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MiR-122-5p suppresses cell proliferation, migration and invasion by targeting SATB1 in nasopharyngeal carcinoma

Y.-H. LIU, J.-L. LIU, Z. WANG, X.-H. ZHU, X.-B. CHEN, M.-Q. WANG

Department of Otorhinolaryngology Head and Neck Surgery, Second Affiliated Hospital of Nanchang University, Nanchang, P.R. China.

Y.-H Liu and J.-L Liu contributed equally to this work

Abstract. – OBJECTIVE: Mounting evidence has suggested that microRNAs (miRNAs) play crucial roles in the progression of nasopharyngeal carcinoma (NPC). However, the molecular mechanism remains not fully understood. We aimed to examine the expression and biological function of miR-122-5p in NPC.

MATERIALS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction was conducted to examine the expression of miR-122-5p. Cell Counting Kit-8 assay, colony formation assay, wound healing assay and transwell assay were performed to measure cell proliferation, colony formation, migration and invasion. Luciferase reporter assay and Western blot assay were used to confirm the target gene of miR-122-5p.

RESULTS: The results showed that miR-122-5p was significantly downregulated in NPC cell lines. Additionally, it was demonstrated that special AT-rich sequence-binding protein 1 (SATB1) was targeted by miR-122-5p. Furthermore, our results revealed that miR-122-5p inhibits cell proliferation, colony formation, cell migration and cell invasion by targeting SATB1.

CONCLUSIONS: Our data suggested that miR-122-5p functions as a tumor suppressor and may be a therapeutic target for NPC.

Key Words:
MiR-122-5p, SATB1, Nasopharyngeal carcinoma, Tumor suppressor, Cell behaviors.

Introduction

Nasopharyngeal carcinoma (NPC) is a commonly diagnosed head and neck cancer and is reported to have an endemic high incidence in southern China[1]. Radiotherapy combined with chemotherapy is a frequently used method for NPC treatment[2]. New treatment agents including Gemcitabine and Camrelizumab have lighted new hope for the patients with NPC[3,4]. However, the molecular mechanism underlying NPC initiation and progression remains largely unknown. MicroRNAs (miRNAs) are a family of non-coding RNAs that were reported to have dual functions, tumor suppressive role or oncogenic role, in the progression of human cancers[5,6]. Accumulating evidence has shown that miRNAs were able to regulate almost all the biological processes by targeting the downstream genes[7,8]. A previous study[9] aiming to investigate the expression of miRNAs in collected tumor tissues and analyzing the associations of these miRNAs with clinical outcome revealed that 41 miRNAs were abnormally expressed in NPC tissues. It also identified a five-miRNA signature to predict the prognosis of NPC patients[9]. MiR-122-5p has been reported to be downregulated in several human cancers including bile duct carcinoma, breast cancer, gastric cancer and hepatocellular carcinoma[10-13]. Xu et al[10] demonstrated that miR-122-5p could inhibit the proliferation and invasion of bile duct carcinoma cells by regulating Aldolase-A, indicating that miR-122-5p may be used as therapeutic target. Xu et al[12] investigating the role of miR-122-5p in gastric cancer revealed that miR-122-5p represses gastric cancer progression by targeting dual specificity phosphatase 4, showing that miR-122-5p has multiple targets in human cancers. Ma et al[13] demonstrated that miR-122-5p is an effector for the biological function of long non-coding RNA ANRIL in hepatocellular carcinoma. However, up to date, the role of miR-122-5p in NPC remains unknown. In the present work, we aimed to investigate

Corresponding Author: Yuehui Liu, MD; e-mail: liuyh_ncu@163.com

the role of miR-122-5p and special AT-rich sequence-binding protein 1 (SATB1) on NPC cell behaviors and the associations between them. We found that miR-122-5p expression was significantly reduced in NPC cell lines compared with the normal cell line. Additionally, the overexpression of miR-122-5p could suppress cell growth, colony formation, migration, and invasion by targeting SATB1. These findings presented novel insights concerning the mechanisms of NPC progression.

Materials and Methods

Cell Line and Cell Culture

NPC cell lines (CNE-1 and C666-1) were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). Human immortalized nasopharyngeal epithelial cell line NP69 was incubated in keratinocyte/serum-free medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum. All these cells were cultured at 37°C humidified incubator containing 5% of CO_2. These cell lines were purchased from Gaining Biological (Shanghai, China).

Cell Transfection

MiR-122-5p mimic, inhibitor, and negative control miRNAs (miR-NC) were purchased from RiboBio (Guangzhou, China). The expression vector for SATB1 and NC vector were purchased from GenScript (Nanjing, China). Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 48 h of transfection, cells were harvested for further experiments.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) based on the provided instructions. To detect the levels of miR-122-5p, extracted RNA was reverse transcribed into complementary DNA (cDNA) using cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR was conducted at ABI 7500 (Applied Biosystems, Foster City, CA, USA) using SYBR Green Mix (TaKaRa, Otsu, Shiga, Japan). The following primers were used: miR-122-5p: (F) 5'-TATTCGCACTGGATACGACACAAAC-3', (R) 5'-GCCCGTGGAGTGTGACAATGGT-3'; U6 snRNA: (F) 5'-GCTTCGGCAGCACATATACTAAAAT-3', (R) 5'-CGCTTCACGAATTTGCGTGTCAT-3'. The relative expression levels were detected using the $2^{-\Delta\Delta Ct}$ method using U6 snRNA as internal control[14].

Protein Extraction and Western Blot

Total protein was isolated using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) according to the supplier's recommendations. The protein concentration was measured using bicinchoninic acid (BCA) protein determination kit (Beyotime, Shanghai, China) according to the standard protocol. After that, an equal amount of protein sample was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane (Beyotime, Shanghai, China). Membranes were incubated with 5% fat-free milk at room temperature for 1.5 h and then incubated with primary antibodies (SATB1: ab109122, GAPDH: ab181602; Abcam, Cambridge, MA, USA) at 4°C overnight. Subsequently, membranes were washed with Tris-Buffered Saline with Tween-20 and incubated with horseradish-peroxidase conjugated secondary antibody (ab6721, Abcam, Cambridge, MA, USA). Blots were visualized using the enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) reagent and analyzed using Image J 1.42 (Bethesda, MD, USA).

Cell Proliferation Assay

Cells were seeded in 96-well plate at the density of 5×10^3 cells/well. Subsequently, 10 µl of Cell Counting Kit-8 (CCK-8) assay solution (Beyotime, Shanghai, China) was added to each well and incubated for an additional 1 h at 37°C. The cell proliferation rate was assessed by measuring the optical density (OD) at 450 nm using a microplate reader.

Colony Formation Assay

Cells were seeded in 6-well plates at the density of 400 cells/well. After incubation for 14 days, cell colonies were washed with Phosphate-Buffered Solution (PBS; Gibco, Grand Island, NY, USA), and fixed with methanol for 15 min at room

temperature. Subsequently, 0.1% crystal violet (Beyotime, Shanghai, China) was used to stain the colonies for 15 min at room temperature. The field contains over 50 colonies and were counted under a microscope.

Cell Migration Assay

Cells were cultured in 6-well plates at the density of 1×10^5 cells/well. A wound was created at cell surface using a pipet tip after growth to 90% confluence. At 0 and 48 h after wound creation, wound width was measured under a microscope with a magnification of 100×. The cell migration capability was expressed by wound closure rate.

Cell Invasion Assay

Cell invasion was analyzed using a transwell chamber (Corning, New York, NY, USA) with 8 μm pores coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). 1×10^5 cells were seeded in the upper chamber in serum-free medium. The medium containing 10% FBS was placed in the lower chamber. After incubation for 24 h, cells on the upper side, which are the non-invasive cells, were removed. The invasive cells were fixed with 10% methanol and stained with 0.1% crystal violet for 15 min at room temperature. Invasion numbers were counted under a microscope from 5 independent fields.

Dual-Luciferase Reporter Assay

TargetScan (http://www.targetscan.org/vert_72/) was applied to predict the putative targets of miR-122-5p. To confirm the association between miR-122-5p and SATB1, we cloned the wild-type (wt) 3'-untranslated region (3'-UTR) of SATB1 into the pMir-report vector (Promega, Madison, WI, USA) and named it as wt-SATB1. Site mutagenesis kit (TaKaRa, Otsu, Shiga, Japan) was used to generate the mutant (mut) type of SATB1 and was named as mut-SATB1. Next, cells were co-transfected with wt-SATB1 or mut-SATB1 and miR-122-5p mimic or miR-NC using Lipofectamine 2000 according to the manufacturer's instructions. The Dual-Luciferase reporter assay kit (Promega, Madison, WI, USA) was used to detect the Luciferase after 48 h of transfection. Data were normalized to Renilla Luciferase activity.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD) and analyzed with SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Student's *t*-test and one-way analysis of variance with Tukey's Post-Hoc test were applied to compare the difference between the groups. $p < 0.05$ was considered statistically different.

Results

MiR-122-5p Was Downregulated in NPC Cell Lines

To investigate the role of miR-122-5p in NPC, we first detected the expression of miR-122-5p in NPC cell lines. We found that miR-122-5p expression was significantly reduced in NPC

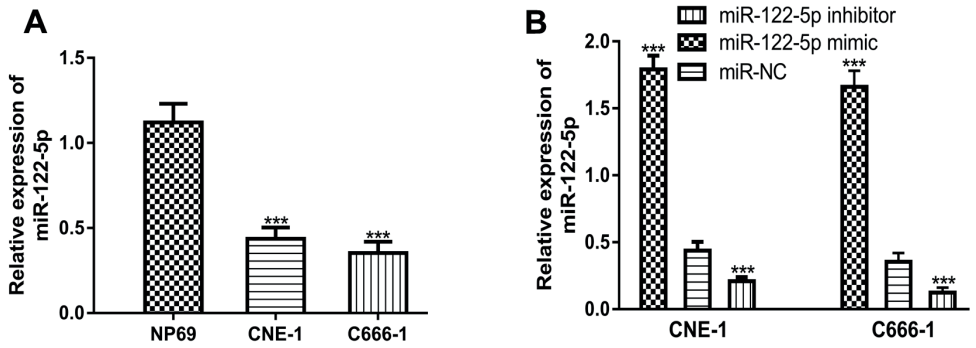

Figure 1. Expression of miR-122-5p in nasopharyngeal carcinoma cell lines. ***A,*** miR-122-5p expression in nasopharyngeal carcinoma cell lines (CNE-1 and C666-1) and human immortalized nasopharyngeal epithelial cell line (NP69). ***B,*** miR-122-5p expression in nasopharyngeal carcinoma cell lines (CNE-1 and C666-1) with synthetic miRNAs transfection. (***p<0.001) miR-122-5p: microRNA-122-5p; miR-NC: negative control miRNA.

cell lines compared with NP69 cell line (Figure 1A). We then introduced synthetic miRNAs into NPC cell lines to regulate miR-122-5p expression. As expected, miR-122-5p mimic transfection could enhance the levels of miR-122-5p, while the introduction of miR-122-5p inhibitor reduced the expression of miR-122-5p (Figure 1B).

MiR-122-5p Overexpression Inhibits NPC Cell Growth, Migration and Invasion

Next, we examined the biological roles of miR-122-5p in NPC. The CCK-8 assay revealed that transfection of miR-122-5p mimic significantly decreased cell proliferation, but proliferation rates of cells transfected with miR-122-5p inhibitor were markedly increased (Figure 2A). To validate the inhibitory effect of miR-122-5p on cell growth, colony formation assay was conducted. As shown in Figure 2B, cells transfected with miR-122-5p mimic displayed fewer colonies compared with miR-NC, while cells transfected with miR-122-5p mimic displayed much more colonies. Additionally, wound-healing assay results showed that cell migration ability was attenuated with miR-122-5p mimics transfection, while the migration ability of cells transfected with miR-122-5p inhibitor was remarkably enhanced (Figure 2C). Furthermore, transwell invasion assay revealed that the invasive numbers in cells transfected with miR-122-5p mimic were fewer than those in the miR-NC group, whereas those in the miR-122-5p inhibitor group was significantly higher than miR-NC group (Figure 2D).

SATB1 Was a Direct Target of MiR-122-5p

The candidate target gene of miR-122-5p was predicted by TargetScan, of which the results suggested that SATB1 contains a binding site for miR-122-5p in its 3'-UTR (Figure 3A). Luciferase activity reporter assay showed that Luciferase activity in cells transfected with wt-SATB1 was markedly inhibited by miR-122-5p mimic (Figure 3B). However, no significant difference was found between miR-122-5p mimic and miR-NC group in cells transfected with mut-SATB1 (Figure 3B). Furthermore, Western blot assay revealed that SATB1 protein expression was remarkably repressed by miR-122-5p mimic in NPC cell lines compared with miR-NC (Figure 3C).

MiR-122-5p Regulates NPC Cell Behaviors by Regulating SATB1

SATB1 in cells transfected with miR-122-5p mimic or SATB1 construct was analyzed by Western blot. SATB1 construct transfection significantly enhanced the expression of SATB1 (Figure 4A). In the meantime, the inhibitory effects of miR-122-5p mimic on SATB1 expression could be reversed by SATB1 construct (Figure 4A). Functional assays showed that SATB1 construct transfection cell proliferation rate analysis markedly increased cell proliferation, colony formation, cell migration, and cell invasion (Figure 4B-E). Meanwhile, we showed that the inhibitory effects of miR-122-5p on NPC cell behaviors could be reversed by SATB1 construct (Figure 4B-E).

Discussion

It is estimated that only about 3% of human genome codes for proteins, while about 75% of them transcribed into non-coding RNAs, including miRNAs and lncRNAs[15]. Some studies[15-20] have revealed the crucial role of miRNAs in NPC tumorigenesis as they were abnormally expressed in cancer tissues and cells. Notably, miR-19b was downregulated in NPC tissues and inhibited NPC cell proliferation and migration[16]. Moreover, miR-19b was found could sensitive the NPC cells to cisplatin by targeting KRAS[16]. MiR-184 was also found to be downregulated in NPC cell lines and act as an inhibitor for NPC cell migration, invasion and metastasis[17]. To the best of our knowledge, miR-122-5p, a tumor suppressive miRNA in several human cancers, has not been investigated in NPC till now. Hence, in this work, we investigated the expression levels of miR-122-5p in NPC cell lines and we found that miR-122-5p expression was significantly reduced in NPC cell lines. Meanwhile, we manipulated miR-122-5p expression in NPC cell lines using synthetic miRNAs. Subsequently, *in vitro* functional experiments revealed that miR-122-5p overexpression inhibits NPC cell proliferation, colony formation, cell migration and cell invasion. On the contrary, the downregulation of miR-122-5p have the opposite effects on NPC cell behaviors. These results illustrated that miR-122-5p functions as a tumor suppressor in NPC. It is well recognized that miRNAs regulate target genes expression through 3'-UTR binding and thus resulted in messenger RNA degradation or translation repression[21]. Through

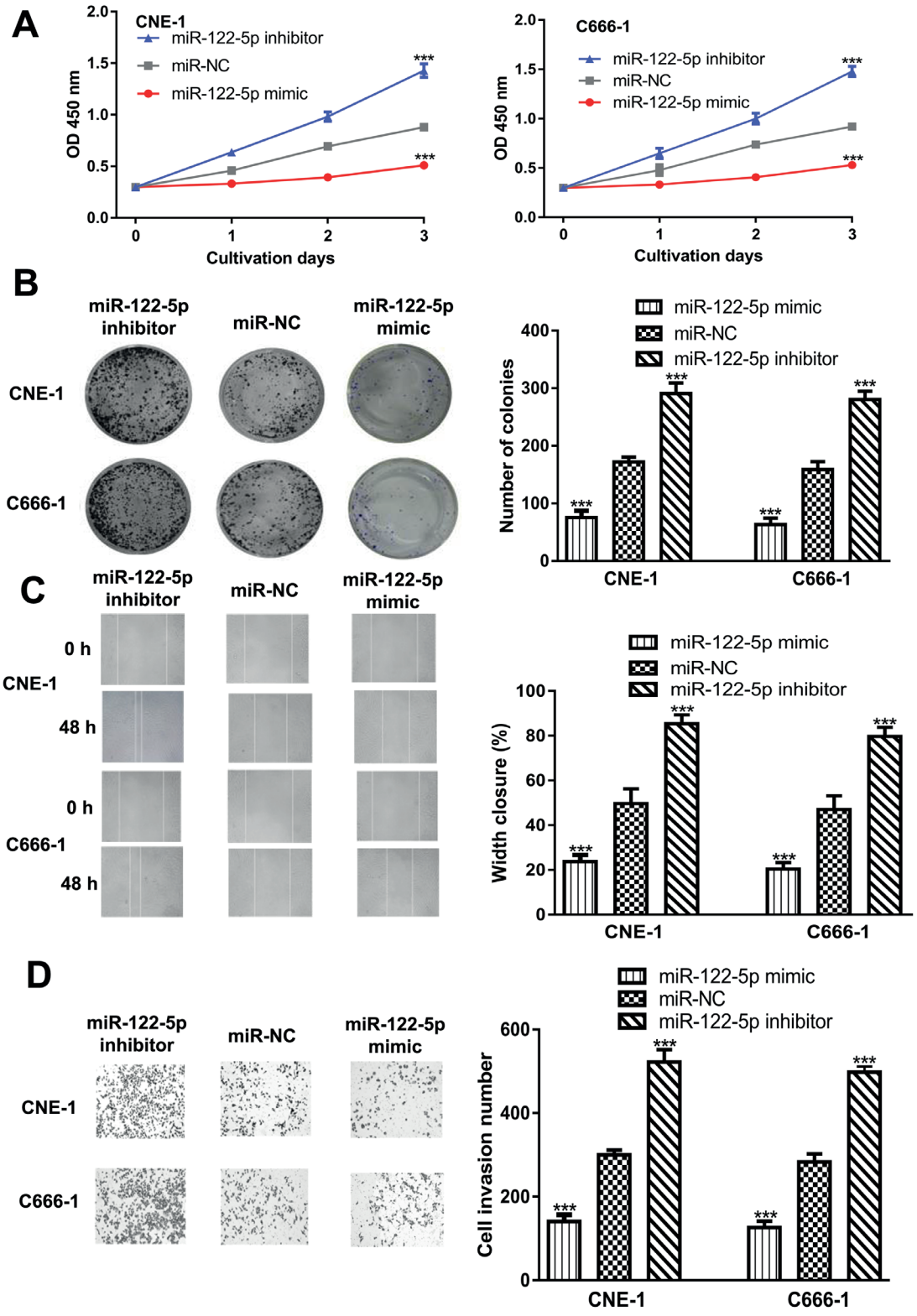

Figure 2. Overexpression of miR-122-5p on nasopharyngeal carcinoma cell behaviors. ***A,*** Cell proliferation, ***B,*** Colony formation, ***C,*** Cell migration, and ***D,*** Cell invasion in nasopharyngeal carcinoma cell lines (CNE-1 and C666-1) with synthetic miRNAs transfection. (***$p<0.001$) miR-122-5p: microRNA-122-5p; miR-NC: negative control miRNA.

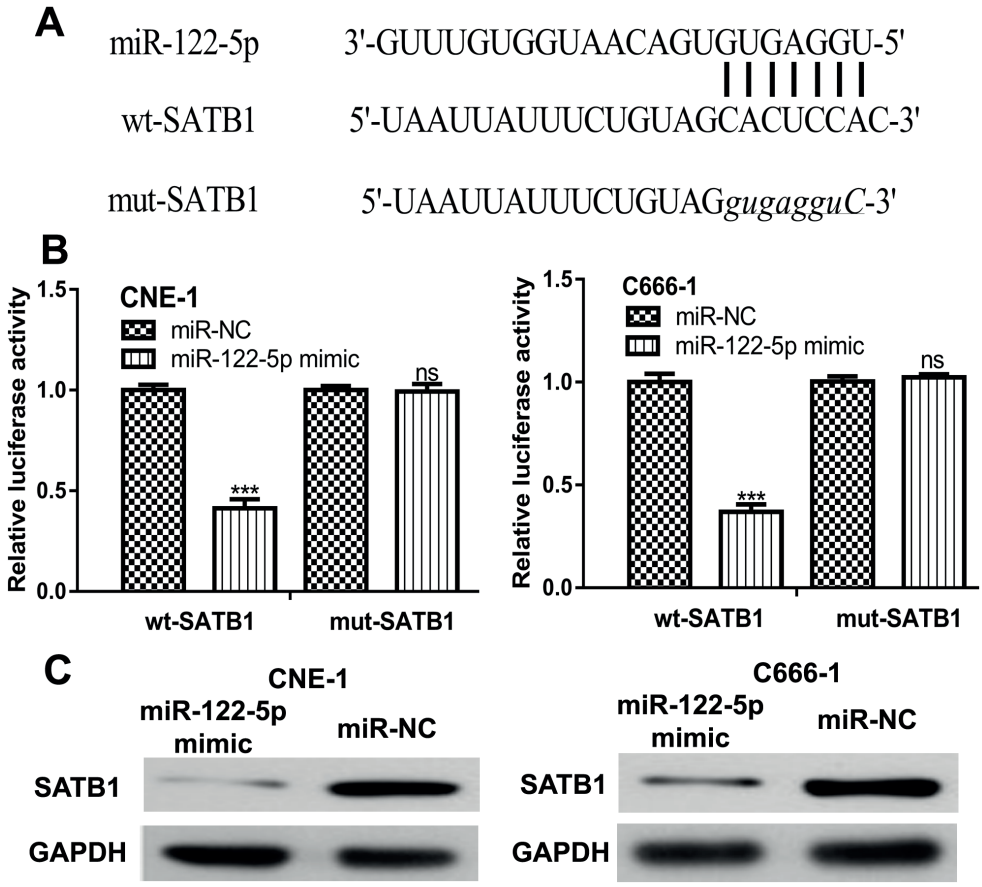

Figure 3. SATB1 was a direct target of miR-122-5p. ***A,*** Prediction binding site between miR-122-5p and 3'-UTR of SATB1. ***B,*** miR-122-5p mimic transfection inhibits the Luciferase activity of nasopharyngeal carcinoma cell lines (CNE-1 and C666-1) transfected with wt-SATB1. ***C,*** SATB1 protein expression in nasopharyngeal carcinoma cell lines (CNE-1 and C666-1) transfected with miR-122-5p mimic or miR-NC. (ns not significant; ***$p<0.001$) miR-122-5p: microRNA-122-5p; SATB1: special AT-rich sequence-binding protein 1; miR-NC: negative control miRNA; UTR: untranslated region; wt: wild-type; mut: mutant.

online prediction software, we found that SATB1 contains a putative binding site for miR-122-5p in its 3'-UTR. SATB1 is a cell type-specific nuclear matrix-associated protein and is reported to regulate hundreds of human genes involved in human cancers[22]. Abnormal expression of SATB1 was found in human cancers including breast cancer, lung and prostate cancer[22-24]. Therefore, the association between miR-122-5p and SATB1 was further investigated. Luciferase activity reporter assay and Western blot assay revealed that SATB1 was a direct target of miR-122-5p. Functional assays revealed that SATB1 was an effector for the inhibitory effects of miR-122-5p on NPC cell behaviors.

Conclusions

We showed that miR-122-5p expression was downregulated in NPC cell lines. MiR-122-5p could inhibit NPC cell proliferation, colony formation, cell migration and cell invasion *in vitro*. Mechanistically, miR-122-5p inhibits NPC cell behaviors by targeting SATB1. Our results provide evidence that miR-122-5p plays crucial roles in NPC progression and may serve as a therapeutic target for NPC treatment in the future.

Conflict of Interest

The Authors declare that they have no conflict of interest.

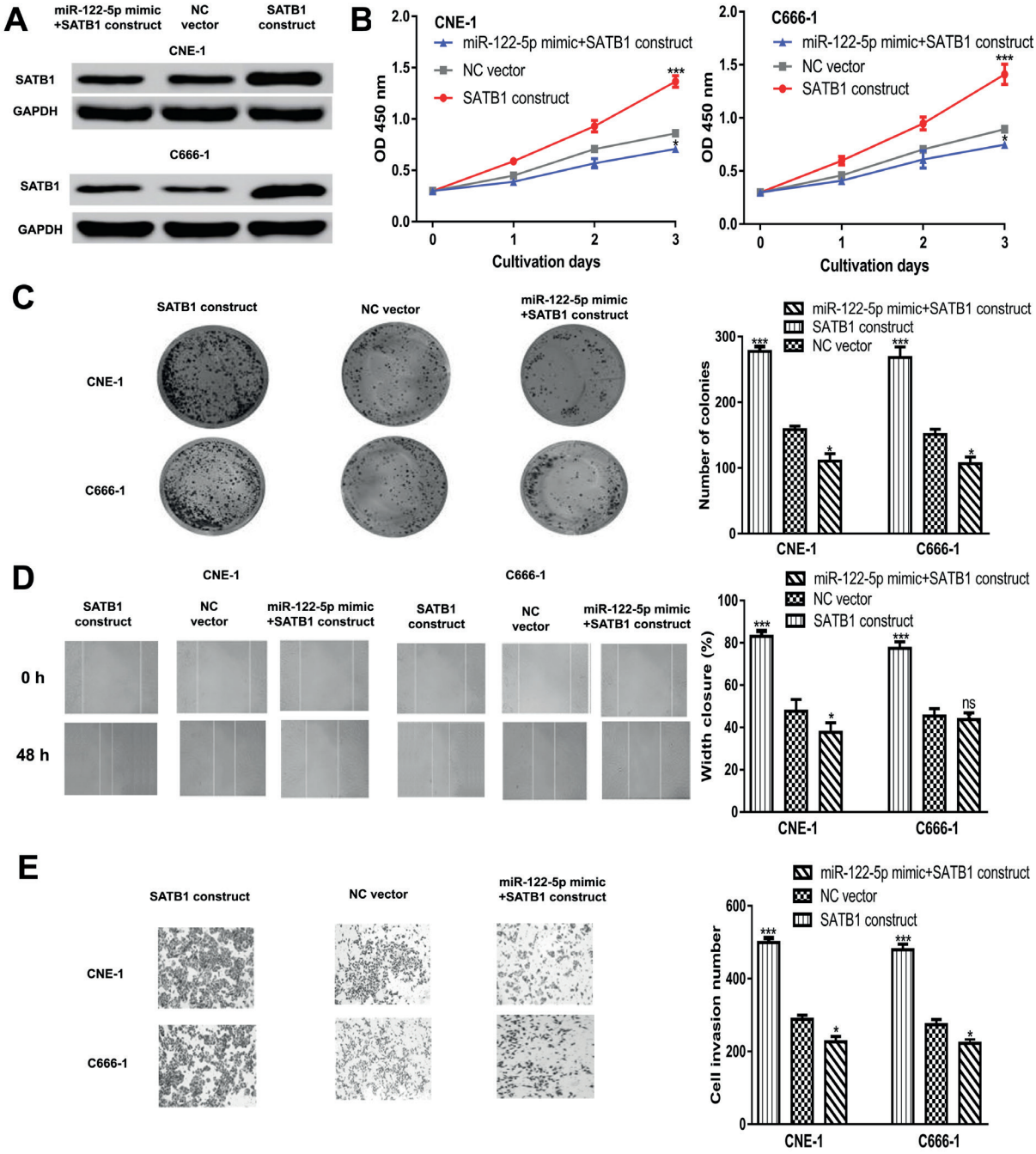

Figure 4. MiR-122-5p affects nasopharyngeal carcinoma cell behaviors through targeting SATB1. ***A,*** SATB1 protein expression, ***B,*** Cell proliferation, ***C,*** Colony formation, ***D,*** Cell migration, and ***E,*** Cell invasion in nasopharyngeal carcinoma cell lines (CNE-1 and C666-1) transfected with SATB1 construct, NC vector, or SATB1 construct and miR-122-5p mimic. (ns not significant; *$p<0.05$; ***$p<0.001$) miR-122-5p: microRNA-122-5p; SATB1: special AT-rich sequence-binding protein 1; NC: negative control.

References

1) Chua MLK, Wee JTS, Hui EP, Chan ATC. Nasopharyngeal carcinoma. Lancet 2016; 387: 1012-1024.
2) Li WF, Chen L, Sun Y, Ma J. Induction chemotherapy for locoregionally advanced nasopharyngeal carcinoma. Chin J Cancer 2016; 35: 94.
3) Hong S, Zhang L. Gemcitabine improves survival in patients with recurrent or metastatic nasopharyngeal carcinoma. Chin J Cancer 2016; 35: 100.
4) Kwong DLW. Camrelizumab for nasopharyngeal carcinoma: a new hope? Lancet Oncol 2018; 19: 1266-1267.
5) Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136: 215-233.
6) Othman N, Nagoor NH. The role of microRNAs in the regulation of apoptosis in lung cancer and its application in cancer treatment. Biomed Res Int 2014; 2014: 318030.
7) Ding X, Park SI, McCauley LK, Wang CY. Signaling between transforming growth factor β (TGF-β) and transcription factor SNAI2 represses expression of microRNA miR-203 to promote epithelial-mesenchymal transition and tumor metastasis. J Biol Chem 2013; 288: 10241-10253.
8) Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, Allgayer H. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 2008; 27: 2128-2136.
9) Liu N, Chen NY, Cui RX, Li WF, Li Y, Wei RR, Zhang MY, Sun Y, Huang BJ, Chen M, He QM, Jiang N, Chen L, Cho WC, Yun JP, Zeng J, Liu LZ, Li L, Guo Y, Wang HY, Ma J. Prognostic value of a microRNA signature in nasopharyngeal carcinoma: a microRNA expression analysis. Lancet Oncol 2012; 13: 633-641.
10) Xu Z, Liu G, Zhang M, Zhang Z, Jia Y, Peng L, Zhu Y, Hu J, Huang R, Sun X. miR-122-5p inhibits the proliferation, invasion and growth of bile duct carcinoma cells by targeting ALDOA. Cell Physiol Biochem 2018; 48: 2596-2606.
11) Uen Y, Wang JW, Wang C, Jhang Y, Chung JY, Tseng T, Sheu M, Lee S. Mining of potential microRNAs with clinical correlation-regulation of syndecan-1 expression by miR-122-5p altered mobility of breast cancer cells and possible correlation with liver injury. Oncotarget 2018; 9: 28165-28175.
12) Xu X, Gao F, Wang J, Tao L, Ye J, Ding L, Ji W, Chen X. MiR-122-5p inhibits cell migration and invasion in gastric cancer by down-regulating DUSP4. Cancer Biol Ther 2018; 19: 427-435.
13) Ma J, Li T, Han X, Yuan H. Knockdown of LncRNA ANRIL suppresses cell proliferation, metastasis, and invasion via regulating miR-122-5p expression in hepatocellular carcinoma. J Cancer Res Clin Oncol 2018; 144: 205-214.
14) Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001; 25: 402-408.
15) Song P, Yin SC. Long non-coding RNA 319 facilitates nasopharyngeal carcinoma carcinogenesis through regulation of miR-1207-5p/KLF12 axis. Gene 2019; 680: 51-58.
16) Zhu HM, Jiang XS, Li HZ, Qian LX, Du MY, Lu ZW, Wu J, Tian XK, Fei Q, He X, Yin L. miR-184 inhibits tumor invasion, migration and metastasis in nasopharyngeal carcinoma by targeting Notch2. Cell Physiol Biochem 2018; 49: 1564-1576.
17) Zhao X, Chu J. MicroRNA-379 suppresses cell proliferation, migration and invasion in nasopharyngeal carcinoma by targeting tumor protein D52. Exp Ther Med 2018; 16: 1232-1240.
18) Ma YX, Zhang H, Li XH, Liu YH. MiR-30e-5p inhibits proliferation and metastasis of nasopharyngeal carcinoma cells by targeting USP22. Eur Rev Med Pharmacol Sci 2018; 22: 6342-6349.
19) Wu RS, Qiu EH, Zhu JJ, Wang JR, Lin HL. MiR-101 promotes nasopharyngeal carcinoma cell apoptosis through inhibiting Ras/Raf/MEK/ERK signaling pathway. Eur Rev Med Pharmacol Sci 2018; 22: 150-157.
20) Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.
21) Han HJ, Russo J, Kohwi Y, Kohwi-Shigematsu T. SATB1 reprogrammes gene expression to promote breast tumour growth and metastasis. Nature 2008; 452: 187-193.
22) Glatzel-Plucinska N, Piotrowska A, Grzegrzolka J, Olbromski M, Rzechonek A, Dziegiel P, Podhorska-Okolow M. SATB1 level correlates with Ki-67 expression and is a positive prognostic factor in non-small cell lung carcinoma. Anticancer Res 2018; 38: 723-736.
23) Wang Q, Yang CS, Ma ZX, Chen JC, Zheng JN, Sun XQ, Wang JQ. Inhibition of prostate cancer DU145 cell growth with small interfering RNA targeting the SATB1 gene. Exp Ther Med 2018; 15: 3028-3033.