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ORIGINAL RESEARCH

MicroRNA-432 Suppresses Invasion and Migration via E2F3 in Nasopharyngeal Carcinoma

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Background: E2F transcription factor 3 (E2F3) is oncogenic and dysregulated in various malignancies. Complex networks involving microRNAs (miRNAs) and E2F3 regulate tumorigenesis and progression. However, the potential roles of E2F3 and its target miRNAs in nasopharyngeal carcinoma (NPC) are rarely reported.
Methods: E2F3 expression was detected in human NPC tissues and cell lines through quantitative real-time PCR. NPC cell proliferation, migration, and invasion were evaluated in vitro by colony forming, cell counting kit-8, wound healing, and Transwell invasion assays. Publicly available database software was used to explore the target miRNAs of E2F3. Dual-luciferase reporter assay was performed to identify the direct relationship. The function of miRNAs in vivo was investigated by using a tumor xenograft model.
Results: E2F3 was upregulated in NPC cell lines and tissues, and its exotic expression promoted NPC cell invasion and migration. E2F3 was identified as a target of miR-432, which restrained NPC cell invasion and migration in vitro and in vivo. Further experiments revealed that miR-432 repressed the invasion and migration potential of NPC cells by modulating E2F3 expression.
Conclusion: miRNA-432 suppressed the malignant biological behavior of NPC cells by targeting E2F3. This study provided further insights into NPC prognosis and treatment.
Keywords: E2F3, nasopharyngeal carcinoma, miR-432, invasion, migration

Introduction

Nasopharyngeal carcinoma (NPC) originates from nasopharyngeal epithelial cells and is an endemic malignancy with a distinct geographical distribution.[1] NPC is most prevalent in Southern China, where its annual incidence has been increasing.[2] Advances in intensity-modulated radiation and comprehensive chemotherapy strategies have greatly improved the locoregional control for most NPCs. However, tumor recurrence and distant metastasis remain massive problems for patient survival.[3,4] Therefore, underlying molecular mechanisms should be determined, and effective therapeutic targets should be applied in individualized NPC treatment.

E2F transcription factor 3 (E2F3), a member of the E2F transcription factor family, is overexpressed in lung, ovarian, bladder, gastric, and prostate cancers.[5–8] The E2F family has distinct roles in the control of cell proliferation, apoptosis, and differentiation.[9–11] miRNAs and E2F3 form an intricate network, and its dysregulation contributes to oncogenesis.[12] Nevertheless, the expression of E2F3 in NPC remains unclear.

MicroRNAs (miRNAs) are a subgroup of small noncoding RNAs that are dysregulated in various cancers, such as breast,[13] lung,[14] liver,[15] esophageal,[16] and colon cancers.[17] MiRNAs regulate gene expression at the posttranscriptional level by pairing

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with the 3′-UTRs of their target mRNAs.[18] MiRNAs play important roles in cell proliferation, differentiation, and migration[19,20] and also function as oncogenes and tumor suppressors in NPC progression and therapeutic response.[21–23]

In this study, we found that E2F3 was expressed in NPC cell lines and tissues and functioned as a target of miR-432, which restrained NPC cell invasion and migration in vitro and in vivo. Our results present insights into the network between miR-432 and E2F3 in NPC development and progression and thus provide new therapeutic targets against NPC.

Materials and Methods

Cell Culture and Clinical Specimens

Five human NPC cell lines (CNE-1, CNE-2, 5-8F, 6-10B, and SUNE-1) were obtained from Jiangsu Cancer Hospital (Nanjing, China) and cultured in RPMI 1640 medium (Corning, Manassas, VA, USA) containing 5% fetal bovine serum (Gibco, Grand Island, USA). An immortalized nasopharyngeal epithelial cell line (NP69) was maintained in keratinocyte/serum-free medium supplemented with growth factors (Gibco, Grand Island, NY, USA). All cells were incubated in the presence of 5% CO_2 at 37 °C. All the cell lines was approved by the Institutional Ethical Review Board of Jiangsu Cancer Hospital. All tissue samples (66 frozen NPC tissues and eight normal nasopharyngeal epithelium tissues) originated from Jiangsu Cancer Hospital (Nanjing, China) and confirmed by pathologists. Research protocols were approved by the Institutional Ethical Review Board of Jiangsu Cancer, and each patient provided written informed consent. miR-432 expression profiles were also evaluated in NPC samples obtained from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo).

RNA Extraction and Quantitative Real-Time PCR Assays

Total RNA was isolated by TRIzol reagent in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA). Real-time PCR reactions were performed using SYBR Green PCR master mix on an ABI7300 real-time PCR machine (Applied Bio-systems). The sequences of the specific primers synthesized by Guangzhou RiboBio (Guangzhou, China) were as follows: E2F3, 5′-TGACCCAAT GGTAGG CACAT-3′ and 5′-CATCTAGGACCACACCGACA-3′; U6, 5′-CTCGCTTCGGCAGCA-CA-3′ and 5′-AACGCTTCACG AATTTGCGT-3′; β-actin, 5′-GGACTTCGAGCAAGAGAT GG-3′ and 5′-AGACTGTGTTGGCGTACAG-3′. U6 and β-actin were used as the normalized controls for miR-432 and E2F3, respectively.

Cell Transfection

MiRNA-432 and negative control (NC) mimics were purchased from RiboBio (Guangzhou, China). Si E2F3 and Si NC were synthesized by RiboBio (Guangzhou, China). CNE-2 and 5-8F cells were cultured in six-well plates after transfection. Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used following the manufacturer's instructions. The cells were collected at 48 h after transfection for further investigation.

Colony Forming Assay

CNE-2 and 5-8F cells were seeded in six-well plates (1×10^3 cells/well) after transfection and then cultured for 10 days to measure their colony-forming activity. The colonies were fixed with paraformaldehyde and stained with 0.1% crystal violet. The number of colonies was then calculated under inverted microscopy. All steps were performed with three independent replications.

Cell Counting Kit 8 Assay

Cell proliferation was determined through the CCK-8 assay every 24 h. After transfection, 3×10^3 cells/well were seeded in 96-well plates and incubated at 37 °C for 1, 2, 3, or 4 days prior to the addition of cell counting kit 8 (CCK8) solution to each well. The optical density of each well was detected at 450 nm. This experiment was performed three times, and the results were all averaged.

Wound-Healing Assay

Wound-healing assay was performed to examine the migration capacity of NPC cells. Transfected CNE-2 and 5-8F cells were cultured in six-well plates and serum starved for 24 h. A wound was created directly by using a 200 μL pipette tube. Cell migration was monitored under an optical microscope at 0 and 24 h. This experiment was repeated three times.

Transwell Invasion Assay

Transwell chambers (Corning, NY, USA) covered with Matrigel (BD Biosciences) were employed to determine cell invasion ability. The transfected cells (CNE-2 and 5-8F) were cultured for 48 h and then collected. The cells (2×10^4 cells/well) were resuspended in 200 μL of serum-free medium and positioned in the upper chamber. The bottom chamber was covered with 500 μL of RPMI-1640 and 20% FBS. After 36 h of hatching, the membranes were

fixed and stained by using 4% paraformaldehyde and crystal violet, respectively. The invaded cells were then counted under a light microscope. All experiments included three independent replications.

Luciferase Reporter Assay

The E2F3 wild-type (Wt) and mutant (Mut) 3′-UTR were constructed and cloned into a pcDNA3.1 luciferase reporter vector. The NPC cells were cotransfected with E2F3 Wt or Mut 3′-UTR vector and miR-432 mimic or NC mimic by using Lipofectamine 2000 reagent (Invitrogen). Luciferase activities were measured using the dual luciferase reporter assay system (Promega) in accordance with the manufacturer's protocol. Each experiment was performed in triplicate.

Tumor Xenograft Model

Six-week-old athymic male BALB/c nude mice were acquired from Nanjing University (Nanjing, China), and 2×10^6 5-8F cells in 20 μL of cell suspension were injected into their footpad. The volume of the xenograft was measured and recorded every 3–4 days. When the tumor volume reached 60 mm^3, 5 nmol miR-432 mimic or miR-Ctrl in 20 μL of saline buffer was subcutaneously injected into the tumor mass in the plantar twice a week. At 6 weeks post injection, all mice were euthanized and dissected. All mice were euthanized and dissected. The mice with popliteal lymph node metastasis were counted and confirmed with GFP. All animal studies were conducted in accordance with National Institutes of Health animal use guidelines and all animal protocols were approved by Nanjing Medical University Animal Care Committee.

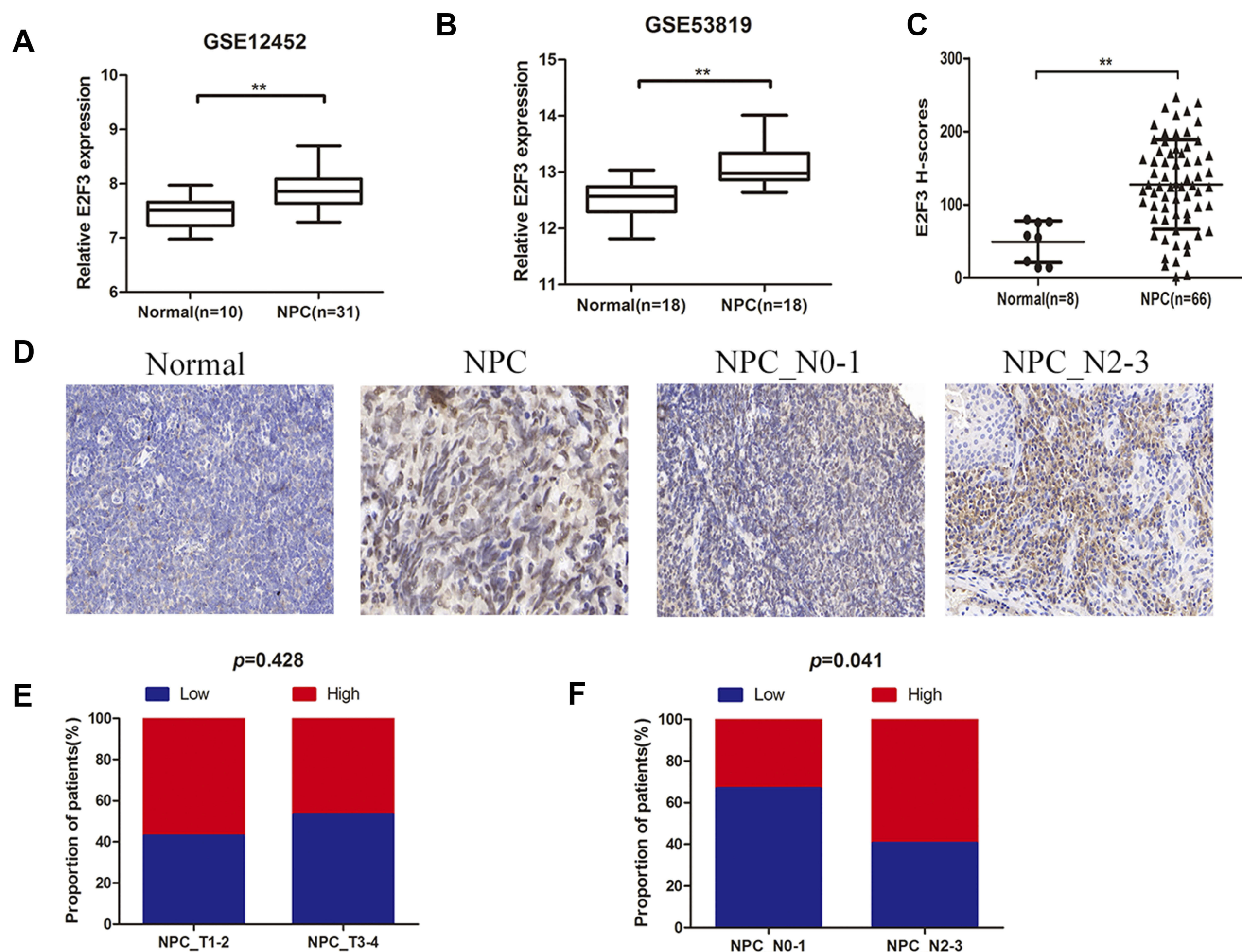

Figure 1 E2F3 was upregulated in NPC cell lines and tissue samples. (**A** and **B**) E2F3 expression profiles in NPC cells and normal nasopharyngeal epithelial samples by GEO datasets GSE 12452 and GSE 53819. (**C**) Relative E2F3 expression in NPC tissues (n = 66) compared with that in normal tissues (n = 8). (**D**) E2F3 expression was detected through immunohistochemistry analysis. (**E**) Relationship between E2F3 expression and the clinical T stage of NPC. (**F**) Relationship between E2F3 expression and the lymph node metastasis of NPC. $^{**}P < 0.01$.

Western Blot Analysis

Transfected NPC cells were harvested and prepared in modified RIPA buffer and PMSF (Beyotime, Shanghai, China). A BCA protein assay kit was used to evaluate protein concentrations. Protein (15 mg) from each sample was separated using 10% SDS-PAGE gels and then transferred onto a nitrocellulose membrane. The membranes were incubated with specific antibodies, namely, anti-E2F3 (1:1000; Cell Signaling Technology, USA) and anti-β-actin (1:2000; Cell Signaling Technology, USA). β-actin was used as the loading control. Immunoreactive bands were visualized with ECL detection reagent (Millipore, Billerica, MA, USA).

Statistical Analysis

All statistical analyses were conducted with SPSS 20.0. Student's *t*-test was performed to determine the significance of mean values between two groups. One-way ANOVA was used to test the difference among multiple groups. Enumeration data were compared using the χ2 test or Fisher's exact test. The Pearson correlation coefficient was used to evaluate the correlation of expression. The results were expressed as mean ± standard deviation from at least three independent experiments. P values < 0.05 were considered significant (*$P < 0.05$, **$P < 0.01$).

Results

E2F3 Was Upregulated in NPC Cell Lines and Tissues

The relative E2F3 expression in NPC cell lines and tissues was detected to explore the correlation between NPC tumorigenesis and E2F3 expression. Data from GEO database confirmed that E2F3 showed higher expression in the NPC tissues than in normal nasopharyngeal samples (GSE12452, GSE53819; Figure 1A and B). Moreover, our results showed that E2F3 was significantly overexpressed in NPC tissues (n = 66) than in normal nasopharyngeal epithelial tissues (n = 8, Figure 1C). The high expression of E2F3 was also positively correlated with lymph node metastasis in patients with NPC (Figure 1D–F). Further analysis indicated that E2F3 overexpression was positively associated with lymph node metastasis but was not significantly associated with gender, age, TNM stage, and tumor size (Table 1). E2F3 expression in the NPC cell lines was also upregulated relative to that in NP69, the immortalized nasopharyngeal epithelial cell line (Figure 2A). These results indicated that E2F3 may be positively related with NPC tumorigenesis.

E2F3 Promoted NPC Cell Invasion and Migration in vitro

As shown in Figure 2A, CNE-2 and 5-8F were chosen as two representative NPC cell lines to determine the effect of E2F3 in NPC tumorigenesis. Both cells were treated with si-E2F3 or si-NC. E2F3 knockdown in the cells was detected through quantitative real-time PCR (qRT-PCR) and Western blot analysis (Figure 2B). As shown in Figure 2C and D, the proliferation and colony formation ability of the two NPC cell lines with E2F3 silencing was nonsignificantly changed compared with those of the control groups. However, wound-healing assay and Transwell invasion assay showed that E2F3 knockdown suppressed NPC cell invasion and migration in vitro (Figure 2E and F). These findings suggested that E2F3 played pro-oncogenic roles in NPC cell lines.

MiR-432 Suppressed E2F3 Expression by Directly Binding to the 3′-UTR

miR-432 was identified as a potential target for E2F3 by utilizing two publically available database software (TargetScan and miRanda) to explore the possible molecular mechanism through which E2F3 exerts its biological function. qRT-PCR was conducted to detect the expression of miR-432 in NPC cells (Figure 3A). The upregulation of miR-432 inhibited E2F3 expression on the protein and mRNA levels (Figure 3B and D), while

Table 1 Correlation Between E2F3 Expression and Clinical Characteristics of NPC Patients

Characteristics	No of Patients (n=66)	E2F3 High Group	E2F3 Low Group	P-value
Gender				
Male	47	24	23	0.786
Female	19	9	10	
Age				
<50	34	19	15	0.325
≤50	32	14	18	
TNM stage				
I – II	6	5	1	0.197
III–IV	60	28	32	
T classification				
T1–T2	21	12	9	0.428
T3–T4	45	21	24	
N classification				
N0–N1	24	8	16	0.041
N2–N3	42	25	17	

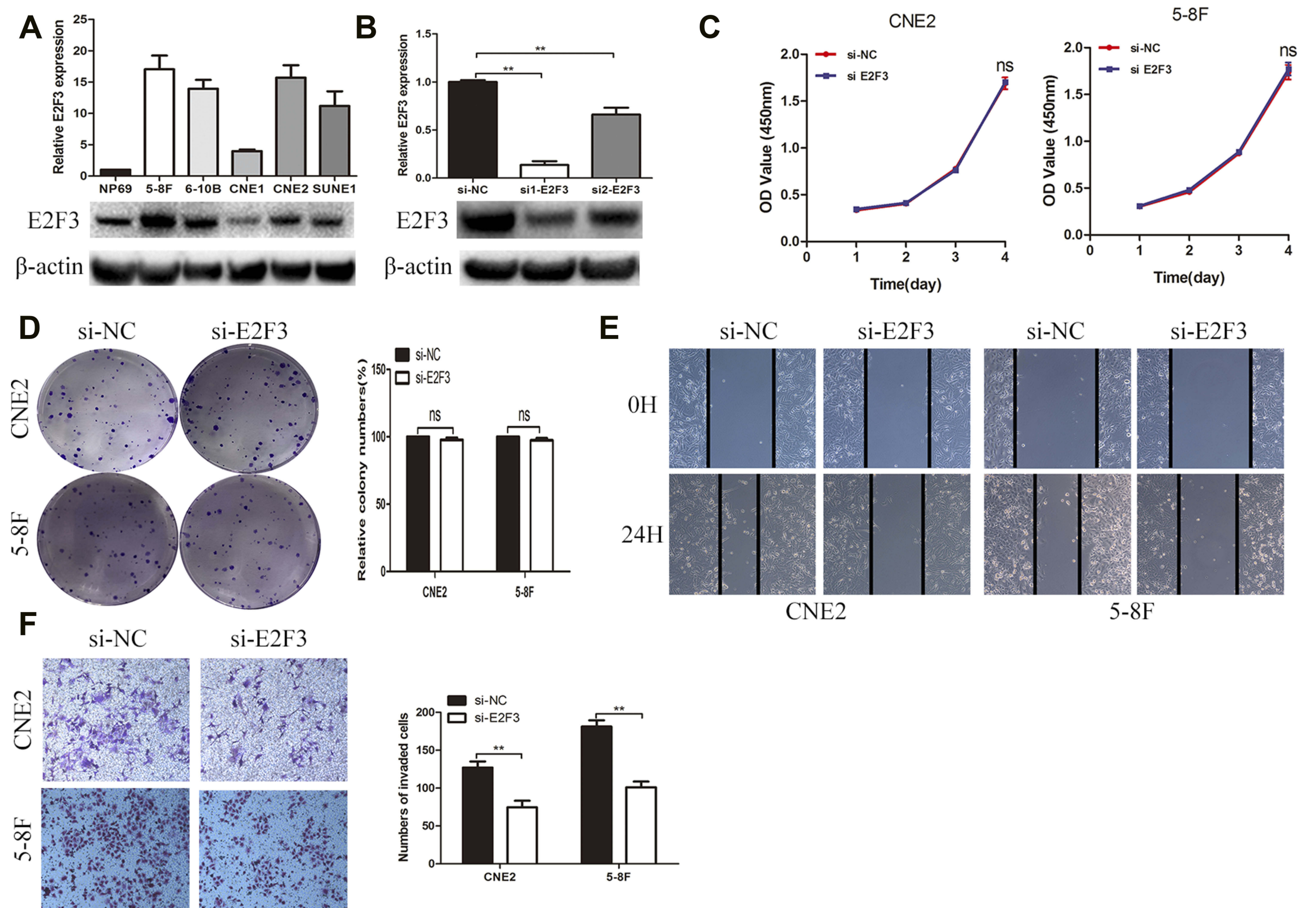

Figure 2 E2F3 promoted NPC cell invasion and migration in vitro. (**A**) Relative E2F3 expression in NP69 and five NPC cell lines analyzed by qRT-PCR. (**B**) qRT-PCR and Western blot analysis were performed to detect the efficiency of si-E2F3. (**C** and **D**) CCK8 and colony-forming assays were used to detect cell proliferation. (**E**) Migration of NPC cells was measured by wound-healing assay. (**F**) Transwell invasion assay was performed to investigate the invasive capacities of NPC cells. Data are presented as mean ± SD.**P < 0.01 compared with the control; ns, not significant.

downregulation of miR-432 increased E2F3 expression (Figure 3C and E). Luciferase reporter vectors containing the Wt or Mut E2F3 3′-UTR sequences were also constructed. As shown in Figure 3F and G, cotransfection with the miR-432 mimic significantly decreased the luciferase activity of the cells transfected with Wt E2F3 3′-UTR. By contrast, inhibition was negative in the cells cotransfected with the Mt E2F3 3′-UTR. These findings revealed that E2F3 was a direct target of miR-432 in the NPC cells.

MiR-432 Inhibited NPC Cell Invasion and Migration in vitro and vivo

The effects of miR-432 on cell migration and invasion were evaluated through the wound-healing assay and the Transwell migration and invasion assay. The exotic expression of miR-432 prominently suppressed NPC cell invasion and migration (Figure 4A and B). To further investigate our data in vivo, we established a model of spontaneous lymph node metastasis. The 5-8F cells transfected with the miR-432 overexpression vector (miR-432 Agomir) or control vector (NC Agomir) were injected into the footpad of each mouse twice a week. After 6 weeks of tumor formation, the mice were sacrificed, and popliteal lymph node metastasis was observed after necropsy. Microscopic observation showed that metastatic popliteal lymph nodes were significantly fewer in the miR-432 Agomir group than in the control group (Figure 4C). These data suggested that miR-432 suppressed tumor metastasis in NPC in vitro and vivo.

MiR-432 Repressed the Invasion and Migration of NPC Cells by Modulating E2F3

CNE2 and 5-8F cells were cotransfected with either the miR-432 inhibitor or the negative control (NC inhibitor) and either

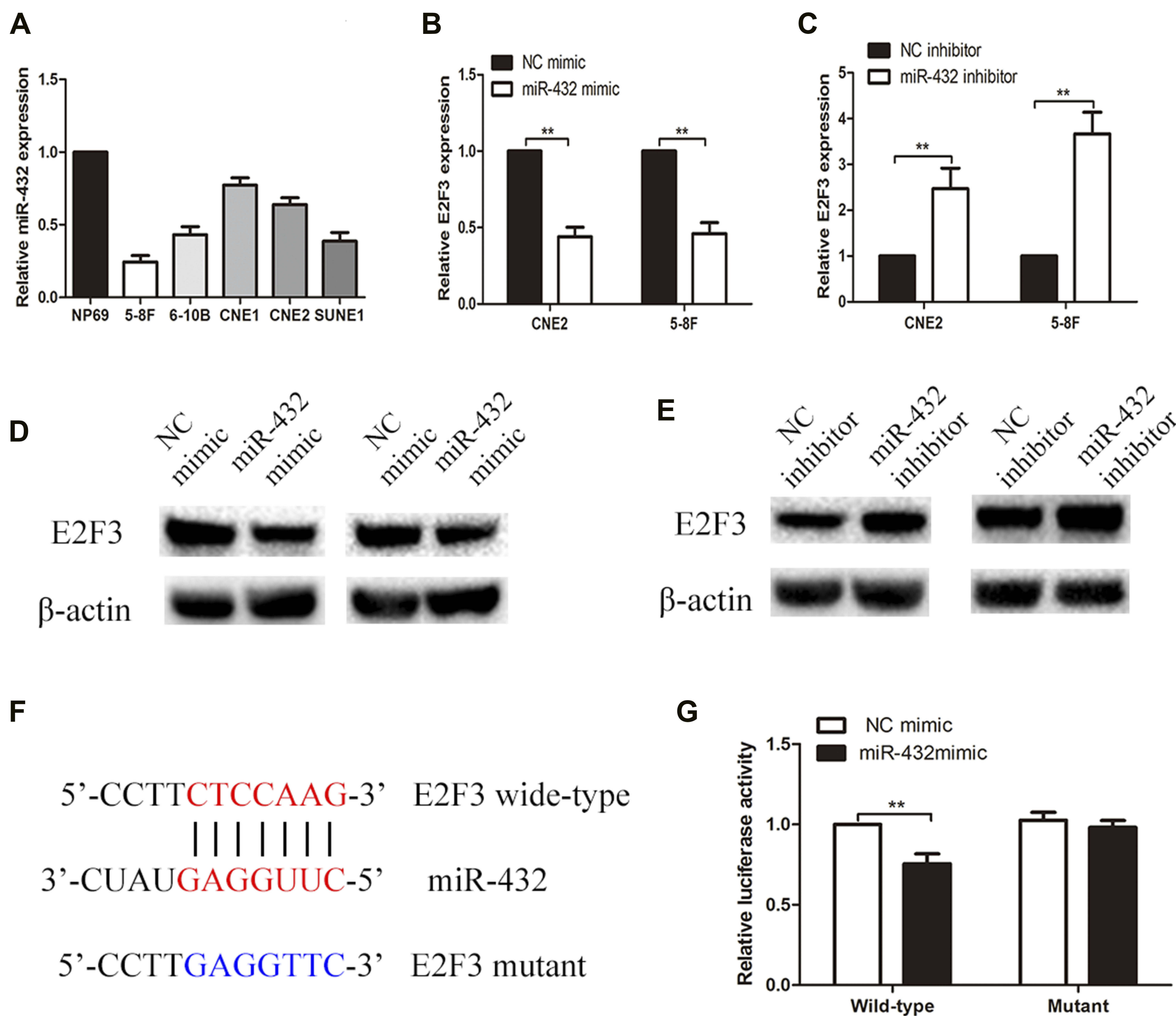

Figure 3 E2F3 acted as a target for miR-432. (**A**) Expression of miR-432 in NPC cells transfected. (**B** and **C**) E2F3 expression in NPC cells transfected with miR-432 mimic or inhibitor was detected through qRT-PCR. (**D** and **E**) E2F3 expression in transfected NPC cells was determined using Western blot analysis. (**F**) miR-432 directly bound to the 3′-UTR of E2F3. (**G**) Luciferase reporter assays showed that miR-432 significantly reduced the luciferase activity of the WT E2F3. ***P*< 0.01 compared with the control

si-E2F3 or si-NC to further determine whether miR-432 could regulate the biological function of NPC cells by modulating E2F3. The downregulation of miR-432 abrogated the suppressive effects on the migration and invasion of NPC cells caused by E2F3 knockdown (Figure 5A and B). These findings confirmed that miR-432 mediated NPC tumorigenesis by inhibiting E2F3.

Discussion

MiRNAs are important regulators in NPC. MiR-203 is a vital determinant of NPC radioresponse, and its degression enhances the radioresistance of NPC cells by targeting IL-8/AKT signaling.[24] MiRNA-34a inhibits the TGF-β-induced invasion, migration, and EMT of NPC cells by directly targeting SMAD4.[25] MiR-10b promotes the migration and invasion of NPC cells, whereas miR-141, miR-145, and miRNA-148a exert tumor suppressive effects in NPC.[26–29] Moreover, miR-432 is dysregulated in the bladder, liver, cervical, and prostate cancers.[30–33] In the present work, miR-432 was found to restrain NPC cell invasion and migration in vitro and in vivo.

E2F3 is located in chromosome 6, NC_000006.12 and has two distinct isoforms, namely, E2F3a and E2F3b.[34] The E2F family acts as a crucial regulator in the control of cell proliferation, apoptosis, and differentiation. E2F3 is

Figure 4 MiR-432 repressed the invasion and migration of NPC cells in vitro and in vivo. (**A** and **B**) Wound-healing assay and Transwell invasion assay were performed to evaluate the invasion and migration abilities of NPC cells. (**C**) 5-8F cells transfected with miR-432 Agomir or NC Argomir were subcutaneously injected into the plantar of each mouse (n = 5), and metastatic popliteal lymph nodes were assessed under a fluoroscope. Data are presented as mean ± SD; $^{**}P < 0.01$ compared with the control.

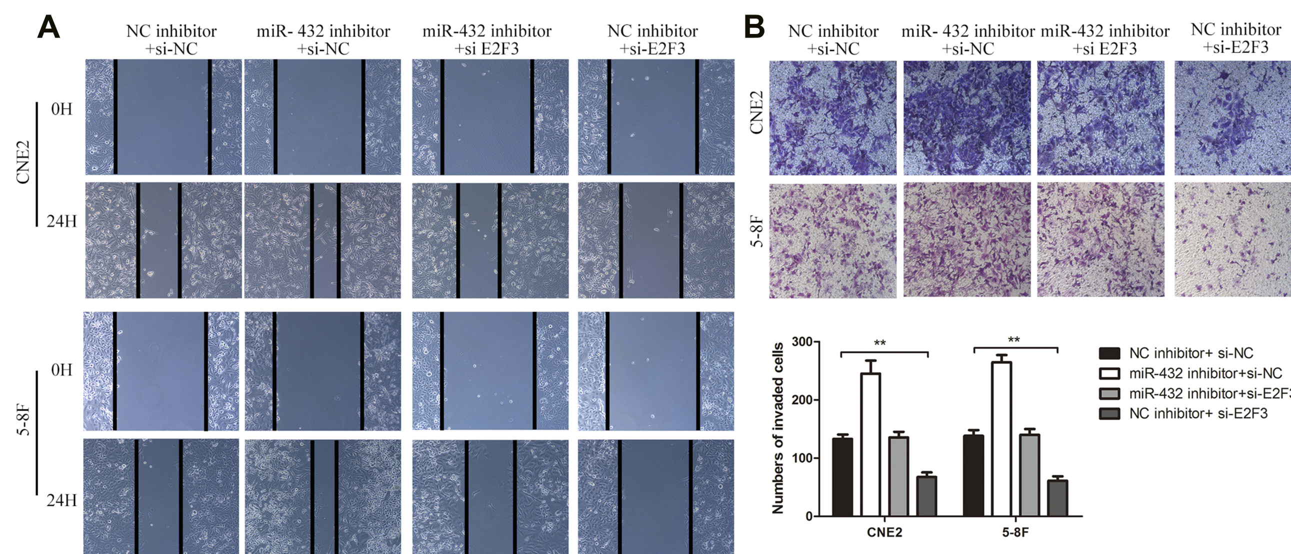

Figure 5 MiR-432 repressed the invasion and migration of NPC cells by repressing the expression of E2F3. (**A**) Wound-healing assay was conducted to analyze the effect of E2F3 and miR-432 on cell migration. (**B**) Transwell invasion assay was performed to measure the effect of E2F3 and miR-432 on cell invasion. ***P* < 0.01 compared with the control.

oncogenic in tumorigenesis. Alterations in the functions and transcriptional activity of E2F3 have been reported in various human malignancies. Trikha et al[35] revealed that E2F3 is a key transcription factor in tumor-associated macrophages and influences the tumor microenvironment and tumor cell metastasis. Oeggerli et al[36] also found that E2F3 is frequently amplified in invasively growing bladder cancer. E2F3 expression appears to activate cell proliferation in a subset of bladder tumors. However, information on the role of E2F3 in NPC progression is lacking. The present results showed that E2F3 was upregulated in NPC tumor tissues and cell lines, and this phenomenon was verified by analyzing GEO datasets. The exotic expression of E2F3 promoted NPC cell invasion and migration in vitro.

A single miRNA can regulate several target mRNAs, whereas multiple miRNAs target individual mRNAs. Regulatory feedback loops, such as ZEB/miR-200, Notch/miR-326, and p53/miR-200, are cases in point.[37–39] E2F1, a member of the E2F transcription factor family, binds to the miR-223 promoter in AML blast cells and represses miR-223 transcription.[40] Thus, we hypothesized that E2F3 may work similarly. Further work identified miR-432 as a potential target of E2F3 by using two publically available database software (TargetScan and miRanda). We performed the luciferase reporter assay to identify the direct relationship between E2F3 and miR-432. The upregulation of miR-432 inhibited E2F3 expression on the protein and mRNA levels. Meanwhile, the effects of E2F3 on NPC tumorigenesis were dependent on miR-432 because the downregulation of miR-432 abrogated the suppressive effects on the migration and invasion of NPC cells caused by E2F3 knockdown. Further exploration is needed to determine the alterations of certain signal molecules and signal pathways. Therefore, miR-432 suppressed E2F3 expression by directly binding to the 3′-UTR of E2F3, and the specific combination could alleviate the cancer-promoting roles of E2F3 in NPC progression.

The relationship between miR-432 and E2F3 has been reported in lung adenocarcinoma and breast cancer.[41,42] However, their functions in tumor metastasis in NPC have rarely been reported. In this study, we first identified that E2F3 was upregulated in NPC tissues and cells. This finding was consistent with previous results. However, we found that the biological functions of E2F3 were different from those previously reported. Our experiments showed that E2F3 knockdown significantly reduced cell invasion and migration but did not exert obvious effects on cell proliferation. These results implied that E2F3 might perform different functions in different cancers. Second, we are the first to prove that E2F3 is the target of miR-432 in NPC cells. Further data revealed that miR-432 repressed the invasion and migration potential of NPC cells by modulating E2F3.

Conclusion

E2F3 was frequently upregulated and functioned as a tumor promoter in NPC. MiR-432 could repress the invasion and migration of NPC cells by directly binding

to the 3′-UTR of E2F3. The present exploration of the regulatory relationship between E2F3 and miR-432 may provide new insights in NPC treatment and prognosis.

Disclosure

The authors report no conflicts of interest in this work.

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