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Circular RNA circ_0008450 upregulates CXCL9 expression by targeting miR-577 to regulate cell proliferation and invasion in nasopharyngeal carcinoma

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ABSTRACT

As a kind of malignant tumor, nasopharyngeal carcinoma (NPC) has attracted increasing attention from researchers. As a member of the circular RNA (circRNA) family, circ_0008450 has been investigated in hepatocellular carcinoma but not in NPC. This study aims to reveal the special biologic role and mechanism of circ_0008450 in NPC. qRT-PCR analysis was conducted to test the level of circ_0008450 in different tissues and cells. Loss/Gain of function assay was utilized to detect the influence of silenced/overexpressed circ_0008450 on the proliferation, apoptosis, migration, and invasion of NPC cells. The mechanism of circ_0008450 was assessed by performing qRT-PCR and luciferase reporter experiments. The results showed that circ_0008450 was elevated in NPC tissues and cells. Silenced circ_0008450 could inhibit cell proliferation, and metastatic properties and increased the number of apoptotic cells. Ectopically expressed circ_0008450 strengthened the abovementioned malignant biological behaviors. Mechanistically, circ_0008450 reduced miR-577-mediated repression of CXCL9, resulting in facilitating the oncogenic functions of NPC. In conclusion, circ_0008450 acts as an oncogene in NPC cells through regulating miR-577/CXCL9 signaling. Our findings might provide a new therapeutic target for treating NPC.

1. Introduction

Nasopharyngeal carcinoma (NPC) arises from the nasal and pharyngeal epithelial cells and prevalent worldwide, especially in Asia and North Africa (Kamran et al., 2015). In 2015, 60,000 new cases were diagnosed in China (Chen et al., 2016). Causes of NPC include viral, environmental, hereditary factors and so on (He et al., 2017). Treatments on NPC depend on the cancer's stage and tumor location. Small localized tumor could be resected with surgery. But in other cases, radiotherapy along with chemotherapy is preferred (Chen and Hu, 2015; Tung et al., 1992).

Circular RNAs (circRNAs), non-coding RNA with closed ring structure, are found to play regulatory and structural roles in embryogenesis, allelic expression, stem cell pluripotency, protein coding gene regulation, cell apoptosis, cycle control, differentiation, growth and senescence (Jeck and Sharpless, 2014; Aufiero et al., 2019; Das et al., 2019; Xu et al., 2018). Increasing evidence indicated that circRNAs are aberrantly expressed in many tumor types and can be biomarkers for cancer diagnosis and prognosis (Bach et al., 2019). For instance, circ_0001649 is reported to be a favorable biomarker for non-small cell lung cancer prognosis and facilitates the biological behaviors of tumor cell by binding to miR-331-3p and miR-338-5p (Liu et al., 2018). For NPC, silencing of circRNA_000543 sensitizes tumor cells to irradiation via targeting platelet-derived growth factor receptor B by sponging miR-9 (Chen et al., 2019).

Circ_0008450, a novel cancer-related circRNA identified previously. Zhang et al. revealed that elevation of circ_0008450 correlates with clinical severity and promotes hepatocellular carcinoma tumorigenesis. They further identified a miRNA, miR-548p, in mediating the oncogenic functions of circ_0008450 (Zhang et al., 2019). The length of circ_0008450 is 497 bp and it is derived from CMTM3 genomic region. Given the important roles of circRNAs in cancer, we here set out to

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discover circ_0008450 levels in NPC specimens and cell lines and further reveal its biological roles in NPC cells. Furthermore, a competing endogenous RNA (ceRNA) mechanism of circ_0008450 in NPC was identified. This work may help to understand the pathogenesis of the molecular alterations of NPC and develop useful indicators that aid novel effective therapies for NPC.

2. Materials and methods

2.1. Patients' tissues

NPC patients from January 2015 to December 2017 underwent tumor resection, were elected in our study (Supplementary Table S1). Seventy freshly NPC specimens with paired adjacent non-tumor specimens were harvested for the subsequent assay. Specimens were stored in -80 °C before total RNA isolation. The research was authorized by the Ethic Committee of Qiqihar Medical University and carried out under the ethical standards of the 1964 Declaration of Helsinki.

2.2. Cell lines and culture

NPC cells including HK-1, 5-8F, C666-1, and SUNE-1 and NP69 cells were acquired from Chinese Academy of Sciences (Shanghai, China). The cells were maintained in RPMI-1640 with 10% FBS (Thermo, USA) and cultured at 37 °C, 5% CO_2 and saturated humidity.

2.3. qRT-PCR and cell transfection

To measure the levels of circ_0008450 in NPC samples and cell lines, qRT-PCR was carried out. Total RNA was isolated by TRIzol followed by the instructions of the manufacturer. Reverse transcription was conducted by a PrimeScript RT Reagent Kit and cDNA amplification with SYBR Premix Ex Taq (TaKaRa, Dalian, China) following the direction of the manufacturer. GAPDH or U6 was used as an endogenous control. Gene expression was calculated by the delta-delta CT method.

Circ_0008450/CXCL9 siRNAs, control-siRNA and miR-577 mimics/inhibitor were chemically synthesized by Genechem (Shanghai, China), and used for transfection into NPC cell lines. To silence the expression of circ_0008450, two siRNAs specifically targeting to the spliced junction site of circ_0008450 was obtained. The target sequences of si-circ_0008450 are shown below: si-circ_0008450-1: 5′- AGACAGAAGG GTCTCTCATTC-3′. si-circ_0008450-2: 5′- ACAAGACAGAAGGGTCTC TCA-3′. For circ_0008450 overexpression, a pcDNA3.1 (+) circRNA mini vector was utilized to clone the whole circRNA sequence and artificial inverted repeats. The whole sequence of circ_0008450 cloned into the vectors is displayed in Supplementary Table S2. CXCL9 was elevated by using a pcDNA 3.1 vector. Lipofectamine 3000 was used for cell transfection (Invitrogen, USA).

2.4. CCK-8 assay

CCK-8 assays were performed by a CCK-8 kit from Beyotime (Beijing, China). In brief, cells in log phase growth were planted in 96-well dishes at a concentration of 1.5×10^3 cells/well and cultivated in RPMI-1640 containing 10% FBS for cell attachment. Cell viability was detected with CCK-8 reagent followed by the protocol of manufacturer at the indicated time point (0, 24, 48, 72 and 96 h).

2.5. Cell apoptosis determination

Cell apoptotic rate was detected by a flow cytometer. Cells were supplied with AnnexinV-FITC and propidiumiodide (PI, Beyotime, Haimen, China) at 22–25 °C for 10 min. Afterwards, using a flow cytometer (BD Biosciences, SanJose, CA, USA) to analyze the apoptotic rate.

2.6. Wound healing assay

5×10^5 cells were planted to each well and incubated overnight. The tip of the pipette was utilized for vertical scratching and the cells were incubated in serum-free medium for 36 h. After taking a photo, the image was processed using Image J software (Rawak Software, Inc., Germany) and percentage of relative wound closure was calculated.

2.7. Transwell experiments

Cell invasion experiment was performed using Matrigel-coated unit (BD Biosciences). Cells were cultured in transwell compartment at a density of 1×10^5 cells/insert in serum-free medium. The lower compartment was supplied with 10% FBS medium. 24 h later, the cells that traversed the filter were fixed, stained, and counted. For cell migration assay, the chambers were not pre-coated with Matrigel and the cells were adjusted to 5×10^4 cells/insert. The other steps were the same as the cell invasion experiment.

2.8. Dual-luciferase reporter gene test

Circular RNA Interactome and starbase V2.0 online databases were utilized to predict the miRNAs which may be target to circ_0008450. NPC cells were co-transfected with wild-type or mutated circ_0008450 reporter plasmids, and with miR-577 mimics or mimics-NC. Circ_0008450 sequence was amplified and subcloned to the pMIR-REPORT™ Vector (ThermoFisher, US), which contained the luciferase coding sequence (5′- …CUCUCAUUCAUCACUUUUAUCUG… -3′). The mutant circ_0008450 was generated by using the Phusion Site-Directed Mutagenesis Kit (Thermo Scientific™) with the wild-type circ_0008450 as the template (5′-… CUCUCAUUCAUCACUUAAUAGUG… -3′). MiR-577 mimics and miR-NC were transfected with these two vectors. After 24 h transfection, a luciferase reporter assay kit (Promega, USA) was applied.

2.9. Western blotting

The lysates from SUNE-1 and HK-1 cells were collected by RIPA (Beyotime Biotechnology, Shanghai, China) and then boiled for 5 min at 100 °C. After that, the proteins were transferred to PVDF membrane and blocked by non-fat milk. The membranes were then incubated with anti-CXCL9 and anti-GAPDH (Abcam, USA). The next day, the bands were probed with HRP-conjugated secondary antibody, followed by visualization with ECL Plus chemiluminescence reagent (Beyotime Biotechnology).

2.10. Data analysis

Data were presented as mean ± standard deviation from three independent experiments. Student's *t*-Test was performed for two groups, and statistical significance was evaluated using one-way ANOVA for more than two groups. The $P < 0.05$ meant the existence of statistical significance.

3. Results

3.1. Circ_0008450 is overexpressed in NPC samples and cell lines

Firstly, we analyzed 70 pairs of NPC and adjacent noncancerous tissues to find out whether circ_0008450 was differentially expressed. As Fig. 1A displayed, the level of circ_0008450 showed a fold change of 3.09, which was markedly increased in NPC tissues than the normal tissues. Further identification in the cell lines of NPC verified elevation of circ_0008450 expression compared with the normal cell line, NP69 (Fig. 1B). These data indicated upregulation of circ_0008450 in NPC samples/cells and circ_0008450 may contribute to cell progression in

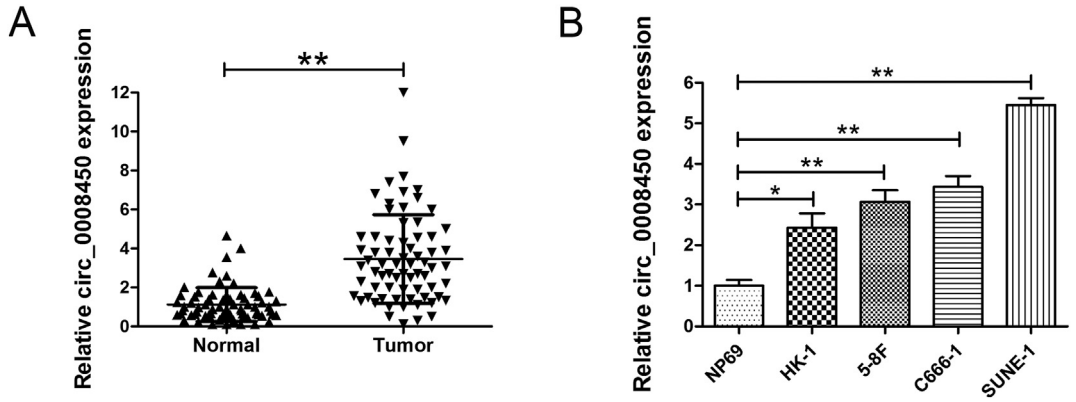

Fig. 1. Relative expression of circ_0008450 in NPC tissues and cell lines. (A) Relative expression of circ_0008450 in NPC tissue samples and their paired non-cancerous tissue samples measured by qRT-PCR. (B) Relative expression of circ_0008450 in NPC cell lines and normal cell line measured by qRT-PCR. *$p < 0.05$, **$p < 0.01$.

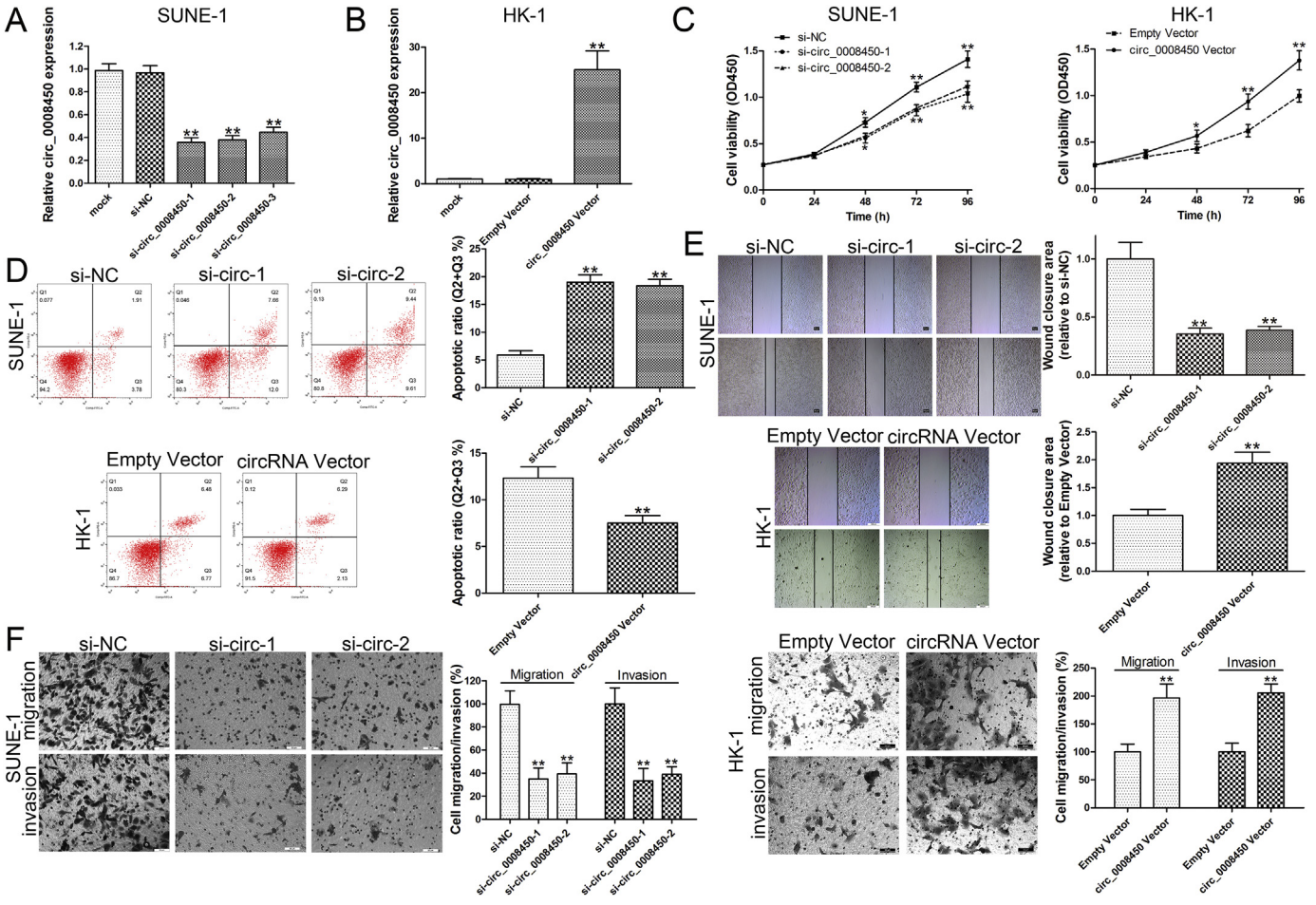

Fig. 2. Circ_0008450 promotes NPC cell progression. (A) Circ_0008450 expression was detected after transfection in SUNE-1 cells by qRT-PCR. (B) Circ_0008450 expression was detected after transfection in HK-1 cells by qRT-PCR. (C) CCK-8 assays were used to detect cell viability of SUNE-1 and HK-1 cells after transfection. (D) Flow cytometric analysis was used to detect cell apoptosis of SUNE-1 and HK-1 cells after transfection. (E) Wound healing assays were used to detect cell migration capacities of SUNE-1 and HK-1 cells after transfection. (F) Transwell assays were used to detect cell migration and invasion capacities of SUNE-1 and HK-1 cells after transfection. *$p < 0.05$, **$p < 0.01$.

this fatal malignancy.

3.2. Circ_0008450 facilitates NPC cell progression

To explore the effects of circ_0008450 on NPC cells, siRNA/plasmid transfection technology was applied on SUNE-1 and HK-1 cells, respectively. Transfection results were confirmed by qRT-PCR. As a result, all of the three elected siRNAs had a good knockdown efficiency. Si-circ_0008450–1 and si-circ_0008450–2 were chosen for the following study for the reason that they have a better knockdown efficiency than si-circ_0008450–3 (Fig. 2A). In HK-1 cell line, overexpression of circ_0008450 was successfully achieved (Fig. 2B). At 48 h, 72 h and 96 h after transfection, the cell viability of SUNE-1 cells in the circ_0008450 depletion groups were significantly decreased. Whereas,

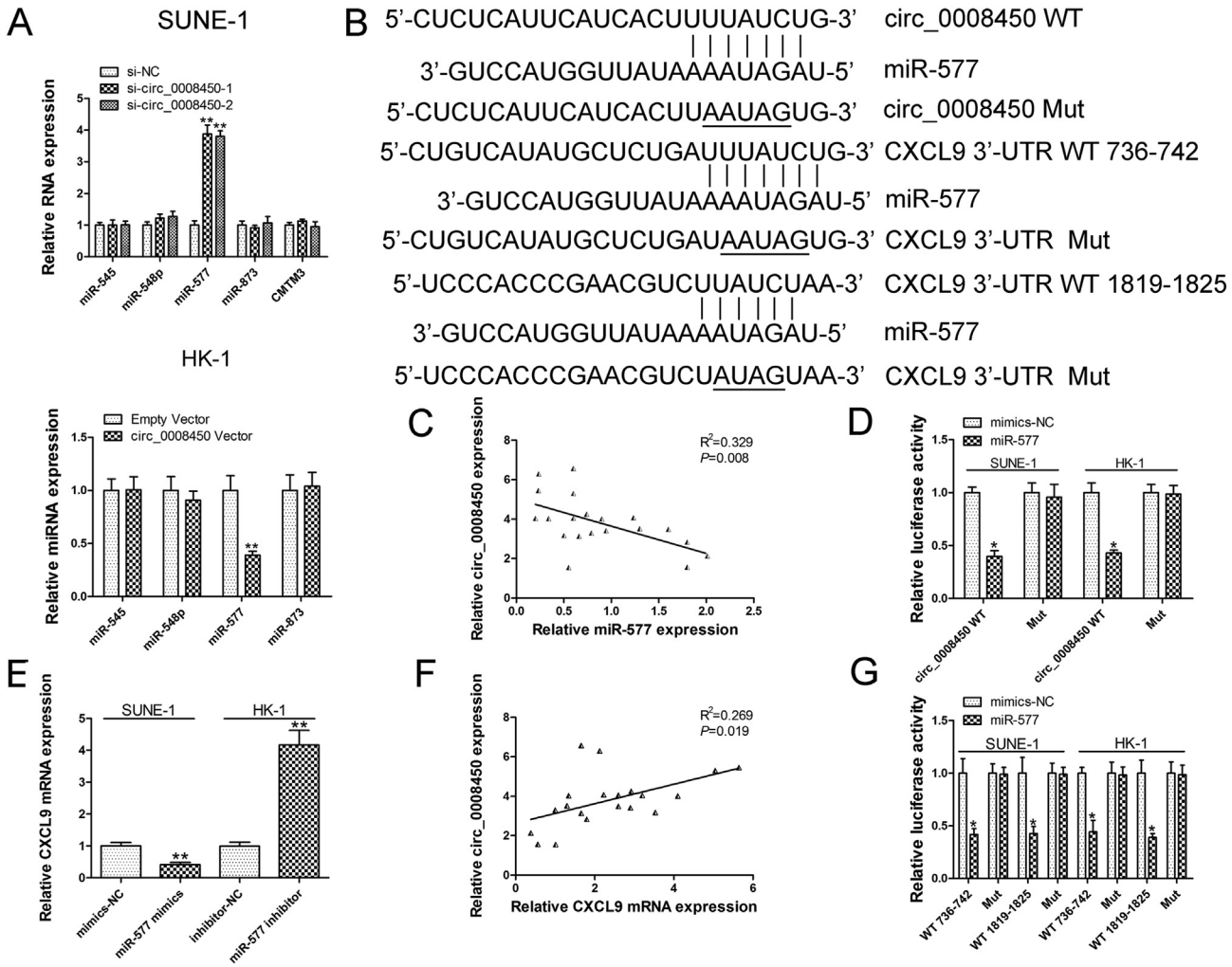

Fig. 3. Circ_0008450 directly binds to miR-577, and miR-577 targeted CXCL9. (A) Relative RNA expression was detected by qRT-PCR after transfection in SUNE-1 and HK-1 cells. (B) The seed sequences of circ_0008450, miR-577, CXCL9 3'-UTR, circ_0008450 mutant, and CXCL9 3'-UTR mutant. (C) Correlation analysis of miR-577 and circ_0008450 expression was explored in NPC tissue samples. (D) Dual-luciferase reporter assays were performed to detect the binding ability between miR-577 and circ_0008450 in SUNE-1 and HK-1 cells. (E) CXCL9 mRNA expression was detected by qRT-PCR after transfection in SUNE-1 and HK-1 cells. (F) Correlation analysis of CXCL9 mRNA and circ_0008450 expression was explored in NPC tissue samples. (G) Dual-luciferase reporter assays were performed to detect the correlation between miR-577 and CXCL9 3'-UTR in SUNE-1 and HK-1 cells. $*p < 0.05$, $**p < 0.01$.

ectopic expression of circ_0008450 enhanced cell proliferation in vitro (Fig. 2C). The results of flow cytometry also showed that the low expression of circ_0008450 had an obvious effect on promoting apoptotic rate of SUNE-1 cells, while the high expression of circ_0008450 significantly downregulated HK-1 cell apoptosis (Fig. 2D). Downregulation of circ_0008450 expression levels could reduce the scratch wound area healed and upregulation of circ_0008450 expression could increase the scratch wound area healed in SUNE-1 and HK-1 cells, respectively (Fig. 2E). Moreover, decreased circ_0008450 expression levels could inhibit the migration and invasion rate in SUNE-1 cell line. Conversely, ectopically expressed circ_0008450 could increase the metastatic properties of HK-1 cells (Fig. 2F).

3.3. Circ_0008450 sponges miR-577 to upregulate CXCL9 expression in NPC

To evaluate the potential mechanisms of circ_0008450 in NPC, we found some miRNAs which may be bound to circ_0008450. We chosen the intersection of Circular RNA Interactome and starbase V2.0 online databases and miR-545, miR-577, and miR-873 were selected. Previous study indicated miR-548p could be sponged by circ_0008450 in hepatocellular carcinoma (Zhang et al., 2019). Thus, miR-548p was also included in this study. In addition, qRT-PCR was used to detect whether circ_0008450 could affect the expression of CMTM3 in NPC. The level of miR-577 was strikingly affected by the expression of circ_0008450 analyzed by qRT-PCR. The other miRNAs and CMTM3 expression were almost unchanged under the manipulation of circ_0008450 (Fig. 3A). The seeding sequence of miR-577 within circ_0008450 is shown in Fig. 3B. Moreover, there is a negative relationship of circ_0008450 and miR-577 in NPC tissues (Fig. 3C). Further dual-luciferase reporter assay results were also performed in SUNE-1 and HK-1 cells, it was showed that the relative luciferase activity was significantly decreased after the cells have been co-transfected with the wild-type circ_0008450 expression vector and miR-577, while the relative luciferase activity was not significantly changed after the cells have been co-transfected with the mutant circ_0008450 expression vector and miR-577 (Fig. 3D). Next, we further identified CXCL9 as a potential downstream target of miR-577 predicted by TargetScan database (Fig. 3B). The relationship between circ_0008450 and CXCL9 was explored. The expression levels of CXCL9 mRNA in SUNE-1 and HK-1 cells at different circ_0008450 expression levels were detected, respectively. The results showed that the CXCL9 mRNA expression in the miR-577 mimics group were strikingly lower than those in the control group. However, the CXCL9 mRNA level in the miR-577 inhibitor group were markedly higher than

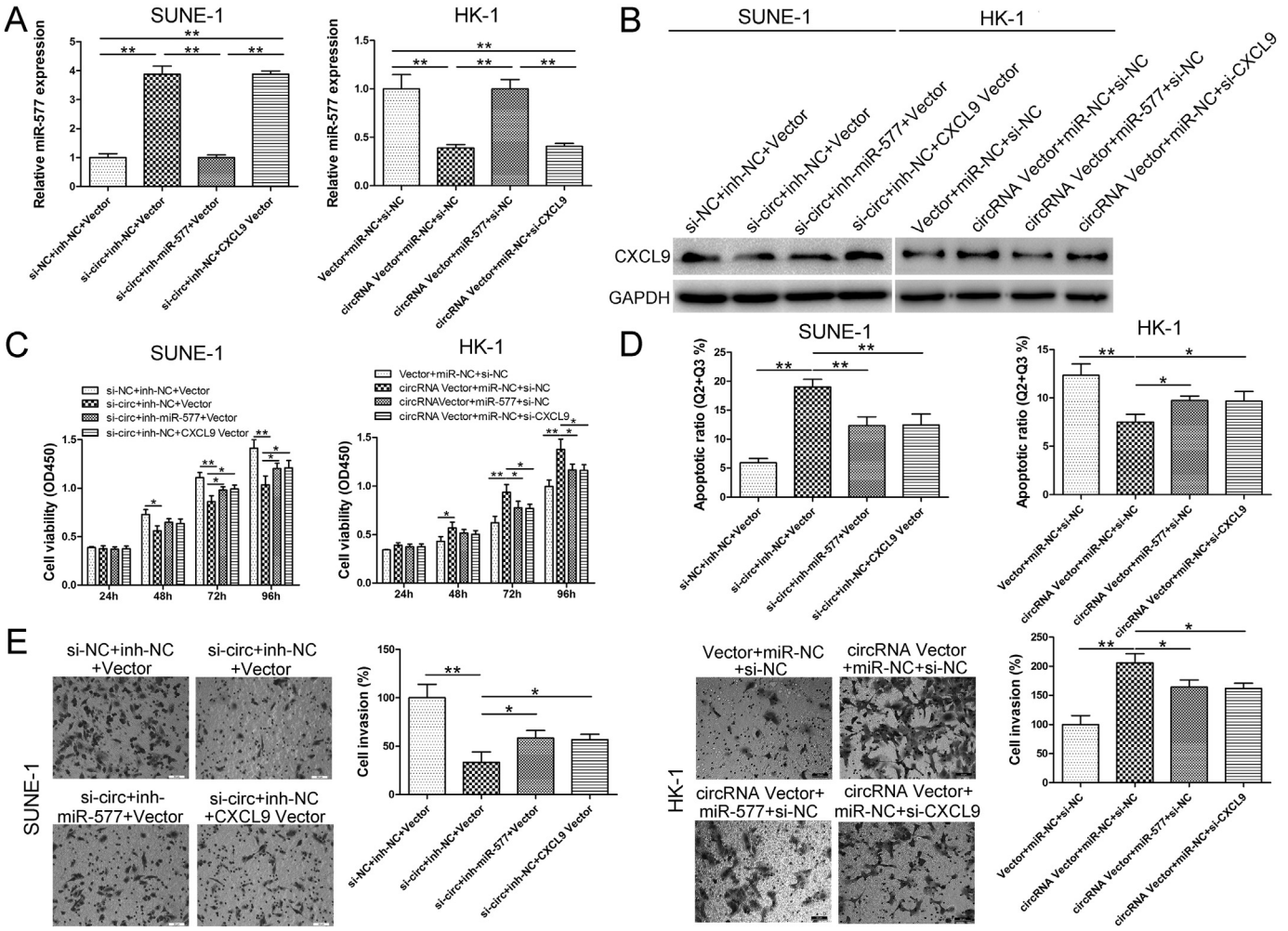

Fig. 4. Circ_0008450 regulates NPC cell growth, apoptosis, and invasion via miR-577/CXCL9 signaling. (A) Relative expression of miR-577 was measured by qRT-PCR after transfection in SUNE-1 and HK-1 cells. (B) The protein level of CXCL9 was measured by Western Blot after transfection in SUNE-1 and HK-1 cells. (C) CCK-8 assay was performed to analyze the viability of SUNE-1 and HK-1 cells after transfection. (D) Flow cytometric assay was performed to detect the apoptosis of SUNE-1 and HK-1 cells after transfection. (E) Transwell assay was performed to detect the migration and invasion of SUNE-1 and HK-1 cells after transfection. $^{*}p < 0.05$, $^{**}p < 0.01$.

the inhibitor-NC group (Fig. 3E). Additionally, we identified a positive relationship between circ_0008450 and CXCL9 mRNA level in NPC tissue specimens (Fig. 3F). Further luciferase reporter experiment verified the interaction of miR-577 and CXCL9 3'-UTR and both of the predicted binding sites were functional (Fig. 3G). This suggests that circ_0008450 has an effect of regulating CXCL9 expression via sponging miR-577.

3.4. Circ_0008450 facilitates cell growth and invasion and inhibits cell apoptosis through miR-577/CXCL9 axis

To confirm whether the biologic role of circ_0008450 is dependent on its modulation of miR-577/CXCL9 axis, rescue experiments were conducted. MiR-577 expression was evidently boosted after knockdown of circ_0008450 in SUNE-1 cell line. However, cotransfected with miR-577 inhibitor restored the level of miR-577 to normal levels. Circ_0008450 vector could decrease the expression of miR-577 significantly. Whereas, this downregulation could be inhibited by miR-577 mimics (Fig. 4A). We next investigated the regulatory actions of circ_0008450 and miR-577 on the expression of CXCL9 by Western blotting. As Fig. 4B indicated, downregulation of circ_0008450 remarkably attenuated the level of CXCL9. Further cotransfected with miR-577 mimics or CXCL9 vector could both restore the expression of CXCL9 in SUNE-1 cells. In HK-1 cell line, circ_0008450 overexpression markedly enhanced the level of CXCL9. Whereas, circ_0008450 vector cotransfected with miR-577 mimics or si-CXCL9 rescued the CXCL9 expression. For the functional assays, the data demonstrated that either silencing of miR-577 or ectopically overexpression of CXCL9 partly reversed the tumor suppressive role caused by si-circ_0008450 in SUNE-1 cell line. What's more, miR-577 mimics and si-MTA3 partially rescued the oncogenic phenotypes of HK-1 cells induced by circ_0008450 vector analyzed by CCK-8, flow cytometric and transwell assay (Fig. 4C–E).

4. Discussion

CircRNAs has been demonstrated to be regulators in various human cancers, including NPC. CircRNAs is critically involved in many human diseases, including cancer (Zang et al., 2018; Ma et al., 2019; Duan et al., 2019; Qiao et al., 2019). For example, circ-FOXM1 promotes non-small cell lung cancer progression as a ceRNA to target PPDPF and MACC1 through interacting with miR-1304-5p (Liu et al., 2019). Elevated circ_0005230 facilitates cholangiocarcinoma cell proliferation and metastasis by binding to miR-1238 and miR-1299 (Xu et al., 2019). NPC is an umbrella concept representing a group of malignant epithelial tumors with various etiopathogenesis and a wide range of histopathological features (Petersson, 2015). Around 42,100 new cases were

diagnosed with NPC and the disease caused 21,320 deaths in China in 2013 (Petersson, 2015). Presently, the relevant study between circRNA and NPC is rare (Chen et al., 2019; Shuai et al., 2018).

Previously, circ_0008450 is found to be upregulated in hepatocellular carcinoma, and its downregulation can impede the ability of proliferation and invasion in tumor cells, suggesting circ_0008450 may serve as a therapeutic agent in liver cancer (Zhang et al., 2019). In the project, we identified and characterized a novel cancer-related circRNA, circ_0008450, which is originated from the CMTM3 genomic region. We unraveled that circ_0008450 was frequently overexpressed in NPC samples and cells. Subsequent functional experiments showed that circ_0008450 functions as an oncogene in NPC for its decreased expression could inhibit NPC cell viability, migration and invasion and induce cell apoptosis. Conversely, gain-of-function assay strengthened the abovementioned malignant biological behaviors. Mechanistically, in contrary with the previous study on liver cancer (Zhang et al., 2019), we found miR-548p expression was almost not affected by circ_0008450, suggesting the tissue specific mechanism of circ_0008450 in different malignancies. What's more, it was found that the expression level of circ_0008450 (circ-CMTM3) had no correlation with CMTM3 expression. Circ_0008450 reduced miR-577-mediated repression of CXCL9, resulting in facilitating the oncogenic functions of NPC. MiR-577 was reported to be a miRNA with tumor suppressive role in malignant tumors, such as non-small cell lung cancer, glioma, colorectal cancer, breast cancer, and papillary thyroid carcinoma (Wang et al., 2018a; Wei et al., 2018; Wang et al., 2018b; Yin et al., 2018; Xue et al., 2017). CXCL9, also termed MIG, is a member of the chemokine γ-subfamily of proteins. The CXCL9 gene is located at chromosome 4q21. CXCL9 is primarily produced in lymphocytes, monocytes/macrophages, and fibroblasts. CXCL9 was identified to be involved in the progression of various malignancies. It could promote prostate cancer progression through inhibition of cytokines from T cells (Tan et al., 2018). Another study indicated that CXCL9 could promote the progression of diffuse large B-cell lymphoma by elevating β-catenin (Ruiduo et al., 2018). In tongue squamous cell carcinoma, CXCL9/CXCR3 axis activates AKT signaling to facilitate cell invasion and metastasis (Li et al., 2018). For NPC, it is reported that serum CXCL9 expression is related to tumor progression and treatment outcome for the patients (Hsin et al., 2013). In this study, we found that overexpressed circ_0008450 could sponge miR-577 to release its repression on CXCL9 at posttranscriptional level. We further identified miR-577/CXCL9 signaling in mediating the oncogenic functions of circ_0008450. Taken together, these results reveal the functions and mechanism of circ_0008450 and provide robust evidence of a crucial regulatory role for this circRNA in NPC progression.

In this study, we identified and characterized a novel dysregulated circRNA in NPC that derived from CMTM3 genomic region, referred to as circ_0008450 (hsa_circ_0008450). We described the expression pattern, and regulatory functions of circ_0008450 in NPC. Furthermore, the mechanism by which circ_0008450 promotes metastasis in NPC was also investigated. Therefore, our study may help to understand the pathogenesis of the molecular alterations of NPC and develop useful indicators that aid novel effective therapies for NPC.

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Declaration of Competing Interest

There is no conflict of interests in this study.

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