

Original Article

Targeting ZFAND5 by EBV-encoded miR-BART19-3p drives malignant phenotypes in nasopharyngeal carcinoma

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Abstract: Nasopharyngeal carcinoma (NPC) is an Epstein-Barr virus (EBV)-associated epithelial malignancy with distinct geographic distribution and a high propensity for local invasion and distant metastasis. EBV-encoded microRNAs have emerged as important viral regulators of host gene expression in NPC; however, the biological function of EBV-miR-BART19-3p remains incompletely understood. In this study, public transcriptomic and microRNA datasets were analyzed to identify dysregulated molecules in NPC, and ZFAND5 was predicted as a potential target of EBV-miR-BART19-3p. EBV-miR-BART19-3p was significantly upregulated in NPC tissues and EBV-positive C666-1 cells, whereas ZFAND5 was downregulated at both the mRNA and protein levels. Kaplan-Meier analysis further showed that higher ZFAND5 expression was associated with better disease-free survival in a public NPC cohort. Dual-luciferase reporter assays confirmed that EBV-miR-BART19-3p directly binds to the 3'-untranslated region of ZFAND5. Functionally, EBV-miR-BART19-3p overexpression enhanced C666-1 cell proliferation, colony formation, migration and invasion while suppressing apoptosis. Conversely, EBV-miR-BART19-3p inhibition or ZFAND5 overexpression suppressed these malignant phenotypes and promoted apoptosis. Importantly, ZFAND5 knockdown partially reversed the tumor-suppressive effects induced by EBV-miR-BART19-3p inhibition. Bioinformatic enrichment analysis indicated that differentially expressed genes in NPC were associated with immune regulation, chemokine signaling, cell adhesion, PI3K-Akt, Wnt and NF-kappaB signaling. These findings suggest that EBV-miR-BART19-3p promotes malignant phenotypes in EBV-positive NPC cells, at least in part, by repressing ZFAND5, providing a potential mechanistic link between EBV-encoded microRNAs and NPC progression.

Keywords: Epstein-Barr virus, miR-BART19-3p, nasopharyngeal carcinoma, ZFAND5, proliferation, apoptosis

Introduction

Nasopharyngeal carcinoma (NPC) is a malignant epithelial tumor arising from the nasopharyngeal mucosa. It shows a distinctive ethnic and geographic distribution, with a particularly high incidence in southern China and Southeast Asia [1, 2]. Most NPCs in endemic regions are non-keratinizing carcinomas and are closely associated with Epstein-Barr virus (EBV) infection [1, 3]. Owing to its anatomically concealed location and non-specific early symptoms, NPC is often diagnosed at an advanced stage, with a high frequency of cervical lymph-node involvement and distant metastasis [1, 4, 5].

Although advances in radiotherapy, chemotherapy, immunotherapy and molecular risk stratification have improved clinical management, recurrent and metastatic NPC remains a major therapeutic challenge [4, 5]. Therefore, identifying EBV-related molecular mechanisms that drive NPC progression may provide new insights into tumor biology and candidate therapeutic vulnerabilities.

EBV is a human gamma-herpesvirus that is etiologically linked to several epithelial and lymphoid malignancies, including NPC [3, 6-8]. In EBV-associated NPC, the virus usually maintains a restricted latent program, allowing viral

products to persist in tumor cells while modulating host gene expression and immune surveillance [6-9]. EBV was the first human virus shown to encode microRNAs, and EBV-encoded microRNAs are mainly derived from the BamHI fragment H rightward reading frame 1 (BHRF1) and BamHI-A rightward transcript (BART) regions [10-12]. Among them, BART microRNAs are abundantly expressed in NPC and regulate both viral and host transcripts through sequence-specific interactions with target mRNAs [12-14].

Accumulating evidence indicates that EBV BART microRNAs contribute to NPC progression by regulating apoptosis, DNA-damage responses, epithelial-mesenchymal transition, invasion, immune evasion and treatment response [13-19]. Our previous bioinformatic study identified a prognostic microRNA signature in NPC and suggested that EBV-miR-BART19-3p may be aberrantly expressed and biologically relevant in this disease [20]. Other studies have also detected EBV-miR-BART19-3p in NPC-related clinical specimens or EBV-associated disease contexts [15-17]. Nevertheless, the functional role of EBV-miR-BART19-3p in NPC and its key host targets remain insufficiently characterized.

ZFAND5, also known as ZNF216, encodes a cytoplasmic zinc-finger protein containing A20-type and AN1-type zinc-finger domains. ZFAND5 has been implicated in proteasome activation, protein degradation, mRNA stability and stress-related cellular responses [21-23]. In cancer, limited evidence suggests that ZFAND5 may act as a tumor-suppressive factor and may be connected to NF-kappaB-related signaling [24-27]. However, whether ZFAND5 is dysregulated in NPC and whether it is directly regulated by EBV-miR-BART19-3p are unknown. The present study therefore investigated the expression pattern, clinical relevance and biological function of the EBV-miR-BART19-3p/ZFAND5 axis in NPC.

Materials and methods

Data sources and processing

Publicly available expression datasets were downloaded from the Gene Expression Omnibus (GEO) database. GSE53819 was used for mRNA expression analysis and included 18 primary NPC tumor tissues and 18 non-cancerous

nasopharyngeal tissues. GSE32960 was used for microRNA expression analysis and included 312 formalin-fixed paraffin-embedded NPC specimens and 18 normal nasopharyngeal tissue samples [28]. GSE102349 was used to evaluate the association between ZFAND5 expression and disease-free survival (DFS) in patients with NPC.

For GSE53819 and GSE32960, processed and normalized expression matrices were used for differential expression analysis. Differentially expressed mRNAs in GSE53819 and differentially expressed microRNAs in GSE32960 were identified using the limma package in R [29]. The Benjamini-Hochberg method was applied to adjust *P* values for multiple testing. Molecules with $|\log_2 \text{fold change (logFC)}| > 0.5$ and adjusted *P* value < 0.05 were considered significantly differentially expressed. Volcano plots were generated to visualize the overall distribution of differentially expressed mRNAs or microRNAs, and box plots were used to display the expression levels of ZFAND5 and EBV-miR-BART19-3p. Differences between NPC and control tissues were assessed using the Wilcoxon rank-sum test unless otherwise specified.

To explore the biological functions and pathways associated with NPC-related transcriptional alterations, Gene Ontology biological process (GO-BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using the differentially expressed genes identified from GSE53819. Terms with adjusted *P* value < 0.05 were considered significantly enriched and were visualized using R.

For survival analysis, patients in GSE102349 were divided into high- and low-ZFAND5 expression groups according to the median ZFAND5 expression level. Kaplan-Meier curves were generated to compare DFS between the two groups, and statistical significance was evaluated using the log-rank test. The hazard ratio (HR) and 95% confidence interval (CI) were estimated using Cox proportional hazards regression analysis.

Cell culture

The human EBV-positive NPC cell line C666-1 was purchased from Shanghai Gaining Biological Technology Co., Ltd. (Shanghai, China).

Table 1. Primer sequences

Target	Primer sequence
EBV-miR-BART19-3p	Forward: 5'-CGCGTTTTGTTTGCTTGGG-3' Reverse: 5'-AGTGCAGGGTCCGAGGTATT-3'
ZFAND5	Forward: 5'-GCTAGTGGTCCAAACAGTCCT-3' Reverse: 5'-TCGGGGTAGTTATTTTGTCTCT-3'
U6	Forward: 5'-CTCGCTTCGGCAGCAT-3' Reverse: 5'-TTTGCCTGTCATCCTTGC-3'
β -actin	Forward: 5'-CATGTACGTTGCTATCCAGGC-3' Reverse: 5'-CTCCTTAATGTACGCACGAT-3'

The immortalized nasopharyngeal epithelial cell line NP69 was purchased from Zhejiang Meisen Cell Technology Co., Ltd. (Zhejiang, China). C666-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, USA), 1% penicillin and 1% streptomycin. NP69 cells were maintained in keratinocyte serum-free medium (HyClone, USA). All cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Target prediction

The human ZFAND5 reference sequence (NM_001102421.3) was obtained from the National Center for Biotechnology Information database. RNAhybrid was used to predict the potential interaction between EBV-miR-BART19-3p and the 3'-untranslated region (3'-UTR) of ZFAND5. The minimum free energy of the predicted microRNA-mRNA duplex was used to assess binding potential.

Cell grouping and transfection

C666-1 cells in the logarithmic growth phase were seeded into six-well plates. After 24 h, cells were transfected using Lipofectamine 2000 reagent (Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The experimental groups included EBV-miR-BART19-3p mimic and mimic negative control (mimic-NC), EBV-miR-BART19-3p inhibitor and inhibitor negative control (inhibitor-NC), pcDNA-ZFAND5 and pcDNA negative control (pcDNA-NC), and EBV-miR-BART19-3p inhibitor plus si-ZFAND5 or siRNA negative control (si-NC). EBV-miR-BART19-3p mimic, inhibitor, corresponding negative controls, the ZFAND5 overexpression plasmid and ZFAND5-targeting siRNA were designed and

synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Cells were harvested 48 h after transfection for RNA, protein and functional analyses.

Quantitative real-time PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, USA). Complementary DNA was synthesized using a PrimeScript RT reagent kit (Takara Bio, Japan). Quantitative real-time PCR (qRT-PCR) was performed on an ABI 7500 real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, USA) using SYBR Premix Ex Taq (Takara Bio, Japan). The qRT-PCR reaction system and cycling conditions were set according to the manufacturer's instructions. U6 was used as the internal control for EBV-miR-BART19-3p, and β -actin was used as the internal control for ZFAND5. Relative expression was calculated using the $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in **Table 1**.

Western blotting

Total protein was extracted from transfected C666-1 cells using RIPA lysis buffer (Beyotime Biotechnology, China), and protein concentrations were determined using a BCA protein assay kit (Beyotime Biotechnology, China). Equal amounts of protein were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. After blocking with 5% skimmed milk for 1 h at room temperature, membranes were incubated overnight at 4°C with primary antibodies against ZFAND5 (SAB2102763, MilliporeSigma, USA; 1:1000), Bcl-2 (#4223T, Cell Signaling Technology, USA; 1:1000), Bax (#2772T, Cell Signaling Technology, USA; 1:1000), caspase-3 (#9664S, Cell Signaling Technology, USA; 1:1000) and β -actin (#4967S, Cell Signaling Technology, USA; 1:1000). After washing with TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (401BW, Promega, USA; 1:5000) for 1.5 h at room temperature. Protein bands were detected using an enhanced chemiluminescence reagent (Millipore, USA), imaged and quantified using ImageJ software.

Immunohistochemistry staining

Paraffin-embedded nasopharyngeal carcinoma (NPC) tissues and paired adjacent noncancerous tissues (n = 10 pairs) were obtained from patients at The Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University. This study was approved by the Institutional Review Board of The Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University (approval number: KY-2025-102-02) and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants. Sections of 4 μm thickness were prepared for immunohistochemistry (IHC) staining using an anti-ZFAND5 antibody (SAB2102763, MilliporeSigma, USA; 1:200). Staining was visualized using 3,3'-diaminobenzidine, and nuclei were counterstained with hematoxylin. Images were captured under identical conditions. ZFAND5 expression was quantified as average optical density (AOD) using ImageJ software. For each sample, the mean AOD value from three randomly selected fields at $\times 400$ magnification was calculated. Tumor tissues and paired adjacent noncancerous tissues were compared using a paired t-test.

Cell proliferation assay

Cell proliferation was assessed using the MTT assay. Transfected C666-1 cells were seeded into 96-well plates at a density of 5×10^3 cells per well and incubated for 24, 48, 72 and 96 h at 37°C with 5% CO_2 . At each time point, 10 μL of MTT solution (5 mg/mL; Sigma-Aldrich, USA) was added to each well and incubated for 4 h protected from light. After removal of the supernatant, formazan crystals were dissolved in 100 μL of dimethyl sulfoxide. Absorbance was measured at 595 nm using a microplate reader.

Colony formation assay

For the colony formation assay, transfected C666-1 cells were seeded into six-well plates at 100 cells per well and cultured at 37°C for 14 days. Colonies were fixed, stained with 0.5% crystal violet for 10 min and counted under a microscope. Colonies containing more than 50 cells were included in the analysis.

Transwell invasion assay

Cell invasion was assessed using Transwell chambers coated with Matrigel matrix (Sigma-Aldrich, USA). Matrigel was diluted 1:8, and 60 μL of the diluted matrix was added to the upper chamber and allowed to solidify. After 24 h of transfection, C666-1 cells were digested, resuspended in serum-free medium and seeded into the upper chamber at 3×10^3 cells per well. RPMI-1640 medium containing 20% fetal bovine serum was added to the lower chamber as a chemoattractant. After 24 h of incubation, non-invading cells on the upper surface were removed with cotton swabs. Invading cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet and counted under a light microscope.

Wound-healing assay

For the wound-healing assay, transfected C666-1 cells were seeded into six-well plates at 5×10^5 cells per well. When confluence reached approximately 90%, a linear wound was generated using a sterile 200-microliter pipette tip. Detached cells were removed by washing with PBS, and cells were cultured in serum-free medium. Wound closure was photographed at 0 and 48 h under an inverted microscope, and the wound-healing rate was calculated.

Apoptosis assay

Cell apoptosis was analyzed using an Annexin V-FITC/propidium iodide (PI) apoptosis detection kit according to the manufacturer's protocol. Briefly, transfected C666-1 cells were collected, washed and stained with Annexin V-FITC and PI for 15 min in the dark. Apoptotic cells were detected using a FACSCalibur flow cytometer (BD Biosciences, USA), and data were analyzed using FlowJo software.

Dual-luciferase reporter assay

Luciferase reporter plasmids containing the wild-type ZFAND5 3'-UTR sequence (ZFAND5-WT) or a mutant ZFAND5 3'-UTR sequence (ZFAND5-MUT) were synthesized by GenePharma (Shanghai, China). The wild-type or mutant ZFAND5 3'-UTR was cloned downstream of the luciferase reporter sequence. Reporter plasmids were co-transfected with

EBV-miR-BART19-3p mimic or mimic-NC into HEK293T or C666-1 cells. Luciferase activity was measured using a dual-luciferase reporter assay system (Promega, USA) according to the manufacturer's instructions. Relative luciferase activity was calculated as firefly luciferase activity normalized to Renilla luciferase activity.

Statistical analysis

All experiments were performed at least three times independently unless otherwise stated. Data are presented as mean $\pm$ standard deviation. Statistical analyses were performed using GraphPad Prism 8.0. Two-group comparisons were analyzed using Student's *t*-test, and paired tumor and adjacent tissue samples were analyzed using a paired *t*-test. Comparisons among multiple groups were performed using one-way ANOVA followed by Tukey's post hoc test. Time-course proliferation data were analyzed using repeated-measures ANOVA. Survival curves were compared using the log-rank test. Two-sided *P* < 0.05 was considered statistically significant.

Results

Bioinformatic and tissue validation of EBV-miR-BART19-3p upregulation and ZFAND5 downregulation in NPC

To identify dysregulated microRNAs involved in NPC, we first analyzed the public GEO microRNA expression dataset GSE32960, which included 312 formalin-fixed paraffin-embedded NPC specimens and 18 normal nasopharyngeal tissue samples. Differential expression analysis was performed using limma with the criteria of $|\log FC| > 0.5$ and adjusted *P* value < 0.05. A total of 225 differentially expressed microRNAs were identified, including 114 upregulated and 111 downregulated microRNAs in NPC tissues compared with control tissues (**Figure 1A**). Notably, EBV-miR-BART19-3p was significantly upregulated in NPC tissues compared with normal controls, as shown by both volcano plot and box plot analyses (**Figure 1A, 1B**).

We next analyzed the mRNA expression dataset GSE53819, which included 18 primary NPC tumor tissues and 18 non-cancerous nasopharyngeal tissues. Using the same threshold of $|\log FC| > 0.5$ and adjusted *P* value < 0.05,

we identified 3,958 differentially expressed genes, including 1,774 upregulated and 2,184 downregulated genes in NPC tissues (**Figure 1C**). Among these genes, ZFAND5 was significantly downregulated in NPC, as demonstrated by volcano plot and box plot analyses (**Figure 1C, 1D**). These findings suggested an inverse expression pattern between EBV-miR-BART19-3p and ZFAND5 in NPC.

GO-BP and KEGG enrichment analyses were then performed using the differentially expressed genes identified from GSE53819. GO-BP analysis showed enrichment in immune- and migration-related biological processes, including leukocyte proliferation, leukocyte migration, B cell activation, chemotaxis, mononuclear cell differentiation, taxis, lymphocyte proliferation, cilium movement and microtubule bundle formation (**Figure 1E**). KEGG pathway analysis showed significant enrichment in cytokine-cytokine receptor interaction, chemokine signaling pathway, ECM-receptor interaction, integrin signaling, cell adhesion molecule interaction, PI3K-Akt signaling pathway, Wnt signaling pathway and NF-kappaB signaling pathway (**Figure 1E**). These pathways are closely related to tumor immune regulation, cell adhesion, migration, invasion and inflammatory signaling.

To further assess the clinical relevance of ZFAND5, Kaplan-Meier survival analysis was performed using the NPC dataset GSE102349. Patients were divided into high- and low-ZFAND5 expression groups according to the median ZFAND5 expression level. Patients with higher ZFAND5 expression showed significantly better DFS than those with lower ZFAND5 expression (*P* = 0.0381; HR = 3.116; 95% CI = 1.00-9.67) (**Figure 1F**). This result supports the potential tumor-suppressive relevance of ZFAND5 in NPC.

To validate the expression pattern of ZFAND5 at the protein level, IHC staining was performed in 10 pairs of NPC tissues and adjacent noncancerous tissues. ZFAND5 protein expression was markedly lower in NPC tissues than in adjacent noncancerous tissues (*P* < 0.0001) (**Figure 1G, 1H**). ZFAND5 staining was predominantly cytoplasmic, with stronger staining intensity in noncancerous tissues and weak or absent staining in tumor tissues. These results were consistent with the transcriptomic analysis.

EBV-miR-BART19-3p/ZFAND5 axis in NPC

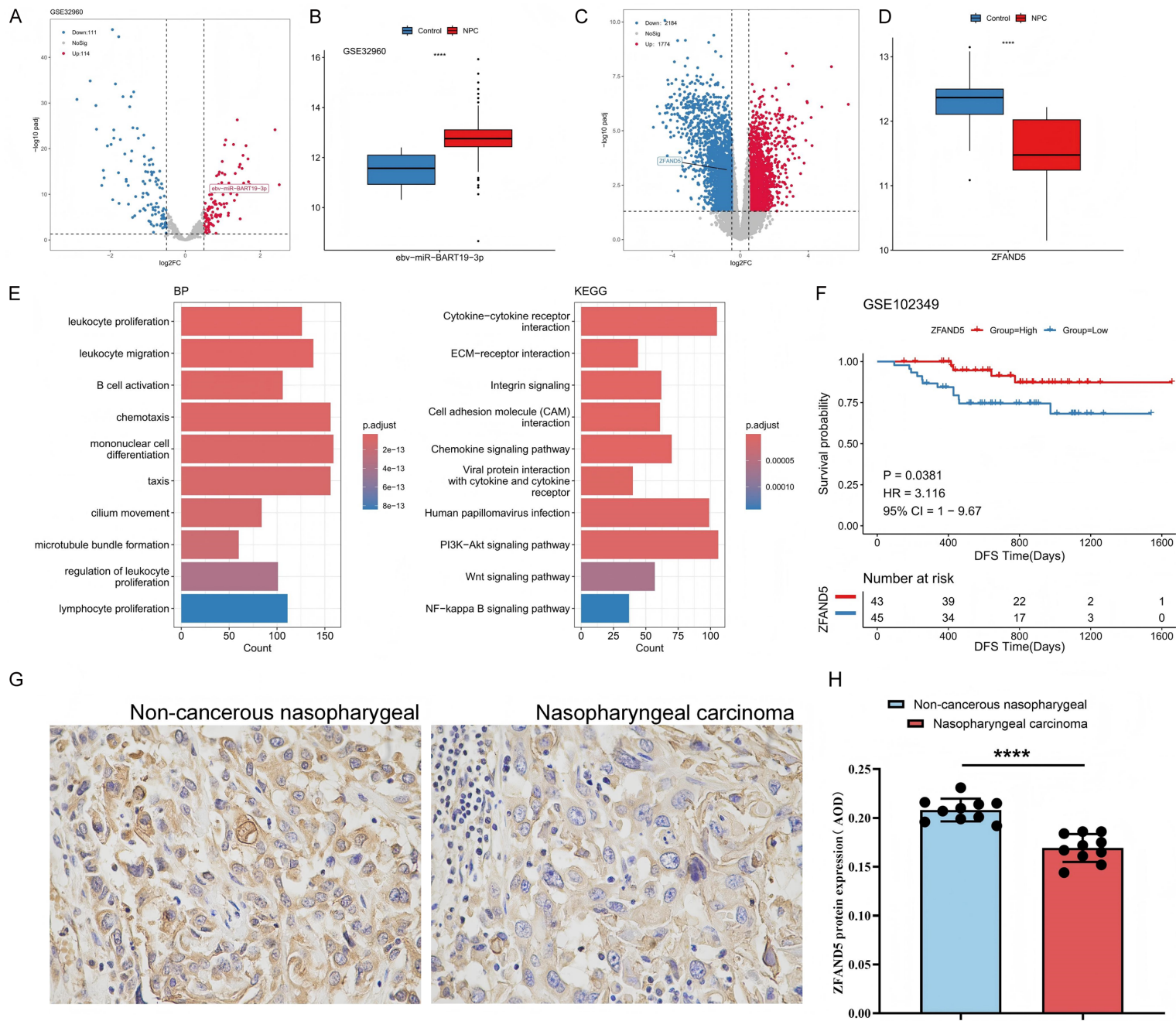


Figure 1. Bioinformatic and tissue validation of EBV-miR-BART19-3p upregulation and ZFAND5 downregulation in nasopharyngeal carcinoma. A. Volcano plot showing differentially expressed microRNAs in GSE32960, including 312 formalin-fixed paraffin-embedded NPC specimens and 18 normal nasopharyngeal tissue samples. Differentially expressed microRNAs were screened using $|\log_{2}FC| > 0.5$ and adjusted P value < 0.05 ; 114 microRNAs were upregulated and 111 microRNAs were downregulated in NPC tissues, and EBV-miR-BART19-3p is highlighted. B. Box plot showing that EBV-miR-BART19-3p expression was significantly increased in NPC tissues compared with control tissues in GSE32960. C. Volcano plot showing differentially expressed mRNAs in GSE53819, including 18 primary NPC tumor tissues and 18 non-cancerous nasopharyngeal tissues. A total of 1,774 genes were upregulated and 2,184 genes were downregulated in NPC tissues, and ZFAND5 is highlighted. D. Box plot showing that ZFAND5 expression was significantly decreased in NPC tissues compared with control tissues in GSE53819. E. GO-BP and KEGG pathway enrichment analyses of differentially expressed genes identified from GSE53819. F. Kaplan-Meier curve showing the association between ZFAND5 expression and disease-free survival (DFS) in GSE102349. G. Representative immunohistochemistry staining of ZFAND5 in adjacent noncancerous tissues and NPC tissues. Images were captured at 400 $\times$ magnification. H. Quantification of ZFAND5 immunostaining intensity by average optical density analysis in 10 paired NPC and adjacent noncancerous tissues. **** $P < 0.0001$.

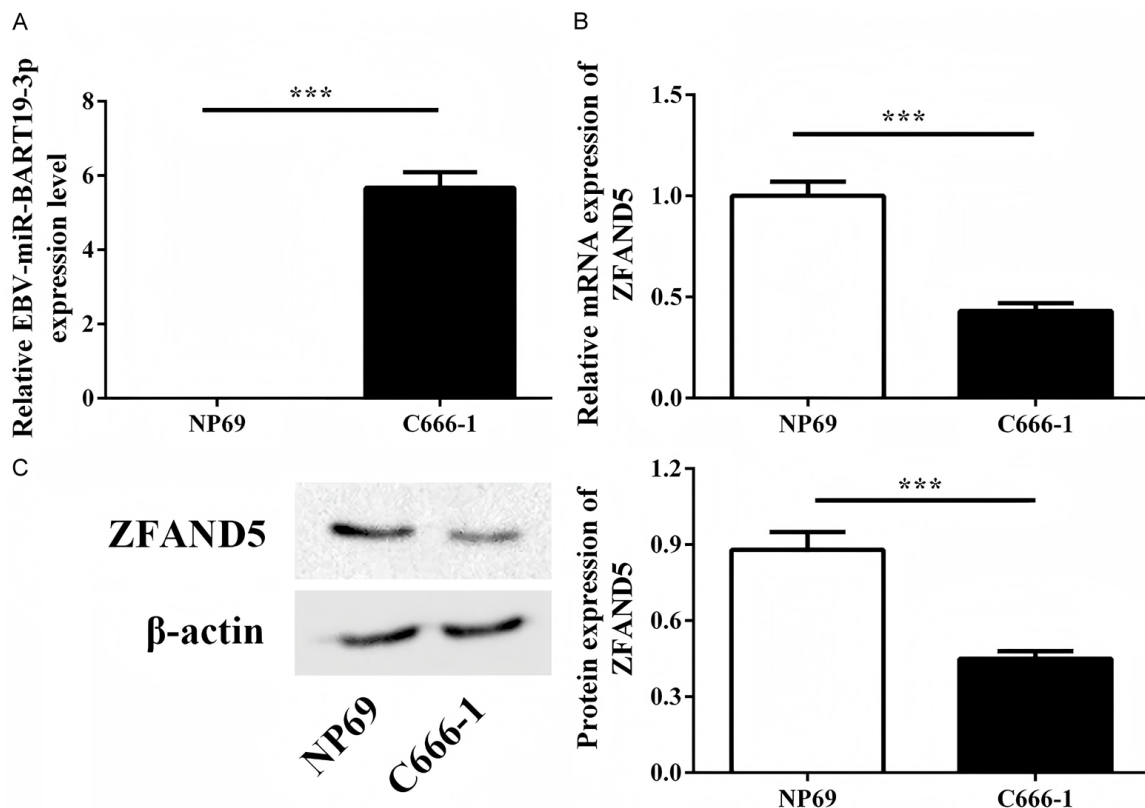


Figure 2. ZFAND5 expression and EBV-miR-BART19-3p expression in cell lines. A. EBV-miR-BART19-3p expression in NP69 and C666-1 cells analyzed using the $2^{-\Delta\Delta Ct}$ method. B. ZFAND5 mRNA expression analyzed using the $2^{-\Delta\Delta Ct}$ method. C. ZFAND5 protein expression analyzed by western blotting. Data are presented as mean $\pm$ SD from three independent experiments; Student's t-test was used. **** $P < 0.001$.

EBV-miR-BART19-3p is highly expressed whereas ZFAND5 is downregulated in EBV-positive C666-1 cells

EBV-miR-BART19-3p was undetectable or expressed at a very low level in NP69 cells, whereas it was clearly expressed in EBV-positive C666-1 cells (**Figure 2A**). In contrast, ZFAND5 mRNA and protein levels were signifi-

cantly lower in C666-1 cells than in NP69 cells (**Figure 2B, 2C**). These data further supported the inverse expression relationship between EBV-miR-BART19-3p and ZFAND5 in NPC cells.

EBV-miR-BART19-3p directly targets ZFAND5

RNAhybrid prediction identified a potential binding site for EBV-miR-BART19-3p in the

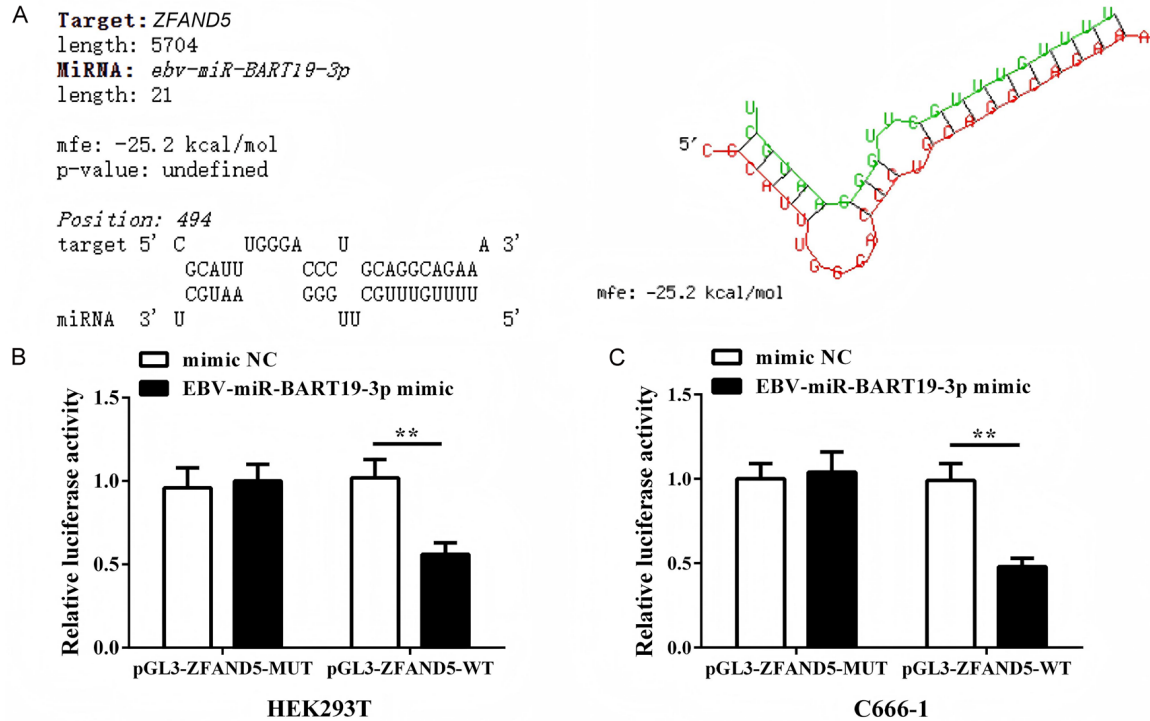


Figure 3. ZFAND5 is a direct target of EBV-miR-BART19-3p. A. Predicted binding sequence of EBV-miR-BART19-3p in the ZFAND5 mRNA 3'-UTR. B. Relative luciferase activity of the wild-type or mutant ZFAND5 3'-UTR reporter in HEK293T cells after transfection with EBV-miR-BART19-3p mimic or mimic negative control. C. Relative luciferase activity in C666-1 cells. Data are presented as mean $\pm$ SD from three independent experiments; Student's t-test was used. ** $P < 0.01$.

3'-UTR of ZFAND5 mRNA (**Figure 3A**). To validate this interaction, wild-type and mutant ZFAND5 3'-UTR reporter plasmids were co-transfected with EBV-miR-BART19-3p mimic or mimic-NC into HEK293T and C666-1 cells. EBV-miR-BART19-3p significantly reduced luciferase activity in cells transfected with the wild-type reporter but had no significant effect on the mutant reporter (**Figure 3B, 3C**). These results confirmed that ZFAND5 is a direct target of EBV-miR-BART19-3p.

EBV-miR-BART19-3p overexpression promotes malignant phenotypes in C666-1 cells

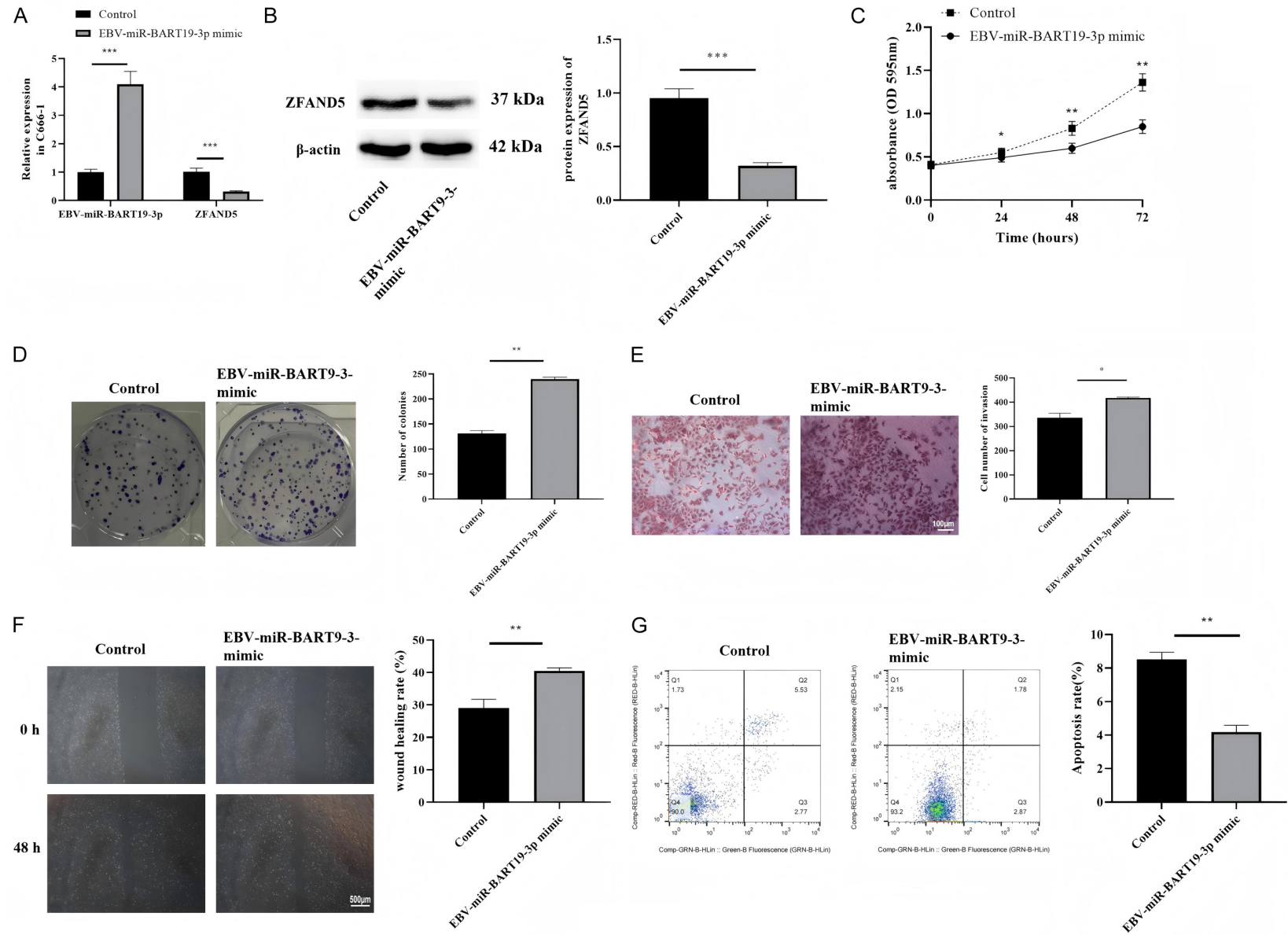
C666-1 cells were transfected with EBV-miR-BART19-3p mimic to assess its functional role. qRT-PCR confirmed increased EBV-miR-BART19-3p expression and reduced ZFAND5 mRNA expression after mimic transfection (**Figure 4A**). Western blotting further showed that ZFAND5 protein expression was decreased (**Figure 4B**). Functionally, EBV-miR-BART19-3p overexpression significantly enhanced cell proliferation and colony formation (**Figure 4C**,

4D). Transwell and wound-healing assays showed increased invasion and migration (**Figure 4E, 4F**). Flow cytometry showed that EBV-miR-BART19-3p overexpression reduced the apoptotic rate (**Figure 4G**). Consistently, Bcl-2 expression was increased, whereas Bax and caspase-3 expression were decreased (**Figure 4H**). These findings indicated that EBV-miR-BART19-3p promotes malignant phenotypes in C666-1 cells.

EBV-miR-BART19-3p inhibition suppresses malignant phenotypes and increases ZFAND5 expression

To determine the effect of EBV-miR-BART19-3p knockdown, C666-1 cells were transfected with EBV-miR-BART19-3p inhibitor. qRT-PCR and western blotting showed that EBV-miR-BART19-3p inhibition significantly decreased EBV-miR-BART19-3p expression and increased ZFAND5 mRNA and protein levels (**Figure 5A, 5B**). Functionally, EBV-miR-BART19-3p inhibition suppressed cell proliferation, colony formation, invasion and migration and increased

EBV-miR-BART19-3p/ZFAND5 axis in NPC



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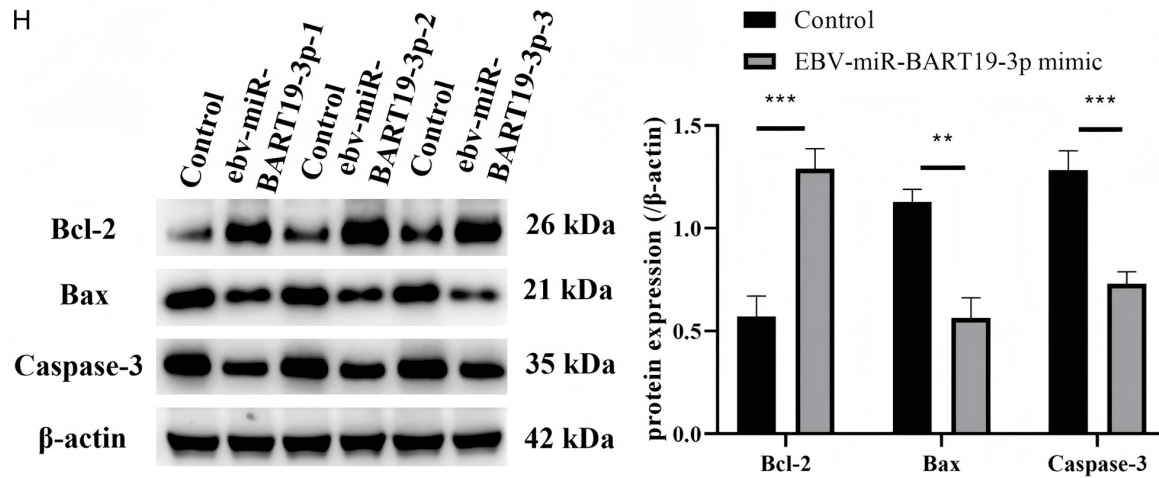
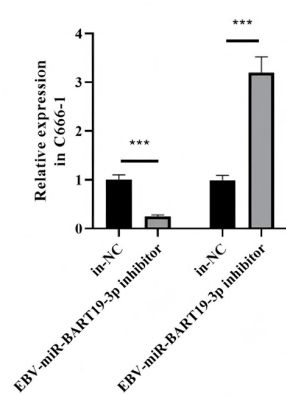
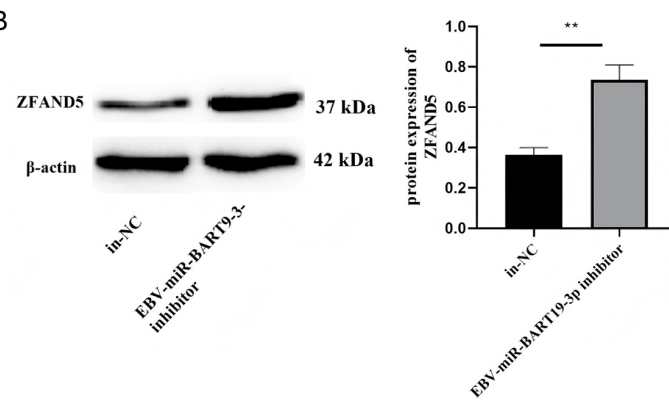


Figure 4. Effect of EBV-miR-BART19-3p up-regulation on C666-1 cell biological behavior. A. EBV-miR-BART19-3p and ZFAND5 mRNA expression after EBV-miR-BART19-3p mimic transfection. B. ZFAND5 protein expression. C. Cell proliferation measured by MTT assay; repeated-measures ANOVA was used for time-course comparisons. D. Colony formation assay. E. Transwell invasion assay (Scale bars: 100 μ m, 200 $\times$). F. Wound-healing assay (Scale bars: 500 μ m, 4 $\times$). G. Flow cytometric analysis of apoptosis. H. Expression of apoptosis-related proteins. Data are presented as mean $\pm$ SD from three independent experiments. Student's t-test was used for two-group comparisons unless otherwise stated. *P < 0.05, **P < 0.01, ***P < 0.001.

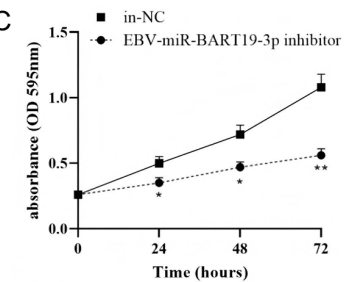
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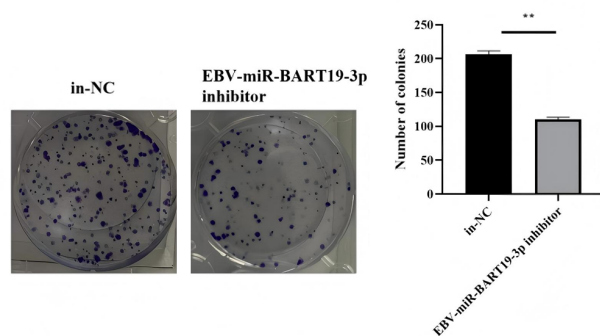
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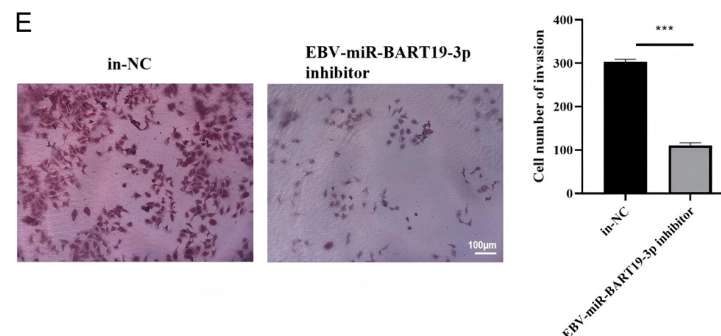
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EBV-miR-BART19-3p/ZFAND5 axis in NPC

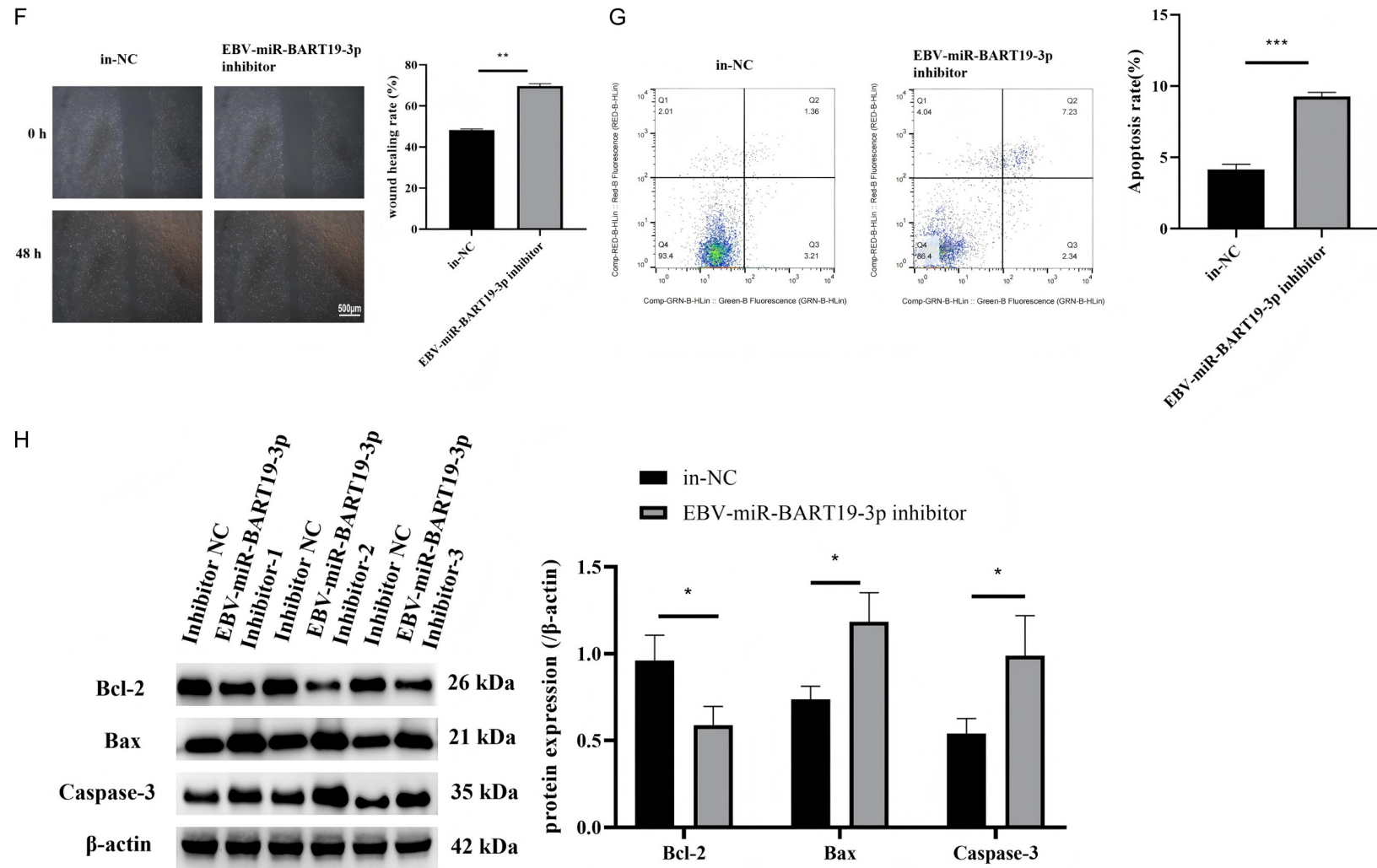


Figure 5. Effect of EBV-miR-BART19-3p knockdown on C666-1 cell biological behavior. A. EBV-miR-BART19-3p and ZFAND5 mRNA expression. B. ZFAND5 protein expression after EBV-miR-BART19-3p inhibition. C. Cell proliferation measured by MTT assay; repeated-measures ANOVA was used for time-course comparisons. D. Colony formation assay. E. Transwell invasion assay (Scale bars: 100 μ m, 200 $\times$). F. Wound-healing assay (Scale bars: 500 μ m, 4 $\times$). G. Flow cytometric analysis of apoptosis. H. Expression of apoptosis-related proteins. Data are presented as mean $\pm$ SD from three independent experiments. Student's t-test was used for two-group comparisons unless otherwise stated. *P < 0.05, **P < 0.01, ***P < 0.001.

apoptosis (**Figure 5C-G**). In addition, Bcl-2 expression was reduced, whereas Bax and caspase-3 expression were increased (**Figure 5H**). These results suggested that EBV-miR-BART19-3p inhibition exerts tumor-suppressive effects in C666-1 cells.

ZFAND5 overexpression inhibits proliferation, invasion and migration and promotes apoptosis

The biological function of ZFAND5 was assessed by transfecting C666-1 cells with pcDNA-ZFAND5. ZFAND5 protein expression was successfully increased after transfection (**Figure 6A**). ZFAND5 overexpression significantly inhibited cell proliferation and colony formation (**Figure 6B, 6C**). It also reduced cell invasion and migration, as shown by Transwell and wound-healing assays (**Figure 6D, 6E**), and promoted apoptosis (**Figure 6F**). Western blotting showed decreased Bcl-2 expression and increased Bax and caspase-3 expression after ZFAND5 overexpression (**Figure 6G**). These findings indicate that ZFAND5 functions as a tumor-suppressive factor in C666-1 cells.

ZFAND5 knockdown reverses the phenotype induced by EBV-miR-BART19-3p inhibition

To determine whether ZFAND5 mediates the biological effects of EBV-miR-BART19-3p, C666-1 cells were co-transfected with EBV-miR-BART19-3p inhibitor and si-ZFAND5. ZFAND5 knockdown significantly reduced ZFAND5 protein expression in cells treated with EBV-miR-BART19-3p inhibitor (**Figure 7A**). Compared with the inhibitor plus si-NC group, ZFAND5 knockdown restored cell proliferation, colony formation, invasion and migration (**Figure 7B-E**). It also reduced apoptosis and partially reversed the expression changes of Bcl-2, Bax and caspase-3 induced by EBV-miR-BART19-3p inhibition (**Figure 7F, 7G**). These results demonstrated that ZFAND5 is an important functional mediator of EBV-miR-BART19-3p in NPC cells.

Discussion

NPC is an EBV-associated malignancy with high metastatic potential and substantial geographic heterogeneity [1-5]. EBV-encoded microRNAs provide a mechanism through which the virus can regulate host gene expres-

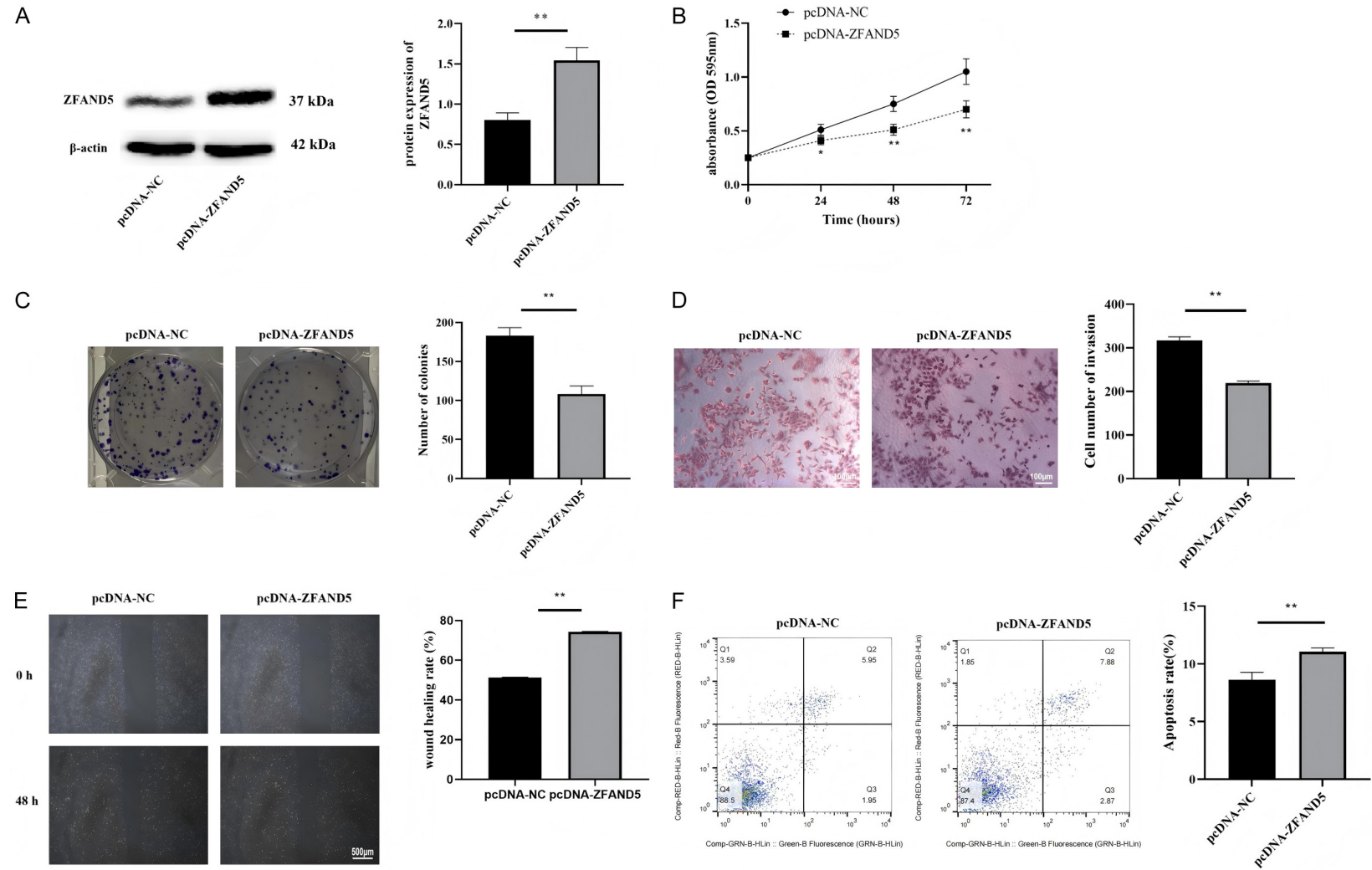
sion while maintaining a restricted latent program [6, 10-12]. In this study, integrative bioinformatic analysis showed that EBV-miR-BART19-3p was upregulated in NPC tissues, whereas ZFAND5 was downregulated. Public survival analysis further indicated that higher ZFAND5 expression was associated with better DFS. These findings, together with IHC validation and in vitro functional assays, support a model in which EBV-miR-BART19-3p contributes to NPC progression by repressing the tumor-suppressive gene ZFAND5.

Several EBV BART microRNAs have been implicated in NPC progression by targeting host genes involved in apoptosis, DNA repair, invasion, metastasis and immune regulation [13-19]. EBV-miR-BART19-3p has been detected in NPC-related clinical samples and other EBV-associated disease contexts, but its functional role has remained unclear [16-18]. Here, EBV-miR-BART19-3p overexpression promoted proliferation, colony formation, invasion and migration and inhibited apoptosis in C666-1 cells. In contrast, EBV-miR-BART19-3p inhibition increased ZFAND5 expression and suppressed malignant phenotypes. These results expand the known biological repertoire of EBV-encoded BART microRNAs and suggest that EBV-miR-BART19-3p may participate in NPC progression through host gene repression.

ZFAND5 is a zinc-finger protein involved in proteasome activation, protein degradation and mRNA stability [21-23]. Although ZFAND5 has been studied mainly in non-NPC contexts, available evidence suggests that it may function as a tumor-suppressive molecule and may intersect with NF-kappaB signaling [24-27]. In the present study, ZFAND5 was consistently downregulated in NPC tissues and C666-1 cells. ZFAND5 overexpression suppressed proliferation, invasion and migration and increased apoptosis, whereas ZFAND5 knockdown reversed the tumor-suppressive effects induced by EBV-miR-BART19-3p inhibition. These findings indicate that ZFAND5 is not only a direct target of EBV-miR-BART19-3p but also a functional mediator of its oncogenic effects in EBV-positive NPC cells.

This study has several limitations. First, all functional experiments were performed in vitro using C666-1 cells. Although the rescue experiments support a causal link among EBV-miR-

EBV-miR-BART19-3p/ZFAND5 axis in NPC



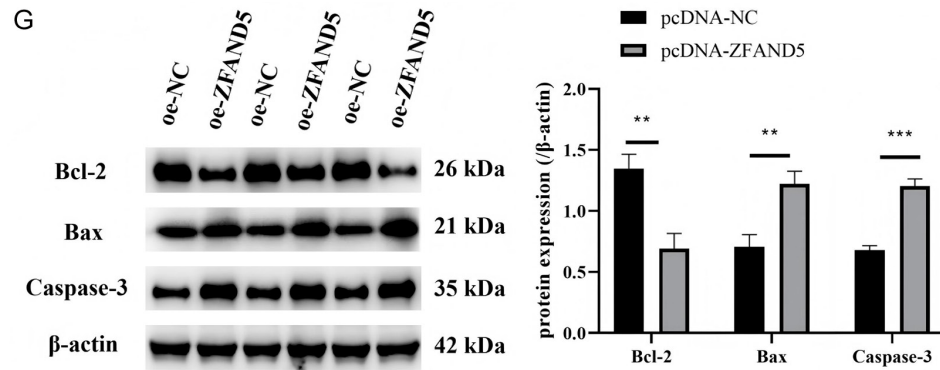
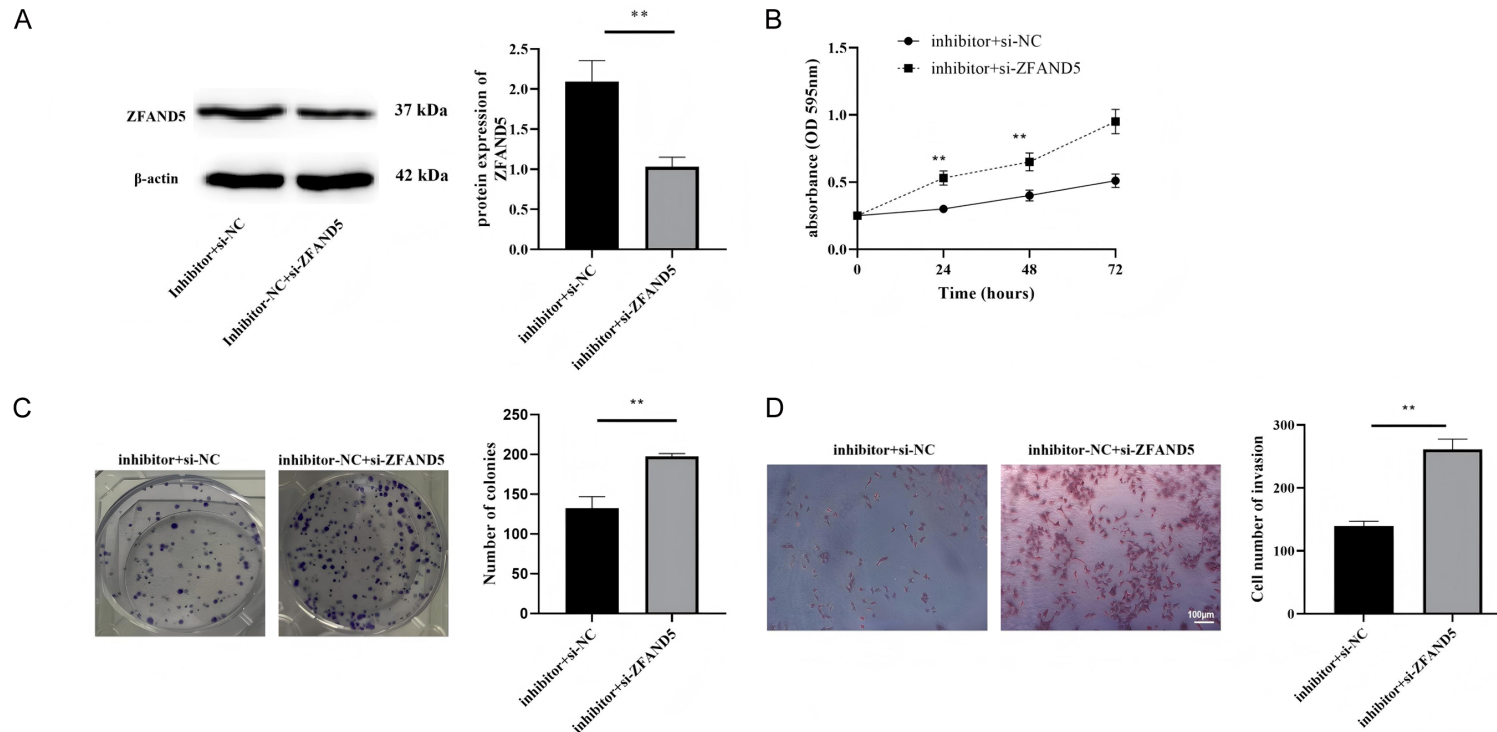


Figure 6. Effect of ZFAND5 overexpression on C666-1 cell biological behavior. A. ZFAND5 protein expression. B. Cell proliferation measured by MTT assay; repeated-measures ANOVA was used for time-course comparisons. C. Colony formation assay. D. Transwell invasion assay (Scale bars: 100 μ m, 200 $\times$). E. Wound-healing assay (Scale bars: 500 μ m, 4 $\times$). F. Flow cytometric analysis of apoptosis. G. Expression of apoptosis-related proteins. Data are presented as mean $\pm$ SD from three independent experiments. Student's t-test was used for two-group comparisons unless otherwise stated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



EBV-miR-BART19-3p/ZFAND5 axis in NPC

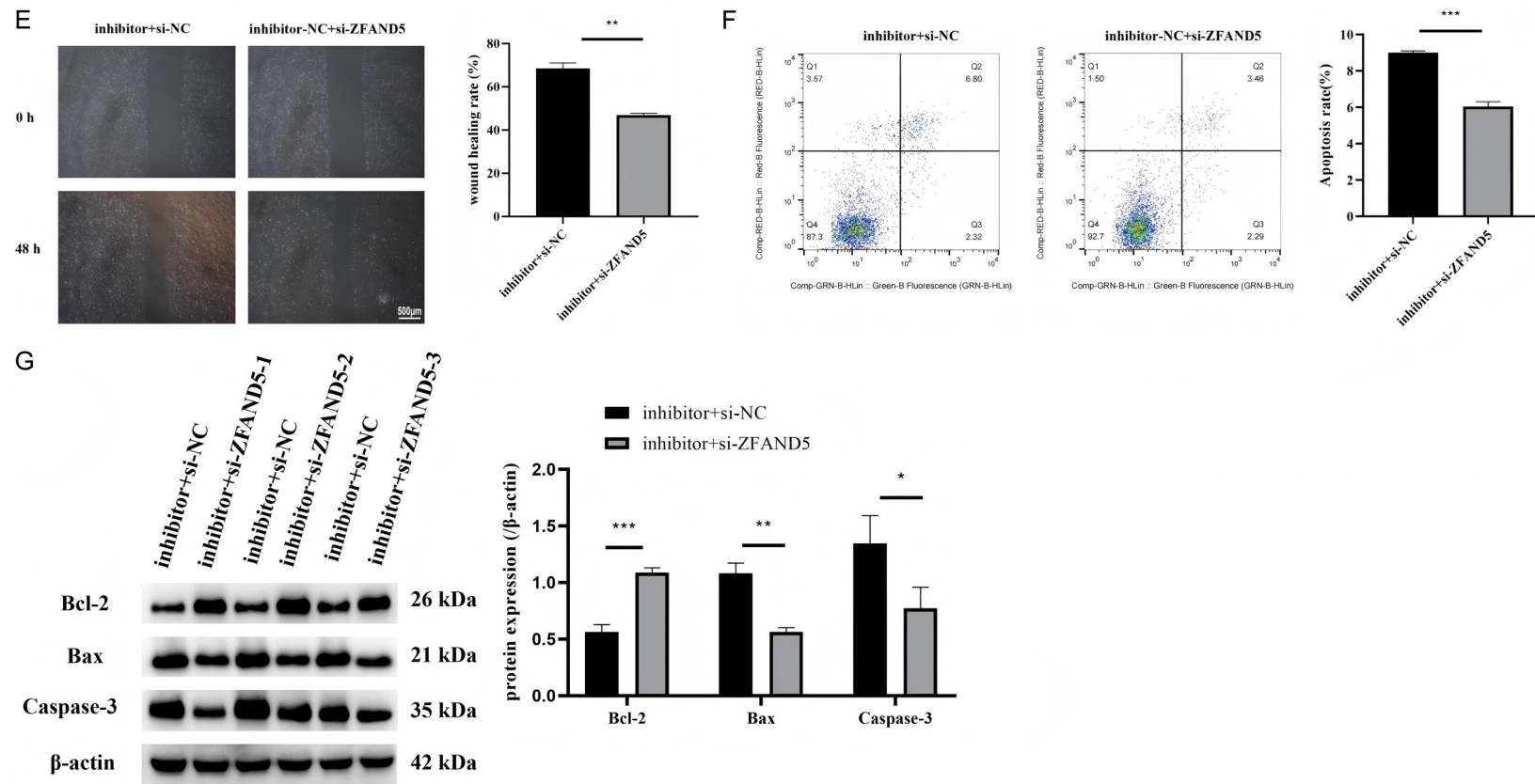


Figure 7. ZFAND5 knockdown reverses the phenotype induced by EBV-miR-BART19-3p inhibition in C666-1 cells. A. ZFAND5 protein expression after co-transfection. B. Cell proliferation measured by MTT assay; repeated-measures ANOVA was used for time-course comparisons. C. Colony formation assay. D. Transwell invasion assay (Scale bars: 100 μ m, 200 $\times$). E. Wound-healing assay (Scale bars: 500 μ m, 4 $\times$). F. Flow cytometric analysis of apoptosis. G. Expression of apoptosis-related proteins. Data are presented as mean $\pm$ SD from three independent experiments. Student's t-test was used for two-group comparisons unless otherwise stated. *P < 0.05, **P < 0.01, ***P < 0.001.

BART19-3p, ZFAND5 and malignant phenotypes, in vivo xenograft experiments are still required to confirm the physiological relevance and therapeutic potential of this axis. Second, although public datasets and IHC staining supported the clinical relevance of ZFAND5, the clinical tissue cohort was limited to 10 paired NPC and adjacent noncancerous tissues, and detailed follow-up and staging information were unavailable for these samples. Larger clinical cohorts with complete clinicopathological and survival data are needed to validate the prognostic value of ZFAND5. Third, GO-BP and KEGG enrichment analyses suggested that immune regulation, cell adhesion, chemokine signaling, PI3K-Akt, Wnt and NF-kappaB pathways may be involved in NPC progression; however, these analyses are exploratory and require experimental validation. Finally, the downstream signaling mechanism of ZFAND5 was inferred from enrichment analysis and previous literature and should be tested in NPC models using pathway-specific assays and rescue experiments.

Conclusion

In summary, this study demonstrates that EBV-miR-BART19-3p is upregulated in NPC and promotes malignant phenotypes in EBV-positive NPC cells by directly targeting and repressing ZFAND5. ZFAND5 is downregulated in NPC tissues, and higher ZFAND5 expression is associated with better DFS in a public NPC dataset. Functional experiments suggest that ZFAND5 acts as a tumor-suppressive factor in C666-1 cells, and ZFAND5 knockdown reverses the inhibitory effects caused by EBV-miR-BART19-3p inhibition. These findings identify the EBV-miR-BART19-3p/ZFAND5 axis as a candidate molecular mechanism in EBV-associated NPC, although further validation in animal models and larger clinical cohorts is warranted.

Disclosure of conflict of interest

None.

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