild-type reporter plasmid significantly at 24 h ($P < 0.0001$) and 48 h ($P = 0.0058$) but not PUMA mutant which indicated that miR-BART5-5p targets PUMA 3′UTR directly (Fig. 1D). Transfection of miR-BART5-5p mimic and inhibitor into cells provided further evidence for a negative relationship between miR-BART5-5p and PUMA (Fig. 1E). The results showed that miR-BART5-5p could accelerate host cells proliferation, promote cell cycle progression and confer resistance to the apoptosis. Moreover, we found that cells with high expression of miR-BART5-5p were less sensitive to apoptosis when treated with doxorubicin (Fig. 1F–H; Fig. S1B–D, F). No change in mRNA level indicated that miR-BART5-5p regulated PUMA expression post-transcriptionally in DLBCL (Fig. S1A).

Interestingly, we found that high levels of miR-BART5-5p tend to reduce p53 expression (Fig. 1H; Fig. S1D). To further investigate the function of p53, cells were treated with p53 inhibitor pifithrin-α (PFT) at a concentration of 10 μM for 48 h. The Western blot analysis indicated that p53 could upregulated PUMA and cleavage PARP (CP) expression (Fig. 1J; Fig. S1E). Flow cytometry showed similar observations that the downregulation of p53 decreased cells apoptosis (Fig. S1G). Moreover, immunofluorescence and Western blot analysis suggested the different distribution of nuclear and cytoplasmic p53. Cytoplasmic p53 was significantly reduced in miR-BART5-5p mimic compared to mimics NC while nuclear p53 was not different (Fig. 1I, K; Fig. S2A). Likewise, cytoplasmic p53 was significantly increased in miR-BART5-5p inhibitor compared to inhibitor NC while nuclear p53 was not different (Fig. 1I, K; Fig. S2A). The results indicated that the cytoplasmic p53 levels could influenced by miR-BART5-5p expression.

The transcription-independent activities of p53 in cytoplasm are involved in the intrinsic mitochondrial pathway and autophagy to regulate apoptosis.[2] Therefore, we next investigated whether the autophagy levels are affected by miR-BART5-5p expression. The expression of LC3, SQSTM1/p62 and Beclin-1 protein were detected. LC3 and Beclin-1 levels were decreased while p62 was increased following the transfection of miR-BART5-5p inhibitor (Fig. S2B). Likewise, LC3 and Beclin-1 were up-regulated while p62 was down-regulated in cells with miR-BART5-5p mimics (Fig. S2B). Immunofluorescence assay was further carried out to measure the LC3 expression intensity and indicated that the intensity of LC3 was increased with elevated miR-BART5-5p (Fig. 1M). To explore the role of autophagy, we used 20 μM autophagy inhibitor chloroquine (CQ) to treat cells for 48 h. The Western blot and flow cytometry analysis showed that autophagy inhibition promotes apoptosis (Fig. 1L; Fig. S2D). Taken together, the results elucidated that miR-BART5-5p could suppress mitochondria-dependent apoptosis and induce autophagy to further reduce host cell apoptosis by decreasing the level of cytoplasmic p53.

Current studies on the pathogenesis of EBV-associated lymphoproliferative disorders were concentrated in the oncogenic roles of EBV latent antigens such as LMP1 and

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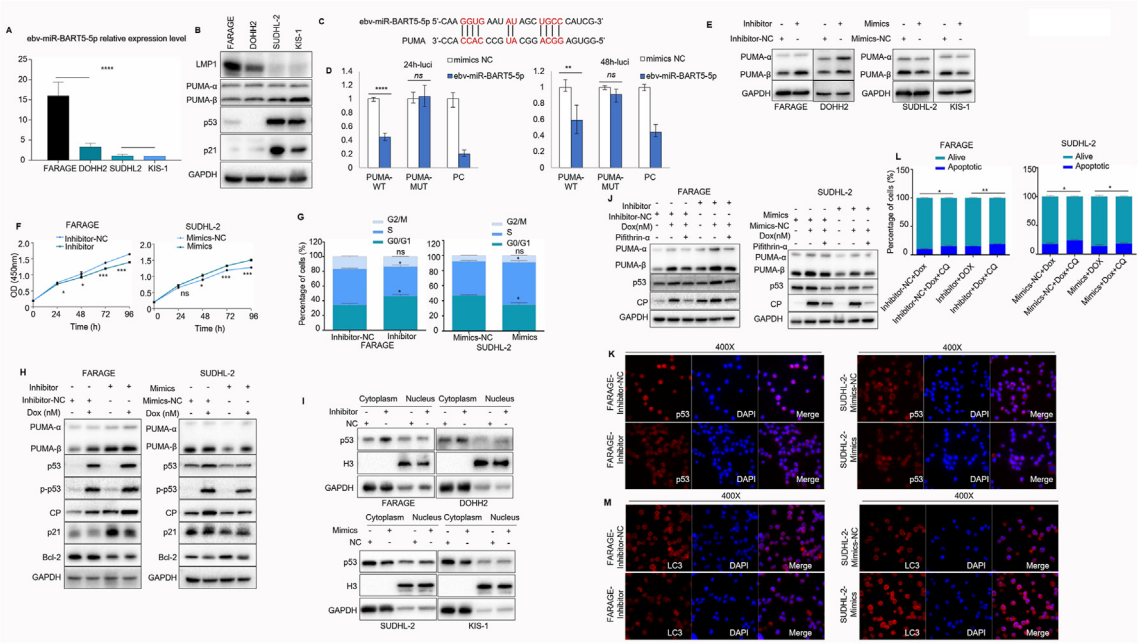

Figure 1 MiR-BART5-5p could promote host cell survival by suppressing mitochondria-dependent apoptosis and inducing autophagy. **(A)** qPCR analysis of ebv-miR-BART5-5p in FARAGE, DOHH2, SUDHL-2 and KIS-1. **(B)** Western blot analysis of LMP1, PUMA-α, PUMA-β, p53 and p21 proteins in FARAGE, DOHH2, SUDHL-2 and KIS-1. **(C)** Computational predictions of ebv-miR-BART5-5p target sites in PUMA 3′UTR. **(D)** The results of dual-luciferase reporter assay in 293T cells showed that miR-BART5-5p had decreased the luciferase activity of PUMA wild-type reporter plasmid significantly at 24 h ($P < 0.0001$) and 48 h ($P = 0.0058$) but not PUMA mutant. **(E)** Western blot analysis of PUMA-α and PUMA-β proteins in FARAGE, DOHH2, SUDHL-2 and KIS-1 after transfection of miR-BART5-5p inhibitor or mimic. **(F)** CCK8 assay in FARAGE, DOHH2, SUDHL-2 and KIS-1 after transfection of miR-BART5-5p inhibitor or mimic. **(G)** Cell cycle analysis in FARAGE, DOHH2, SUDHL-2 and KIS-1 after transfection of miR-BART5-5p inhibitor or mimic. **(H)** Western blot analysis of PUMA-α, PUMA-β, p53, p-p53, cleaved PARP, p21 and Bcl-2 proteins in cells treated with doxorubicin of FARAGE, DOHH2, SUDHL-2 and KIS-1 after transfection of miR-BART5-5p inhibitor or mimic. **(I)** Western blot analysis of cytoplasmic p53 in FARAGE, DOHH2, SUDHL-2 and KIS-1 after transfection of miR-BART5-5p inhibitor or mimic. **(J)** Western blot analysis of PUMA-α, PUMA-β, p53 and cleaved PARP in cells treated with doxorubicin and/or pifithrin-α of FARAGE, DOHH2, SUDHL-2 and KIS-1 after transfection of miR-BART5-5p inhibitor or mimics. **(K)** Immunofluorescence analysis of the nuclear and cytoplasmic distribution of p53 in FARAGE and SUDHL-2 after transfection of miR-BART5-5p inhibitor or mimics. **(L)** Apoptosis assay in cells treated with doxorubicin and/or CQ of FARAGE, DOHH2, SUDHL-2 and KIS-1 after transfection of miR-BART5-5p inhibitor or mimic. **(M)** Immunofluorescence analysis of LC3 in FARAGE and SUDHL-2 after transfection of miR-BART5-5p inhibitor or mimics. CP, cleaved PARP; Dox, doxorubicin; MUT, mutant; NC, negative control; PC, positive control; PFT, pifithrin-α; WT, wild-type. For A and D, mean and SEM values from $n = 3$ replicates are *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, non-significant using one way Anova with multiple comparison test and two-tailed paired Student t test respectively.

EBNA2.[3,4] Minimal research exists on the carcinogenic effect of EBV encoded miRNAs in EBV-positive DLBCL. In this paper, we confirmed that PUMA is one of the cellular targets of EBV encoded miRNAs which confer growth advantages to tumor cells in EBV-positive DLBCL. Our results showed that increased miR-BART5-5p could not only inhibit PUMA directly through post-transcriptional regulation, but also reduced cytoplasmic p53 expression to further contribute to reducing PUMA expression. Our date showed a consistent result while the specific mechanism has not been further studied yet. This study contributes to our understanding of miRNAs in the pathogenesis of EBV-associated lymphoproliferative disorders which provided a novel idea for targeted therapy.

Author contributions

WX and JL were responsible for supervising and conceiving the project. TW and JG participated in designing and carrying out most of the experiments. LW, JW, HS, YK and YX were responsible for analyzing data and interpreting results. TW and JG participated in writing the article and making the figures. JL, JL and WX drafted and revised the

paper. All authors contributed to the article and approved the submitted version.

Conflict of interests

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.07.003.

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