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ORIGINAL RESEARCH

Knockdown of EBV-encoded circRNA circRPMS1 suppresses nasopharyngeal carcinoma cell proliferation and metastasis through sponging multiple miRNAs

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Background: Epstein-Barr virus (EBV)-produced non-coding RNAs, including circular RNA (circRNA), regulate host cell gene expression and play important roles in development of nasopharyngeal carcinoma (NPC). EBV-encoded circRNA circRPMS1 consists of the head-to-tail splicing of exons 2-4 from the *RPMS1* gene. Its roles and mechanism on NPC remain unknown.

Purpose: In this study, we investigated the biological functions and molecular mechanisms of circRPMS1 in tumor proliferation, apoptosis, invasion, metastasis and as a potential biomarker for NPC diagnosis and prognosis.

Patients and methods: NPC tissues and the adjacent tissues were collected. Cell proliferation assay, cell apoptosis assay, cell invasion assay, luciferase reporter assay, RNA immunoprecipitation and tumor xenograft in nude mice were performed to analyze the circRPMS1 functions.

Results: We found that EBV-encoded circRPMS1 was increased in metastatic NPC and was associated with short survival time. Knockdown of circRPMS1 inhibited cell proliferation, induced apoptosis and repressed cell invasion in EBV-positive NPC cells. Further mechanism investigation revealed that circRPMS1-meadiated NPC oncogenesis through sponging multiple miRNA and promoting epithelial-mesenchymal transition (EMT). The inhibitors of miR-203, miR-31 and miR-451 could reverse the effects of circRPMS1 knockdown on NPC cells.

Conclusion: The findings indicate circRPMS1 as a potential therapeutic target for EBV-associated NPC. Our findings provide important understanding for the further elucidation on the therapeutic use of circRNA in NPC.

Keywords: Epstein–Barr virus, circular RNA, nasopharyngeal carcinoma, miRNA, epithelial-mesenchymal transition

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Introduction

Nasopharyngeal carcinoma (NPC) arises from pharyngeal mucosal space and is prevalent in Asian and North African population.[1] Despite significant medical advancement, metastasis of NPC remains a main cause of death for NPC patients. The potential mechanism of NPC metastasis is not well known, but Epstein–Barr virus (EBV) infection is strongly associated with tumor metastasis.[2,3] Previous studies have demonstrated that long noncoding RNAs (lncRNAs) and microRNAs (miRNAs) play epigenetic regulatory roles in NPC metastasis.[4,5] EBV can produce lncRNAs and miRNAs and they target each other to form an interconnected, complex molecular regulatory

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network.[6,7] EBV encodes 44 mature miRNAs that regulate host cell gene expression and play important roles in the development of NPC.[4,8,9] Recently, deregulation of circular RNAs (circRNAs) is also associated with NPC progression. For example, high circRNA_000543 expression displayed poorer overall survival in NPC patients, and circRNA_000543 knockdown sensitized NPC cells to irradiation.[10] These results highlight the importance of circRNAs in mediating NPC processes. Interestingly, it is found that EBV also encodes circRNA, and contributes to viral oncogenesis.[11]

EBV-encoded circRNA circRPMS1 consists of the head-to-tail splicing of exons 2–4 from the *RPMS1* gene and localized in both cytoplasm and nuclei.[12] Its roles and mechanism on NPC remain unknown. In this study, we investigated the biological functions and molecular mechanisms of circRPMS1 in tumor proliferation, apoptosis, invasion, metastasis and as a potential biomarker for NPC diagnosis and prognosis.

Materials and methods

Human tissues collection

This project was approved by the Ethic Committee of Xiangya Hospital, Central South University, and written informed consent was obtained from each patient. These included subjects have not received any treatment before operation. NPC tissues (N=117) and the adjacent tissues (N=88) were collected from Xiangya Hospital, Central South University between September 2009 and December 2018. EBV test was performed in each patient with NPC to determine the virus infection. Each sample was immediately flash-frozen in liquid nitrogen until RNA extraction.

Cell culture

The cell lines used in the present study included EBV-negative normal nasopharyngeal epithelial cell lines NP69, EBV-positive NPC cell line C666-1, EBV-negative NPC cell line HNE2. These cell lines were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cell-to-cell contact method with a recombinant EBV was used to construct EBV-infected HNE2 cell line (HNE2-EBV$^+$).[13]

All cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Scientific, Shanghai, China) supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified atmosphere with 5% CO_2 at 37°C.

Cell transfection

CircRPMS1 siRNA plasmids were constructed and purchased from GenomediTech (Shanghai, China). miRNAs inhibitors were purchased from RiboBio (Guangzhou, China). Transfection was performed using Lipofectamine 3000 (Life Technologies, Shanghai, China) according to the manufacturer's instructions.

Cell proliferation analysis

A BrdU Cell Proliferation Assay Kit (Biovision, Milpitas, CA, USA) was used to measure the cell proliferation rate according to the manufacturer's instructions. Briefly, the cells were seeded in a 96-well plate and incubated overnight. After transfection, the cells were further cultured for 24 hrs. Then, 10 μM BrdU was added to the medium and incubated for 4 hrs. The absorbance at 450 nm was read and recorded to calculate the relative proliferation rate. The experiment was done three times with triplicate samples.

Cell apoptosis analysis

An Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China) was used for analysis of apoptosis according to the manufacturer's instructions. Briefly, cells with indicated treatment were resuspended in 195 μL Annexin V-FITC binding buffer and then mixed well with 5 μL Annexin V-FITC and 10 μL Propidium Iodide for 15 mins incubation. The cells were analyzed by using a BD FacScanto II flow cytometer (BD Biosciences, San Jose, CA, USA). Unstained cells and fluorescence minus one controls were used for cytometry and gating set up.

Cell invasion assays

Transwell assays were performed to evaluate the invasive cells. 5×10^4 cells were seeded in serum-free medium for 6 hrs. Then, 1640 medium containing 10% FBS was added to the bottom chamber. After culture for 24 hrs, the cells on the lower surface of the filter were fixed, stained, and counted under a microscope.

Quantitative PCR analysis

Trizol reagent (Invitrogen, CA, USA) was used to extract total RNA from cells. SuperScript™ IV First-Strand Synthesis System (cat no. 18091050, Thermo Scientific, Beijing, China) was used to reverse transcript cDNA from 500 ng of

RNA according to the manufacturer's protocol. The expression of circRPMS1 was measured by BeyoFast™ SYBR Green qPCR Mix (Beyotime, Shanghai, China) according to manufacturers' instructions. Expression of β-actin was used as an endogenous control. QPCR was performed at the condition: 95.0°C for 2 mins, and 40 circles of 95.0°C for 15 s and 60°C for 20 s in LightCycler 480 (Roche, Basel, Switzerland). The primers were used as following: 5′- AGCCCTTCTTCGTTATGCAC-3′(forward), 5′- TGAGGAATACCTCGTTGTCTTCCG-3′(reverse) for circRPMS1. 5′-CACACTGTGCCCATCTATGAGG-3′(forward), 5′-TCGAAGTCTAGGGCGACATAGC-3′(reverse) for β-actin. miRNAs expression was measured by All-in-One™ miRNA qRT-PCR (GeneCopoeia, Shanghai, China) according to the manufacturer's instructions. The qPCR data were analyzed using $2^{-\Delta\Delta Ct}$ method.[14]

Western blot

Protein was extracted and determined their concentrations by BCA Protein assay kit (Beyotime, Shanghai, China). Proteins were separated by 10% SDS/PAG and transferred into PVDF membrane. The membranes were immunoblotted with the primary antibodies (anti-E-cadherin, 1:2000 dilutions; anti-Vimentin, 1:1000 dilutions; anti-N-cadherin, 1:2000 dilutions; anti-GAPDH, 1:4000 dilutions). All the antibodies were purchased from Cell signaling Technology (Danvers, MA, USA). Membranes were then incubated with peroxidase-conjugated secondary antibody, and specific bands were detected with a Bio-Rad imaging system.

Luciferase reporter assay

The wild type sequence and mutants in binding sites of circRPMS1 was cloned downstream of FL reporter vector. The 50 miRNA mimics were obtained from GeneCopoeia (Shanghai, China). C666-1 cells (5×10^3 cells/well) were seeded and cultured in 96-well plates for 24 hrs. The cells were co-transfected with FL reporter, Renilla luciferase reporter, and miRNA mimic for 48 hrs. Luciferase activity was measured using a Luc-Pair™ Duo-Luciferase HS Assay Kit (GeneCopoeia, Shanghai, China). Relative luciferase activity was normalized to Renilla activity.

RNA immunoprecipitation (RIP)

A Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Danvers, MA, USA) was used in this assay as previously described.[15] Briefly, C666-1 cells were treated with formaldehyde to cross-link RNA complexes. The supernatant was incubated with a circRPMS1-specific probes dynabeads (GeneCopoeia) mixture overnight at 30°C. On the next day, the dynabeads was washed and incubated with 200 μL of lysis buffer. Finally, RNA was extracted from these complexes and was used for qPCR.

Tumor xenograft in nude mice

Animal experiments were approved by the Ethical Committee for Animal Research of Xiangya Hospital, Central South University and followed the guidelines on the welfare and use of animals (Laboratory animal—Guideline for ethical review of animal welfare, GB/T 35892-2018, China). The C666-1 cells transfected with circRPMS1 si2# were subcutaneously injected into nude mice (5 mice per group, male, 2 months-old). The tumor sizes were measured every week. The tumor volume was calculated using the formula: $0.5\times L\times W^2$ where L and W are the long and short diameter of the tumor, respectively.

Statistical analysis

Data were analyzed in GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). The expression of circRPMS1 greater or equal to the mean was defined as high expression, otherwise were defined as low expression in NPC patients. Overall survival analysis and disease-free survival (DFS) were performed by Kaplan–Meier curves and log-rank test for significance. Student's *t*-test with two tails was used to assess the statistical significance in two groups and one-way ANOVA with post hoc Bonferroni test was used in three or more groups. Correlations were analyzed by Pearson correlation test. *p*-value less than 0.05 was considered statistically significant.

Results

EBV encoding circRPMS1 is upregulated in patients with NPC

CircRPMS1 has been identified as an EBV encoding circRNAs. To investigate the role of circRPMS1 in NPC, we measured its expression in NPC tissues and adjacent tissues. We found that circRPMS1 was significantly increased in EBV-positive NPC tissues compared with adjacent tissues, and circRPMS1 did not express in EBC-negative NPC tissues (Figure 1A). In addition, the expression of circRPMS1 was higher in patients with metastasis than in those without metastasis (Figure 1B). Further

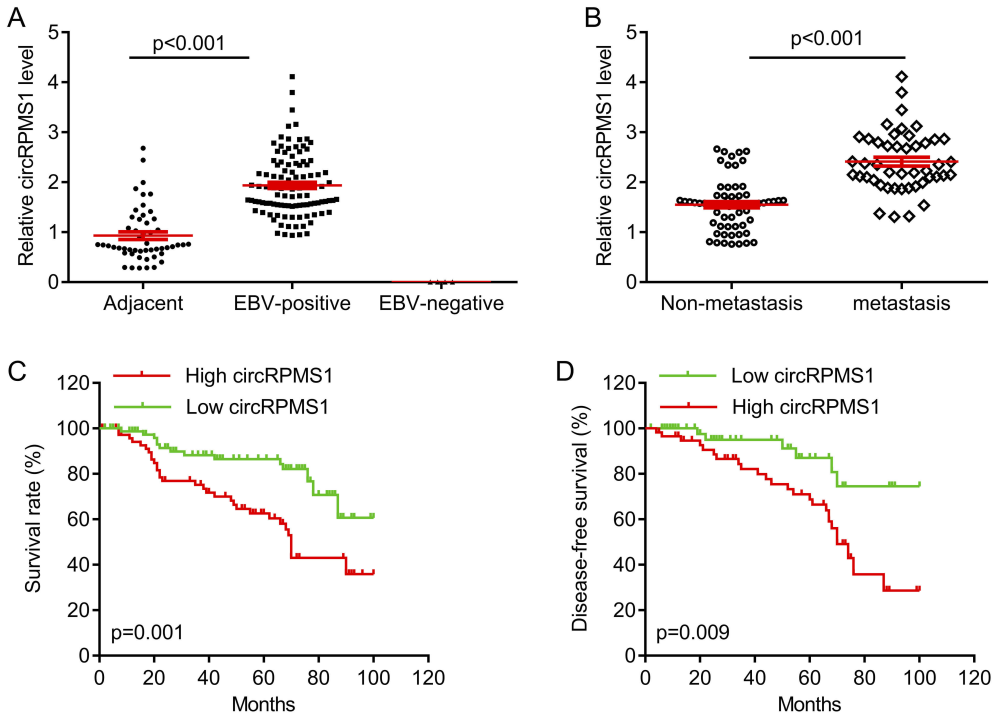

Figure 1 CircRPMS1 is upregulated in NPC.
Notes: (**A**) The expression of circRPMS1 in EBV-positive and -negative NPC tissues and adjacent tissues. (**B**) The expression of circRPMS1 in patients with metastatic NPC or without metastasis. (**C**) Kaplan–Meier curves of overall survival in NPC patients with low or high circRPMS1 expression. (**D**), Kaplan–Meier curves of disease-free survival in NPC patients with low or high circRPMS1expression.
Abbreviations: EBV, Epstein-Barr virus; NPC, nasopharyngeal carcinoma.

survival analyses revealed that the NPC patients with low expression of circRPMS1 had longer overall survival (p=0.001, Figure 1C) and DFS (p=0.009, Figure 1D) than the patients had high circRPMS1. These results suggest that circRPMS1 is closely associated with the development of NPC.

The effects of circRPMS1 on cell proliferation, apoptosis, and invasion in NPC cells

Given that circRPMS1 is an EBV encoding circRNA, we measured its expression in several cell lines. We found that circRPMS1 was expressed in EBV-positive cell lines C666-1 and HNE2-EBV$^+$, but not in NP69 and HNE2 (Figure 2A). We knocked down circRPMS1 in C666-1 and HNE2-EBV$^+$ cells (Figure 2B). Downregulation of circRPMS1 significantly inhibited C666-1 and HNE2-EBV$^+$ cells proliferation and induced apoptosis compared with negative control (Figure 2C–F). In addition, circRPMS1 knockdown also attenuated C666-1 and HNE2-EBV$^+$ cells invasive capacities (Figure 2G–H). Furthermore, the inhibitory effects of circRPMS1 knockdown on tumor growth also confirmed in vivo. Nude mice were injected subcutaneously with C666-1 cells, expressing either control or circRPMS1 si2# plasmids. We showed that the tumor volumes in mice injected with C666-1 cells expressing circRPMS1 si2# were smaller than in the mice injected with control cells (Figure 2I).

CircRPMS1 serves as a sponge for multiple miRNAs in NPC

It has been reported that circRNAs act as miRNA sponge to exhibit their functions. We tested whether circRPMS1 binds to miRNAs in NPC cells. A miRNA mimic library was constructed and used to identify the binding miRNAs. MiRNA mimics were co-transfected with circRPMS1 luciferase reporters in C666-1 cells (Figure 3A). Five out of 50 miRNAs including miR-203, miR-200, miR-31, miR-9, and miR-451, reduced at least half of the luciferase reporter activities compared with the control (Figure 3A). Further assay confirmed that circRPMS1 targeted to miR-203, miR-31, and miR-451 (Figure 3B), suggesting that circRPMS1 may function as a sponge to these three miRNAs. In addition, RIP

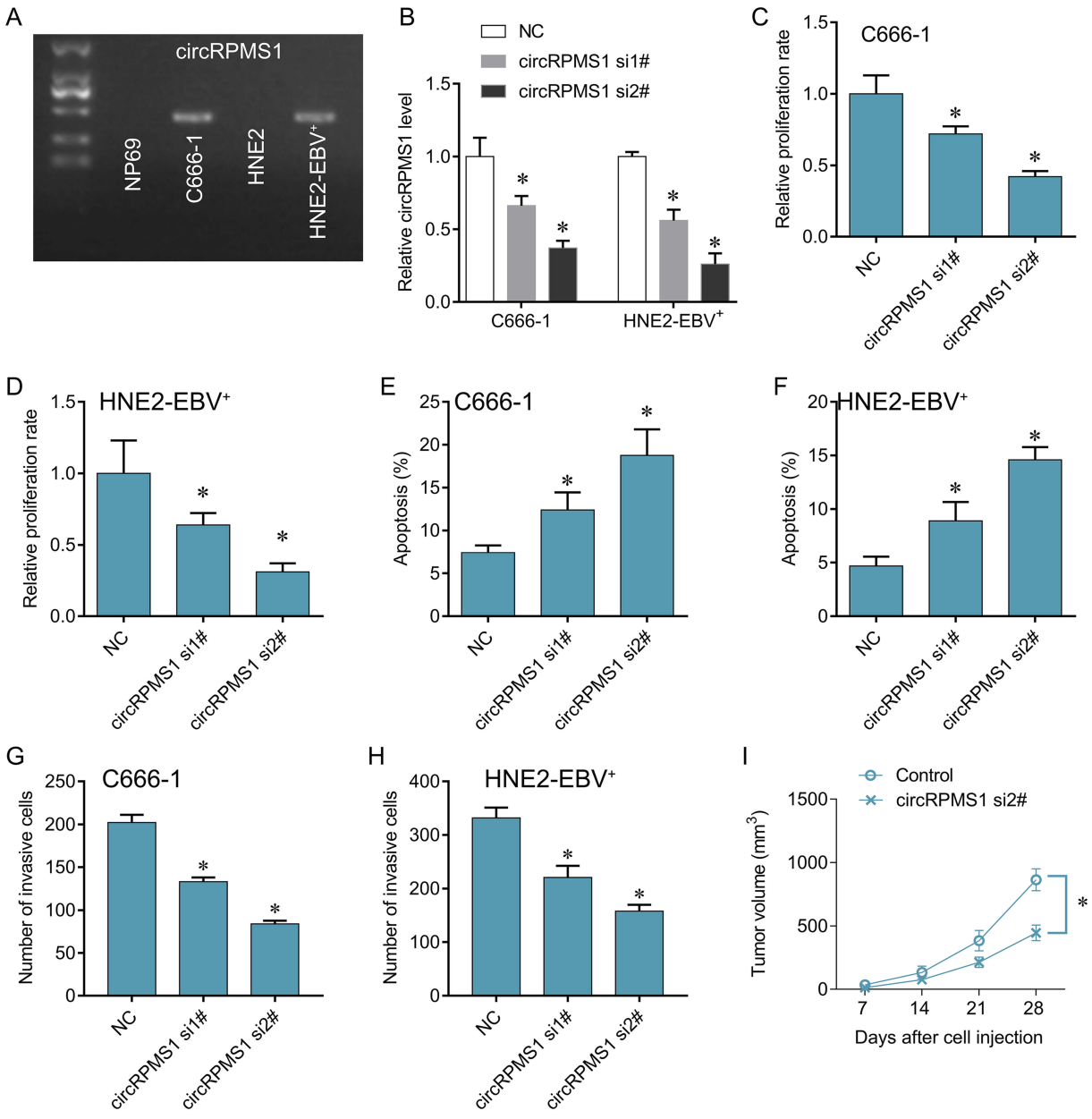

Figure 2 Silencing circRPMS1-inhibited NPC cell proliferation.

Notes: (**A**) RT-PCR for circRPMS1 in NP69, C666-1, HNE2, and HNE2-EBV$^+$ cells. (**B**) qRT-PCR for circRPMS1 in C666-1 and HNE2-EBV$^+$ cells transfected with control vector or circRPMS1 siRNA plasmid. (**C, D**) Brdu assay showed that silencing circRPMS1- inhibited proliferation of C666-1 (**C**) and HNE2-EBV$^+$ (**D**) cells. (**E** and **F**) Flow cytometry showed that silencing circRPMS1 induced apoptosis in C666-1 (**E**) and HNE2-EBV$^+$ (**F**) cells. (**G, H**) invasion assay showed that silencing circRPMS1 inhibited the invasive ability of C666-1 (**G**) and HNE2-EBV$^+$ (**H**) cells. (**I**) C666-1 cells were transfected with circRPMS1 si2# plasmids or with the control and were injected into BALB/c nude mice. The tumor volumes were measured every week. *p<0.05 vs control.

Abbreviations: EBV, Epstein-Barr virus; NPC, nasopharyngeal carcinoma; NC, normal control.

assay revealed that miR-203, miR-31, and miR-451 were copurified circRPMS1 (Figure 3C). We also found that the levels of miR-203, miR-31, and miR-451 were significantly decreased in EBV-positive NPC tissues compared with adjacent tissues (Figure 3D), and were negatively correlated with circRPMS1 expression (Figure 3E–G). These results suggested that circRPMS1 bond with miR-203, miR-31, and miR-451.

CircRPMS1 knockdown inhibits NPC cells growth and epithelial-mesenchymal transition (EMT) through sponging miR-203, miR-31, and miR-451

To investigate the downstream mechanism by which circRPMS1 exerted its functions in NPC cells, the inhibitor of miR-203, miR-31, and miR-451 was co-transfected with

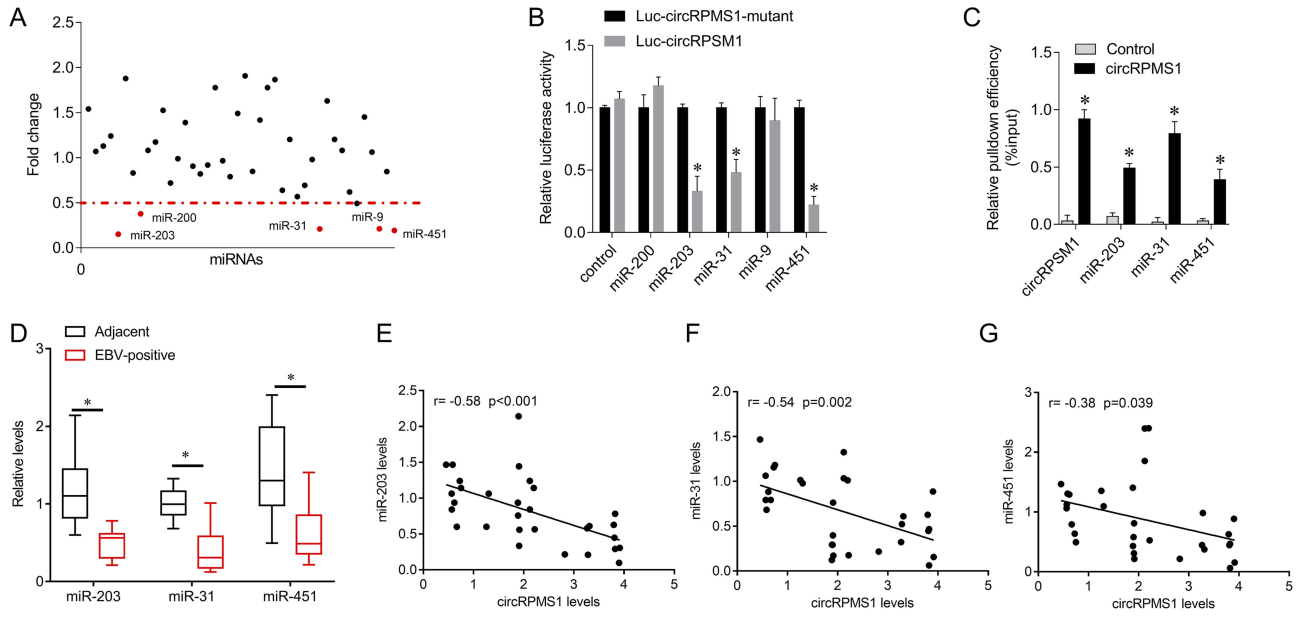

Figure 3 CircRPMS1 served as a sponge for multiple miRNAs in NPC cells.
Notes: (**A**) Luciferase reporter assay for a miRNA library to identify circRPMS1 targeted miRNAs. The luciferase activity reduced by 50% is indicated by red dots. (**B**) C666-1 cells were co-transfected Luc-circRPMS1 or Luc-circRPMS1 mutant with miRNA mimics. Luciferase reporter gene assay was performed to measure luciferase activity. (**C**) RNA pull-down assay for the amount of circRPMS1 and miR-203, miR-31 and miR-451 in C666-1 cells. (**D**) Scatter plots illustrating qRT-PCR analysis of expression fold change for miR-200, miR-31, and miR-451 in EBV-positive NPC tissues compared with adjacent tissues. (**E–G**) CircRPMS1 expression was negatively correlated with miR-203, miR-31, and miR-451 expression in NPC patients. *p<0.05 vs control.
Abbreviations: EBV, Epstein-Barr virus; NPC, nasopharyngeal carcinoma.

circRPMS1 si2#. The results showed that miR-203, miR-31, and miR-451 inhibitors could significantly reverse circRPMS1 knockdown-mediated inhibition of proliferation, induction of apoptosis, and suppression of invasive abilities (Figure 4A–C). In addition, downregulation of circRPMS1 led to upregulation of E-cadherin, and reduction of N-cadherin and vimentin in C666-1 cells. However, miR-203, miR-31, and miR-451 inhibitors treatment reversed the upregulation of E-cadherin, as well as the downregulated Vimentin and N-cadherin (Figure 4D and E). These results demonstrated that downregulation of circRPMS1 inhibited NPC cells' EMT and aggressiveness through sponging miR-200, miR-31, and miR-451.

Discussion

In this study, we found that EBV-encoded circRPMS1 was increased in metastatic NPC and was associated with short survival outcome. Knockdown of circRPMS1-inhibited cell proliferation induced apoptosis and repressed cell invasion in EBV-positive NPC cells. Further mechanism investigation revealed that circRPMS1-mediated NPC oncogenesis through sponging multiple miRNA and promoting EMT.

EBV is an oncogenic herpes virus that is closely associated with the initiation and development of NPC and other malignant tumors, such as B cell lymphomas to NK or T cell lymphoma.[16] EBV expresses a number of viral proteins and miRNA which are able to manipulate cell cycle and cell death processes to promote survival of the tumor cells.[17] For example, EBV-miR-BART8-3p induces EMT and promotes metastasis through directly targeting RNF38 via activation of NF-κB and Erk1/2 signaling pathways in NPC cells.[18] EBV-miR-Bart1-5P directly targets AMP-activated protein kinase α1 and regulates the AMPK/mTOR/HIF1 pathway and promotes NPC cell anomalous aerobic glycolysis and angiogenesis.[19] These EBV-encoded miRNAs also become valuable biomarkers for early and recurrent detection of NPC.[20,21] Recently, Toptan et al, identify EBV and Kaposi's sarcoma herpesvirus encodes circRNAs, including four circBART isoforms.[11] EBV circBARTs are expressed in all forms of EBV tumor latency, indicating that they play important roles in tumor cell reproductive fitness for EBV-positive cancers.[11] In addition, the circRNAs are highly resistant to exonuclease degradation and may be retained even in formalin-fixed, paraffin-embedded specimens.[11] Thus, virus-encoded circRNA also raises the possibility of new diagnostic tests to detect these viruses. Our recent results revealed that high levels of circRPMS1 were associated with metastasis and poor survival outcome in NPC patients, suggesting that circRPMS1 may be a useful biomarker for NPC diagnosis and prognosis.

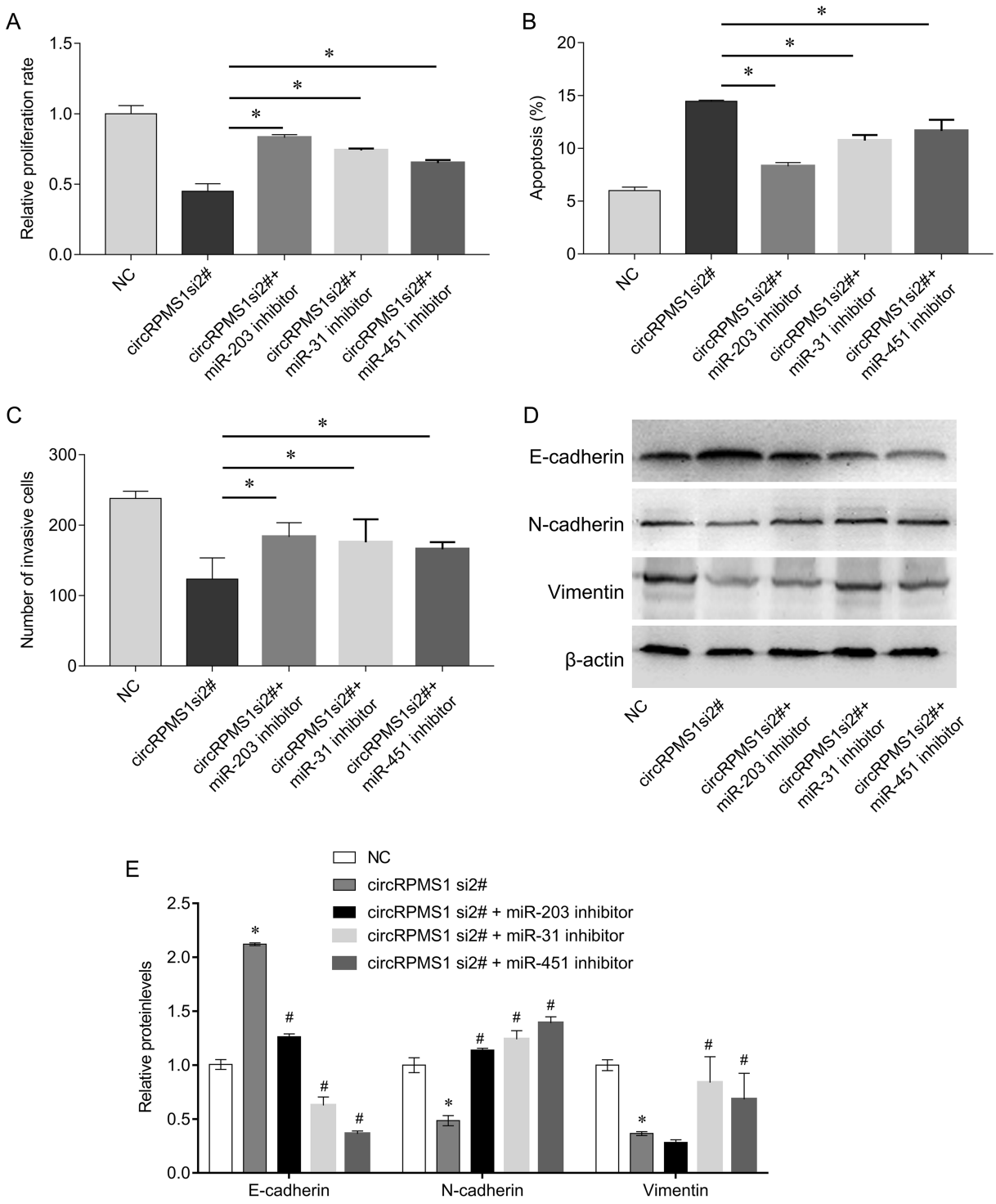

Figure 4 MiR-200, miR-31, and miR-451 inhibitors attenuate circRPMS1 knockdown-mediated inhibitory effects in C666-1 cells.

Notes: (**A**) Decreased cell proliferation in circRPMS1 knockdown cells was rescued by inhibitors of miR-203, miR-31, and miR-451 in C666-1 cells. (**B**) circRPMS1 knockdown-induced apoptosis was reduced by inhibitors of miR-203, miR-31, and miR-451 in C666-1 cells. (**C**) circRPMS1 knockdown-inhibited invasive ability was increased by inhibitors of miR-203, miR-31, and miR-451 in C666-1 cells. (**D**) circRPMS1 knockdown inhibited the expression of vimentin, N-cadherin, and induced E-cadherin expression; but these effects were abolished by inhibitors of miR-203, miR-31, and miR-451. (**E**) Quantification of the Western blot bands figure part **D**. *p<0.05 vs NC group. #p<0.05 vs circRPMS1 si2# group.

Abbreviation: NC, normal control.

We further investigated the biological functions of circRPMS1 in NPC cells. Knockdown of circRPMS1-inhibited cell proliferation, induced apoptosis and repressed cell invasion in EBV-positive NPC cells. Previous studies have shown that EBV-encoded proteins and miRNAs can mediate NPC uncontrollable growth and

metastasis.[22,23] Huang et al, also showed that overexpression of circRPMS1 promoted migration of AGS cells.[12] These results indicate that circRPMS1 is a viral oncogene in EBV-positive cancer. CircRPMS1 expresses in both cytoplasm and nucleus,[12] suggesting circRPMS1 could function as a miRNA sponge in the cytoplasm and regulated gene transcription via specific RNA–RNA interaction. Huang et al, predicted that ebv-miR-BART13-5p have three binding sites of circRPMS1.[12] In this study, we confirmed that circRPMS1 directly targeted three miRNAs, miR-203, miR-31, and miR-451 in NPC cells. The expression of circRPMS1 was negatively correlated with these miRNAs. The inhibitors of miR-203, miR-31, and miR-451 could reverse the effects of circRPMS1 knockdown on NPC cells. miR-203, miR-31, and miR-451 have been demonstrated as a tumor suppressor in several cancers, including NPC[24–26] and also acts as EMT inhibitors in esophageal squamous cell carcinoma, liver cancer, and lung cancer.[27–29] In line with these results, we here found that circRPMS1 knockdown-inhibited EMT, which was reversed by the inhibitors of miR-203, miR-31, and miR-451. However, how circRPMS1 regulates miRNAs expression remain unknown.

Our study revealed that circRPMS1 is frequently activated in EBV-positive NPC and its high level predicted a poor survival outcome. CircRPMS1 may sponge multiple miRNAs that function as a tumor suppressor to promote EMT, and subsequently enhance growth and invasion of NPC cells in vitro and in vivo. These findings indicate circRPMS1 as a potential therapeutic target for EBV-associated NPC. Our findings provide an important understanding for the further elucidation on the therapeutic use of circRNA in NPC.

Ethics approval and informed consent

This study was approved by Ethics Committee of Xiangya Hospital, Central South University.

Author contributions

All authors contributed toward data analysis, drafting and revising the paper, gave final approval of the version to be published and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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