

ORIGINAL ARTICLE

Downregulation of miR-204 facilitates the progression of nasopharyngeal carcinoma by targeting CXCR4 through NF- κ B signaling pathway

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Summary

Purpose: Nasopharyngeal carcinoma (NPC) is a highly invasive and metastatic cancer. The alterations of miRNA downregulation and pathway have been reported to be implicated in NPC progression. Here, we aimed to explore miR-204 role and mechanism in NPC development.

Methods: We examined the expression level of miR-204 in NPC tissues and NPC cells (HONE-1, 6-10B, HNE1) using reverse-transcription quantitative PCR (RT-qPCR) analysis. MTT, and transwell assays were used to analyze the effects of miR-204 on the proliferation, invasion and migration of NPC cells. Luciferase reporter gene assays were used to confirm the target gene of miR-204 in NPC cells.

Results: The results showed that miR-204 was downregulated, while CXCR4 was upregulated in NPC samples and cells with important functional consequences. Also, miR-204 expression was inversely correlated to CXCR4 expression and it was also associated with the clinicopathologic

features. Ectopic expression of miR-204 was significantly suppressed, whereas downregulation of miR-204 facilitated the capacities of NPC cells proliferation, invasion and migration. Besides, it was also found that miR-204 mimic strongly decreased CXCR4 expression and miR-204 inhibitor increased CXCR4 expression. Furthermore, luciferase assay results demonstrated that CXCR4 was the direct target of miR-204. Conversely to miR-204 effect, knockdown of CXCR4 showed an inhibitory effect on NPC cell progression. Mechanistic investigations revealed that miR-204 regulated NF- κ B signaling via CXCR4.

Conclusion: Taken together, our findings suggested that miR-204 regulated NPC progression by targeting CXCR4 through NF- κ B signaling pathway.

Key words: CXCR4, miR-204, nasopharyngeal carcinoma, NF- κ B signaling pathway

Introduction

NPC is a malignant tumor that occurs on the top and side walls of the nasopharyngeal cavity. It is one of the high-grade malignant tumors in China, and its incidence ranks first among malignant tumors of the ear, nose and throat [1,2]. In the process of tumorigenesis and development, multiple genetic and epigenetic abnormalities synergistically disrupt the function of normal cells, leading to the pathogenesis of NPC [3,4]. Therefore, to further identify new targets may help elucidate

the specific molecular mechanisms involved in the development and progression of NPC.

MiRs serve transcriptional functions by binding mRNA and contribute to the progress of various tumors, including invasion, migration, proliferation and apoptosis [5]. Although many miRs have long been discovered, the exact role of miRs in tumorigenesis and their mechanisms remain unclear. Currently, some miRs have been reported to regulate NPC progression and development, including

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miR-506, 29c, 193a and miR-122 [6-9]. They form a complex network with their target genes which play vital roles in the pathogenesis of NPC.

MiR-204 is dysregulated in tumors' progression and development, and its role in cancers seems controversial. For instance, it serves as a tumor suppressor in liver cancer [10], while inhibition of miR-204 exerts protection against ischemia-reperfusion injury [11]. Also, miR-204 was down-regulated in various cancers, such as breast cancer [12-14], ameloblastoma [15], melanoma [16], and gastric cancer [17]. Moreover, previous studies showed that miR-204 was decreased in NPC [18,19]. However, miR-204 function and its mechanism in NPC progression have not been reported.

CXCR4 is expressed abnormally in many cancers and has been involved in tumorigenesis [20]. Also, NF- κ B pathway modulated the tumorigenesis of many cancers via regulating CXCR4 [21]. However, the potential molecular mechanism regulating the development of NPC by regulating CXCR4 is not well understood. CXCR4 was proved to be the target of various RNAs in regulating of many kinds of cancers. MiR-133a regulated colorectal cancer progression via targeting CXCR4 [22]. However, whether CXCR4 is the target of miR-204 in NPC has been fully unknown.

In this study, we stated that miR-204 exhibited a suppressive effect on NPC cell viability, invasion and migration. However, CXCR4 showed the opposite effect on NPC progression and acted as the target of miR-204. Taken together, the inhibitory effect of miR-204 was achieved by suppressing CXCR4 and NF- κ B signaling pathway, which provide valuable clues for understanding the pathogenesis of NPC.

Methods

Histology

Sixty fresh NPC tissues and the equal number of normal tissues that were pathologically reassessed were

resected from the NPC patients. None of the patients had received chemotherapy or radiotherapy. The Ethics Committee of Shandong Provincial Hospital affiliated to Shandong University has approved this study. Written informed consents were obtained from all individuals participating in the study.

Cell culture

The normal nasopharyngeal epithelial cell line NP69 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in CM2-1 medium. The three NPC cell lines (HONE-1, 6-10B, HNE1) were purchased from ATCC and maintained in RPMI-1640 medium. The mediums were added with 10% fetal bovine serum (FBS) and an antibiotic cocktail. All the cells were maintained at 37°C and 5% CO₂ conditions.

Cell transfection

MiR-204 mimic or inhibitor were used for increasing or decreasing miR-204 expression. CXCR4 siRNA was used for silencing CXCR4. All these were provided by Gene Pharma (Shanghai, China). Lipofectamine 3000 reagent (Invitrogen, USA) was used for the transfection for 48h.

QRT-PCR assay

TRIzol reagent (Invitrogen) was applied for extracting total RNA. Taqman miR assay was applied for determining mature miR expression. U6 served as an internal control. SYBR Green PCR master mix was applied for evaluating miR-204 and CXCR4 mRNA level and their expression was quantified by 2^{- $\Delta\Delta$ CT} method. The primers are shown in Table 1.

Cell proliferation

Cell proliferation was assessed by cell viability using MTT assay. HONE-1 cells were transfected with miR-204 mimic or inhibitor for 48h. Then the cells were placed in 48-well plates with fresh RPMI 1640 medium and cultured for 24, 48, 72 and 96h. Then 10% MTT solution (5g/L) was added and incubated for another 4h. After DMSO replaced the MTT solution, a microplate reader was used for measuring the absorbance at 490nm.

Migration and invasion assays

Transwell assay was used to detect cell migration and invasion. For migration, the upper chamber was covered with non-coated membrane, while it was covered with Matrigel-coated membrane for invasion assay. For both assays, HONE-1 cells were plated in the upper chamber and the medium containing serum was added in the lower chamber as a chemoattractant. After the cells were incubated for 24h, the non-migrated or invaded cells were removed by cotton swab and the cells in the lower chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution (Beyotime).

Proteins analysis

RIPA lysis buffer containing proteinase inhibitors (Beyotime, China) was used for extracting total protein

Table 1. Primer sequences for RT-qPCR

Genes	Sequence
miR-204	F: 5'-ACACTCCAGCTGGGTTCCTTTGTCATCC-3' R: 5'-CTCAACTGGTGTCTGGA-3'
U6	5'-CTCGCTTCGGCAGCACA-3' R: 5'-AACGCTTCACGAATTTGCGT-3'
CXCR4	F: 5'-GCAGCAGGTAGCAAAGTGAC-3' R: 5'-GAAGTGTATATACTGATCCCTCCA-3'
GAPDH	F: 5'-TCGGAGTCAACGGATTTGGT-3' R: 5'-TTCCCGTTCTCAGCCTTGAC-3'

from NPC tissues and cells. BCA protein kit (Beyotime, China) was applied to test the protein concentrations. The proteins were separated by SDS-PAGE and transferred to negative control (NC) membranes. Then the membranes were blocked by 5-10% skim milk for 2h at room temperature. Subsequently, primary antibodies were added and incubated overnight at 4°C and secondary antibodies were incubated for another 2h at room temperature. Finally, chemiluminescence kit (ECL, Millipore) was performed to detect the protein bands. GAPDH served as internal control.

Dual-luciferase reporter assay

The amplified fragments of wild type or mutant type CXCR4 were cloned into the psiCHECK™-2 vector. Thereafter, the CXCR4 reporter and miR-204 mimic were co-transfected into the HONE-1 cells. After transfection for 48h, the cells were collected and the luciferase activities were assessed.

Statistics

Statistical analyses were conducted by SPSS 19.0 software. The experiments were performed in triplicate and the data were presented as mean ± standard deviation (SD). Student's *t*-test or Tukey's *post hoc* test were used for the comparison of differences between two groups or multiple independent groups. Spearman's correlation test was applied for exploring the relationships between miR-204 and CXCR4. $P < 0.05$ was considered as statistically significant.

Results

MiR-204 was downregulated in NPC

To understand the function of miR-204 in NPC, we first detected the expressional level of miR-204 in NPC tissues from 60 NPC patients. The results showed that miR-204 expression was reduced in NPC tissue samples compared with normal control tissues (Figure 1A). Then we tested its expression in three NPC cell lines. As shown in Figure

1B, miR-204 expression was decreased in all NPC cell lines compared with normal cell line NP69. Because miR-204 was moderately expressed in HONE-1 cells, we selected HONE-1 cells to increase or decrease miR-204 for further analysis.

Downregulation of miR-204 facilitated NPC cell progression

To further explore the effect of miR-204 on the development of NPC, we increased or decreased miR-204 expression in HONE-1 cells. As shown in Figure 2A, miR-204 expression was raised after overexpression in HONE-1 cells, and reduced after silencing miR-204. Thereafter, we investigated cell viability by MTT assay and the results showed that miR-204 mimic inhibited cell viability, and miR-204 inhibitor promoted cell viability (Figure 2B). Subsequently, we carried out transwell assay to measure the capacities of cell migration and invasion. The findings displayed that the migratory and invasive ability of HONE-1 cells were inhibited by miR-204 mimic, while they enhanced by miR-204 inhibitor (Figure 2C-2D). Collectively, downregulation of miR-204 facilitated NPC cell progression and development.

Knockdown of CXCR4 suppressed NPC cell progression

To investigate the mechanism of miR-204, we paid attention to CXCR4. Firstly, we tested CXCR4 expression in NPC tissues and cells. As displayed in Figure 3A, CXCR4 was increased in NPC tissues. Similarly, its expression was high in NPC cells (Figure 3B), which was opposite to miR-204 expression. To further explore the effect of CXCR4 on the development of NPC, we decreased CXCR4 expression in HONE-1 cells. As shown in Figure 3C, CXCR4 expression was decreased after knockdown CXCR4 both in mRNA and protein level. Thereafter, we investigated cell viability by MTT assay and the results showed that CXCR4 siRNA inhibited cell

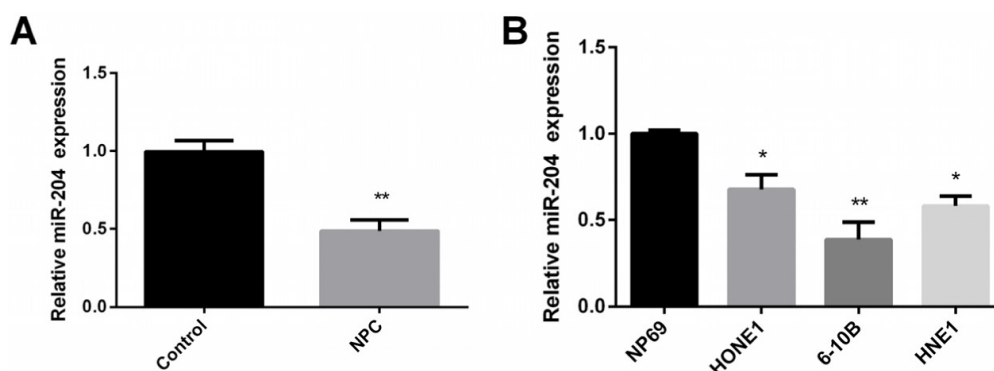


Figure 1. MiR-204 expression in NPC tissues and cells. A: MiR-204 expression in NPC tissues and normal control tissues by qRT-PCR (n=60). miR-204 expression was reduced in NPC tissue samples compared with normal control tissues. B: MiR-204 expression in three NPC cell lines and normal cells by qRT-PCR. miR-204 expression was decreased in all NPC cell lines compared with normal cell line NP69. * $p < 0.05$, ** $p < 0.01$.

viability (Figure 3D). Subsequently, we carried out transwell assay to measure the capacities of cell migration and invasion. The findings displayed that the migratory and invasive ability of HONE-1 cells were inhibited by CXCR4 siRNA (Figure 3E,3F). Collectively, knockdown of CXCR4 suppressed NPC cell progression and development.

CXCR4 was the target of miR-204 in NPC cells

Because CXCR4 and miR-204 have opposite effect on the progression of NPC, we explored

the relationship between miR-204 and CXCR4. As shown in Figure 4A, CXCR4 was a predicted target of miR-204. Then, we used dual-luciferase reporter assay to further confirm whether CXCR4 was the direct target of miR-204. The results displayed that miR-204 mimic reduced the luciferase activity of CXCR4 3'UTR-WT, while the luciferase activity of CXCR4 3'UTR-MuT didn't change by miR-204 mimic (Figure 4B). These results demonstrated that CXCR4 was the direct target of miR-204. Besides, we detected the effect of miR-204 on CXCR4 ex-

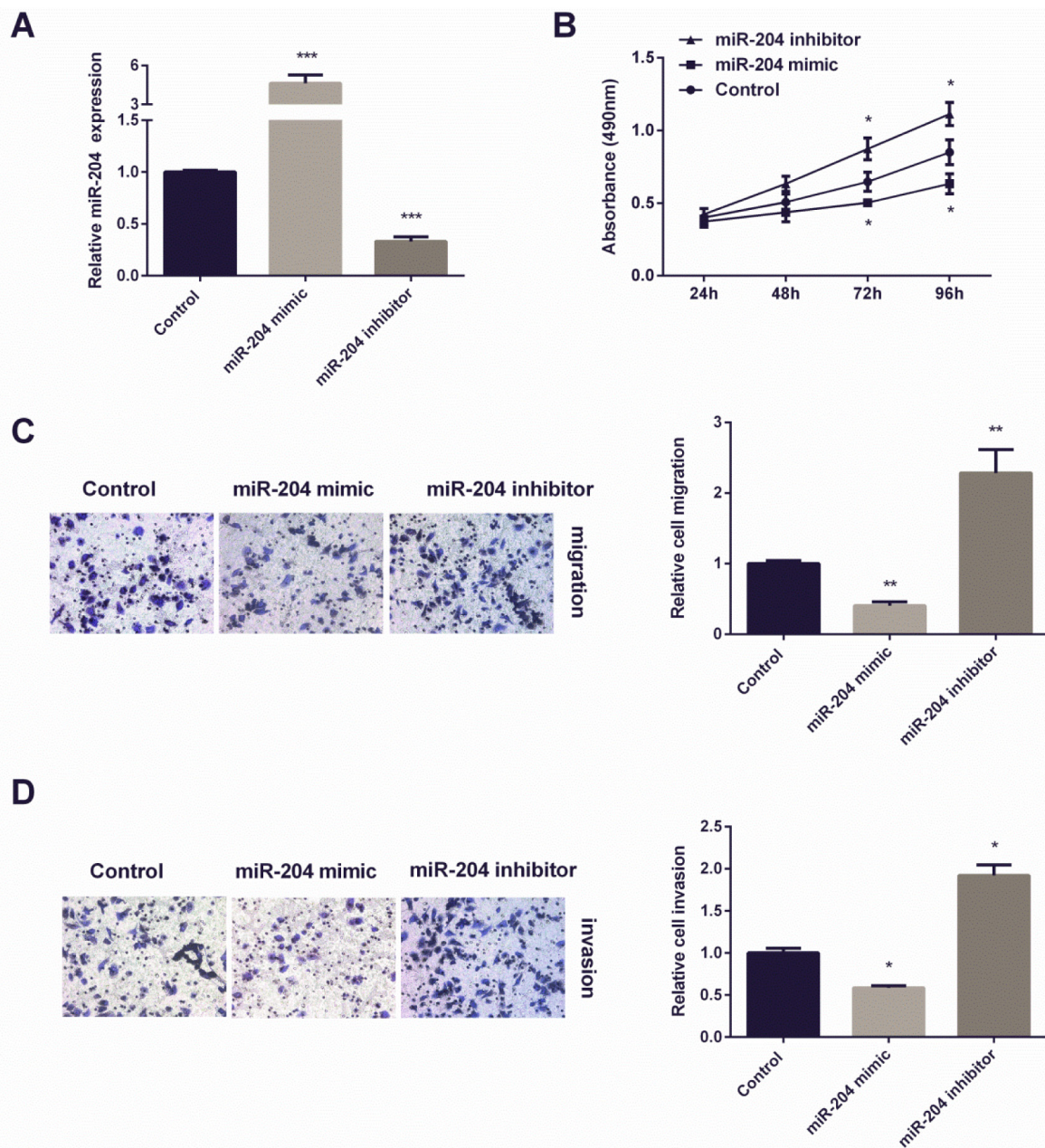


Figure 2. Effects of miR-204 downregulation on NPC cell proliferation, invasion and migration. **A:** MiR-204 mimic or inhibitor were transfected into HONE-1 cells, and detected miR-204 expression by qRT-PCR. **B:** Detection of cell viability by MTT assay. miR-204 mimic inhibited cell viability, and miR-204 inhibitor promoted cell viability. **C:** Detection of cell migration by transwell assay showing that miR-204 mimic inhibited cell migration and miR-204 inhibitor promoted cell migration. **D:** Detection of cell invasion by transwell assay showing that miR-204 mimic inhibited cell invasion and miR-204 inhibitor promoted cell invasion. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

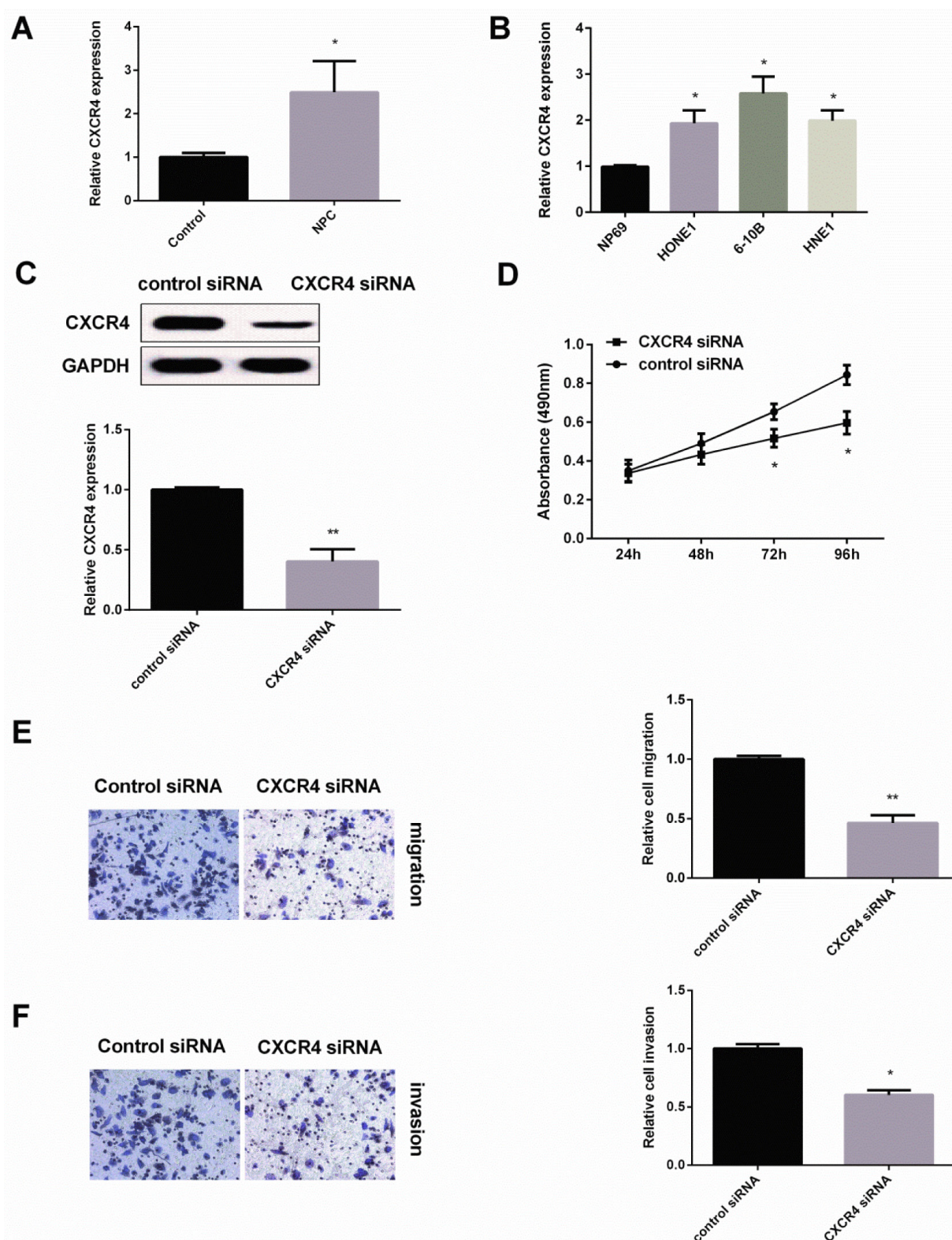


Figure 3. Effects of CXCR4 siRNA on NPC cell proliferation, invasion and migration. **A:** CXCR4 expression detected in NPC tissues and normal control tissues by qRT-PCR (n=60). CXCR4 was increased in NPC tissues compared to normal control tissues. **B:** CXCR4 expression measured in three NPC cell lines and normal cells by qRT-PCR: the results showed that the expression of CXCR4 was higher in NPC cells compared to normal cells. **C:** HONE-1 cells were treated with CXCR4 siRNA, and CXCR4 protein level and mRNA expression were measured by Western blot and qRT-PCR. CXCR4 expression was decreased after knockdown CXCR4 both in mRNA and protein level. **D:** HONE-1 cells were treated with CXCR4 siRNA, and cell viability was detected by MTT assay. CXCR4 siRNA inhibited cell viability. **E:** HONE-1 cells were treated with CXCR4 siRNA, and cell migration was detected by transwell assay. The migratory ability of HONE-1 cells was inhibited by CXCR4 siRNA. **F:** HONE-1 cells were treated with CXCR4 siRNA, and cell invasion was detected by transwell assay. The invasive ability of HONE-1 cells was inhibited by CXCR4 siRNA. *p<0.05, **p<0.01.

pression. The findings showed that overexpression of miR-204 inhibited CXCR4 expression, while silencing miR-204 increased CXCR4 expression both in protein level and mRNA level (Figure 4C,D). Moreover, miR-204 and CXCR4 expression were inversely correlated (Figure 4E). MiR-204 might regulate NPC progression via repressing CXCR4.

Downregulation of miR-204 inhibited EMT and NF- κ B signaling pathway

Epithelial-mesenchymal transition (EMT) is well known to take part in the metastatic process of cancers. Here, we investigated the role of miR-204 in EMT. The results showed that miR-204 upregulation facilitated E-cadherin expression

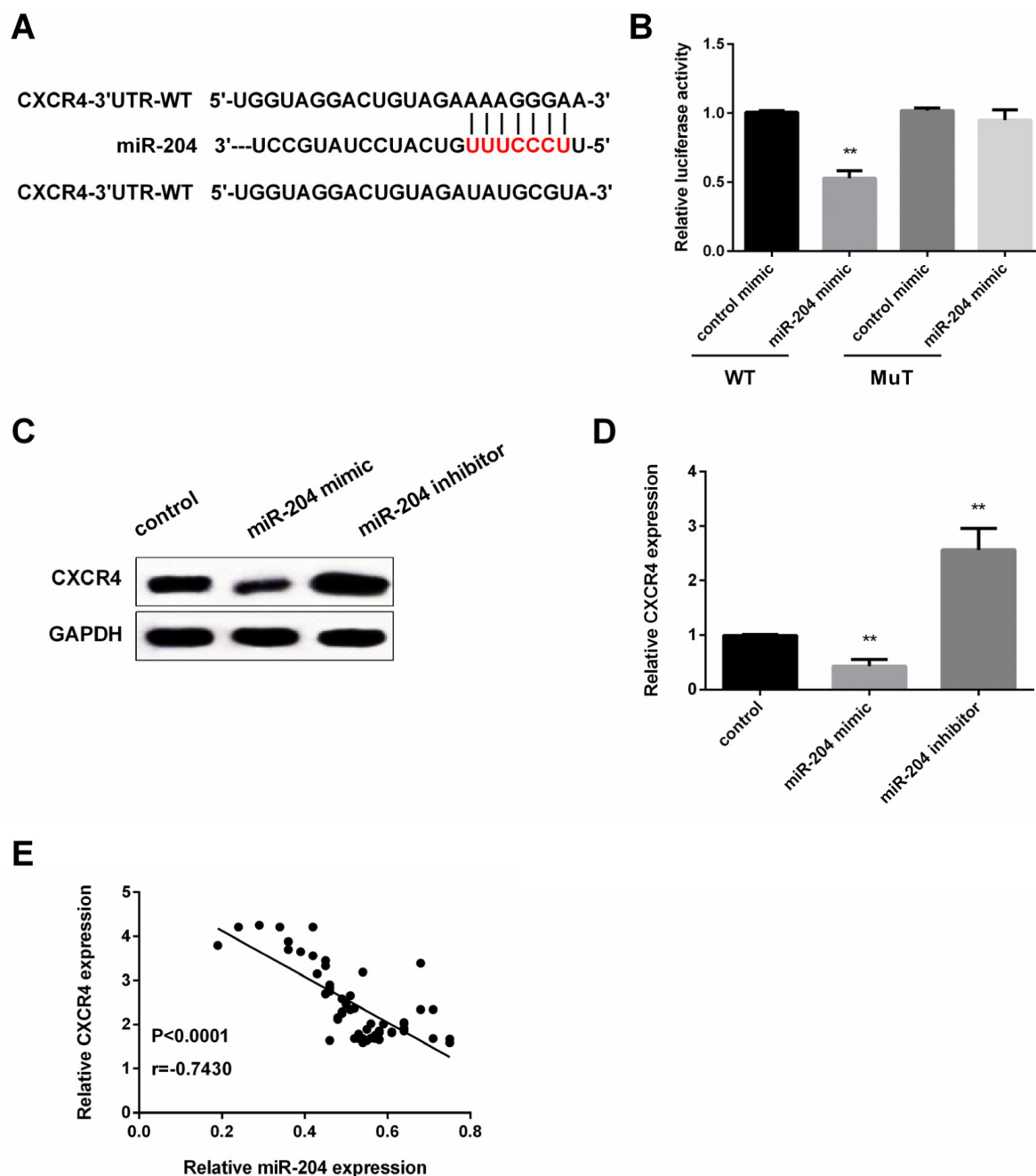


Figure 4. CXCR4, the target of miR-204 in NPC. **A:** The binding sites of miR-204 and CXCR4. **B:** HONE-1 cells were treated with miR-204 mimic and CXCR4 3'-UTR reporter, and the luciferase activity was detected by luciferase assay. The results displayed that miR-204 mimic reduced the luciferase activity of CXCR4 3'UTR-WT, while the luciferase activity of CXCR4 3'UTR-MuT didn't change by miR-204 mimic. **C-D:** HONE-1 cells were treated with miR-204 mimic or inhibitor, and CXCR4 expression was measured by Western blot and qRT-PCR. The findings showed that overexpression of miR-204 inhibited CXCR4 expression, while silencing miR-204 increased CXCR4 expression both in protein level and mRNA level. **E:** Inverse correlation of miR-204 and CXCR4. The results showed that miR-204 and CXCR4 expression were inversely correlated. ** $p < 0.01$

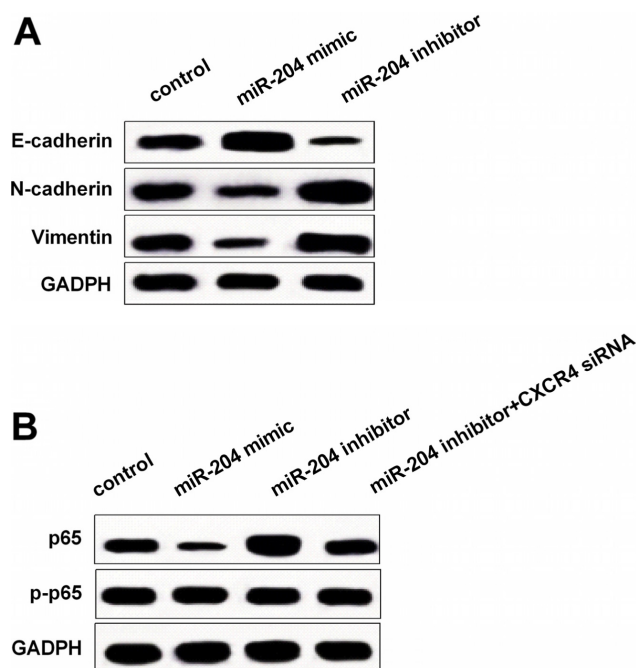


Figure 5. Activation of NF- κ B signaling pathway by downregulation of miR-204. **A:** HONE-1 cells were treated with miR-204 mimic or inhibitor, and E-cadherin N-cadherin, Vimentin expression was tested by Western blot. The results showed that miR-204 upregulation facilitated E-cadherin expression and suppressed N-cadherin and Vimentin expression. However, miR-204 downregulation has the opposite effect. **B:** HONE-1 cells were treated with miR-204 mimic, inhibitor, or combined with CXCR4 siRNA and p65 and p-p65 expression was tested by Western blot. The results showed that miR-204 upregulation inhibited the phosphorylation of p65 and I κ B α , which were the downstream genes of NF- κ B signaling pathway. Moreover, miR-204 downregulation facilitated the activation of NF- κ B signaling pathway.

and suppressed N-cadherin, Vimentin expression. However, miR-204 downregulation decreased E-cadherin expression and increased N-cadherin, Vimentin expression (Figure 5A). Then, we explored how miR-204 affected NF- κ B signaling pathway by Western blot. As Figure 5B displays, miR-204 upregulation inhibited the phosphorylation of p65 and I κ B α , which were the downstream genes of NF- κ B signaling pathway. Moreover, miR-204 downregulation facilitated the activation of NF- κ B signaling pathway. Furthermore, CXCR siRNA overturned the promotion effect of miR-204 inhibitor. Taken together, downregulation of miR-204 enhanced the activation of NF- κ B signaling pathway in NPC cells by inhibiting CXCR4.

Discussion

MiR-204 has been proved to take part in various cancers. However, its role in NPC has not been reported. In the present study, we showed that miR-

204 was decreased in NPC tissues and cells while miR-204 downregulation was associated with poor prognosis of NPC patients. These results suggested that miR-204 might represent a potential target for NPC therapy. Therefore, further investigation of the prognostic value of miR-204 is warranted.

MiR-204 dysregulation modulated the progression and development of multiple tumors. For instance, miR-204 was downregulated in breast cancer and limited cell growth and metastasis [13]. Also, downregulation of miR-204 promoted pancreatic cancer cell proliferation and invasion [23]. Also, miR-204 facilitated osteosarcoma cell apoptosis and suppressed osteosarcoma cell invasion and migration [24]. Similar to the above results, we found that miR-204 was downregulated in NPC tissues and cells. Moreover, downregulation of miR-204 promoted NPC cell viability, invasion and migration.

A mounting number of studies has shown that NF- κ B signaling pathway plays an important role in the development of various human cancers. Here, we demonstrated that overexpression of miR-204 inhibited the downstream genes of NF- κ B signaling pathway, suggesting that miR-204 regulated NPC progression via NF- κ B signaling pathway.

NPC is a complex disease. Thus, understanding the molecular regulatory mechanism will help explore effective treatments. A previous study showed that miR-506 was decreased in NPC and modulated cell growth and metastasis by targeting LHX2 through Wnt/ β -catenin signaling [6]. Besides, miR-122 inhibited NPC cell proliferation via PI3K/AKT signaling pathway [25]. Furthermore, miR-34a suppressed cell migration and invasion in NPC through TGF- β pathway via targeting SMAD4 [26]. Our study showed that downregulation of miR-204 promoted NPC cell proliferation, invasion and migration by directly targeting CXCR4 through NF- κ B signaling pathway. These results provide an effective and promising therapeutic strategy for NPC with miRs as the regulatory target.

CXCR4 was proved to act as an oncogene during tumor progression [27,28]. CXCR4 was inhibited by miR-193 and contributed to colon cancer cell migration [29]. Hersi and his colleagues reported that miR-9 suppressed head and neck cancer cells growth and invasion by targeting CXCR4 [30]. Here, we state that CXCR4 was the target of miR-204. Downregulation of miR-204 enhanced NPC cell proliferation, invasion and migration by suppressing CXCR4 expression, followed by promoting NF- κ B signaling pathway.

Taken together, we found that miR-204 was decreased in NPC tissues and cells and it was associated with clinicopathological features of NPC

patients. Moreover, downregulation of miR-204 facilitated NPC cell proliferation, invasion and migration, while upregulation of miR-204 contributed to the opposite effect. Furthermore, miR-204 negatively regulated CXCR4 expression. Collectively, we revealed that downregulation of miR-204 enhanced

NPC progression through NF- κ B signaling pathway by suppressing CXCR4.

Conflict of interests

The authors declare no conflict of interests.

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