

MiR-212-3p inhibits cell proliferation and promotes apoptosis by targeting nuclear factor IA in bladder cancer

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Accumulating evidence suggest that microRNAs play crucial roles in the development and progression of bladder cancer (BC). Here, we found that miR-212-3p was significantly down-regulated and negatively correlated with nuclear factor IA (NFIA) in human BC tissues. Bioinformatics analysis predicted that NFIA was a target gene of miR-212-3p. Then BC cell lines, T24 and J82 cells were transfected with miR-212-3p mimics or siNFIA to obtain miR-212-3p overexpression or NFIA knockdown cell lines, respectively. Quantitative real-time PCR was used to determine the expression of miR-212-3p and NFIA. Western blot analysis was utilized to detect NFIA expression. MTT assay showed either miR-212-3p overexpression or NFIA knockdown significantly inhibited the BC cell proliferation. Double staining with Annexin V-APC and 7-AAD showed the total number of apoptotic BC cells were remarkably increased after miR-212-3p overexpression or NFIA knockdown. Collectively, our results indicated that miR-212-3p targeting NFIA might serve as a promising target for BC.

Keywords. Apoptosis; bladder cancer; miR-212-3p; NFIA; proliferation

1. Introduction

Bladder cancer (BC) is the ninth most frequently diagnosed cancer around the world (Al-Ahmadie *et al.* 2016), with higher incidence in Southern and Western Europe, North America, Northern Africa and Western Asia (Antoni *et al.* 2017). Despite a dramatic decline in mortality rates, BC still ranks the eighth in cancer-related death in European Union (Matsushita *et al.* 2015). Until now, patients with BC are usually treated by radical cystectomy, systemic cisplatin-based chemotherapy and immunotherapy. However, the five-year survival rate still remains poor (Kamat *et al.* 2016). Therefore, it is urgently needed to well understand the pathological molecular mechanisms underlying the development and progression of BC.

There is substantial empirical literature showing that dysregulation of microRNAs (miRNAs) links many malignancies leading to oncogenesis and metastasis (Lin and Gregory 2015). As an abundant class of small non-coding RNAs (~19–24 nt long), miRNAs can recognize the special motif of their target mRNAs and modulate gene expression via post-transcriptional, RNA interference and other pathways (Chen *et al.* 2015). Till now, several miRNA family members were found to contribute to BC physiological and pathological processes, such as miR-144-5p (Matsushita *et al.* 2015), miR-23b/27b (Chiyomaru *et al.* 2015), miR-145 (Matsushita *et al.* 2016) and miR-411 (Jin *et al.* 2018). The pre-miR-212 is processed by Dicer, leading to

production of yield sense (miR-212-3p) and antisense strand (miR-212-5p) (Hansen *et al.* 2016). Hyperexpression of miR-212-3p decreases glioblastoma multiforme cells viability via pair to 3'UTRs of SGK3 (Liu *et al.* 2015). It has been reported that emergence of miR-212-3p binding motif mutation in CD80 is associated with the occurrence of gastric cancer in a Chinese population (Wu *et al.* 2014). However, the role of miR-212-3p is as yet undefined in BC.

The nuclear factor I (NFI) is a family of transcription factors that consists of four members, NFIA, NFIB, NFIC and NFIX (Messina *et al.* 2010). This family of site-specific (5'-TTGGC(N)₅GCCAA-3') DNA-binding proteins has been implicated in viral replication and activating or suppressing gene expression (Lee *et al.* 2017). NFIA is located in the 1p31 locus. In recent years, NFIA has emerged as a critical regulator in cancer development (Bernard *et al.* 2009). Zhao *et al.* (2017) showed the miR-191/NFIA signal transduction pathway in non-small cell lung cancer. A previous study identified that long non-coding RNA RP5-833A20.1 inhibition of NFIA as an important intrinsic mechanism suppressing carcinogenesis (Kang *et al.* 2016). In addition, NFIA plays important roles in glial development, including maintaining the glial progenitor cell status, controlling specification of neuronal-glial fate, and governing terminal differentiation (Lee *et al.* 2017). It also promotes glioma cell proliferation, survival and growth via negative modulation of p53, p21 and PAI1 (Lee *et al.* 2014).

In this study, we found that miR-212-3p expression was inversely correlated with NFIA in BC tissues. Bioinformatics analysis revealed that miR-212-3p could potentially target NFIA. Therefore, we decided to focus our study on the crosstalk of miR-212-3p and NFIA in the development and progression of BC. This study may be helpful to deepen our understanding of the molecular basis of BC.

2. Materials and methods

2.1 Tissue samples

A total of 32 pairs of fresh BC tissues and adjacent normal tissues were collected at the Department of Urology, the First Hospital of Jiaxing (Zhejiang, China) and immediately snap-frozen in liquid nitrogen until RNA extraction. All patients signed the informed consent and their clinicopathologic characteristics were summarized in table 1. This study was approved by the Ethics Committee of the First Hospital of Jiaxing (Zhejiang, China).

2.2 Cell lines and culture

Human BC cell lines, T24 and J82 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human embryonic kidney cell line 293T (HEK293T) were purchased from the Cell Bank of Shanghai Institute of Cell Biology (Chinese Academy of Sciences, Shanghai, China). All cell lines were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and maintained in a humidified incubator containing 5% CO₂ at 37°C.

2.3 Quantitative real-time PCR

Total RNA was extracted from tissues or cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's

instructions. For detection of miR-212-3p, cDNA was synthesized using a miRNA Reverse Kit (TaKaRa, Dalian, China) using the SYBR Premix Ex Taq kits (TaKaRa, Dalian, China). For detection of the NFIA mRNA level, cDNA was synthesized using the PrimeScript RT Reagent kit (TaKaRa). Quantitative real-time PCR was performed on ABI Prism 7500 HUT (ABI, MA, USA). The primers used in the experiments were as follows: miR-212-3p forward, 5'-GGTAACAGTCTCCAGTCA-3' and reverse, 5'-GCAATTGCAC TGGATACG-3'; U6 forward, 5'-GCTTCGGCAGCACATAT ACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTG CGTGTCAT-3'; NFIA forward, 5'-TGGCCAAGTTACGG AAAGAT-3' and reverse, 5'-GCGCTCGCCATCAGTACT-3'; GAPDH forward, 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse, 5'-GAAGATGGTGATGGGATTTTC-3'. The relative expression levels of miR-212-3p and NFIA were normalized, respectively, to U6 and GAPDH by using the 2^{-ΔΔCt} method (Livak and Schmittgen 2001).

2.4 Gene transfection

Human miR-212-3p mimic (miR-212-3p) and the corresponding negative control mimic (miR-NC), as well as NFIA siRNA (siNFIA) and its negative control siRNA (siNC), were purchased from RiboBio Co., Ltd. (Guangzhou, China). For gene transfection, T24 and J82 cells were plated into six-well plates and transfected with 100 nM miR-212-3p or siNFIA as well as their corresponding negative control using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.5 Cell proliferation assay

The cell proliferation was evaluated using the MTT assay. Briefly, the transfected cells at a density of 2.5 × 10³ cells/well were seeded into 96-well plates and cultured for consecutive 5 days. Subsequently, cells in each well were incubated with 100 μL MTT (10 mg/mL, Sigma) at 37°C for 2 h. Next, 200 μL of DMSO was added to each well after the cell supernatant was removed. Finally, the optical density (OD) value at a wavelength of 595 nm was measured using a microplate spectrophotometer (BioTek Instruments, Inc. Winooski, VT, USA).

2.6 Cell apoptosis analysis

Flow cytometry was performed to determine cell apoptosis after 48 h transfection. Briefly, BC cells were harvested with Trypsin-EDTA, washed twice with PBS and underwent dual stained with an Annexin V-APC and 7-AAD Apoptosis Detection kit (Keygen, Nanjing, China) according to the manufacturer's instruction. The

Table 1. Clinicopathological features of BC patients

Characteristics	Number of cases (<i>n</i> = 32)
Age (years)	
≤60	24
>60	8
Sex	
Male	18
Female	14
Tumor size (cm)	
≤3	20
>3	12
Tumor node metastasis (TNM) stage	
0/I	28
II/III	4

percentage of early and late apoptotic cells was quantified between different groups using FlowJo software (TreeStar, San Carlos, CA, USA).

2.7 Luciferase reporter assay

Possible miR-212-3p binding sites were obtained from a miRNA database (http://www.targetscan.org/vert_71/). Of which, NFIA was predicted and selected as a potential target gene of miR-212-3p. Subsequently, luciferase reporter assay was performed to confirm this prediction. In brief, the wild-type (WT) of NFIA mRNA and its homologous mutant (MUT) sites were amplified and cloned into psiCHECK-2 vector (Promega Corp., Madison, WI, USA) to obtain WT NFIA and MUT NFIA plasmids, respectively, according to the manufacturer's instructions. For luciferase assays, approximately 1×10^5 HEK293T cells were seeded into 24-well plates and co-transfected with 100 ng luciferase reporter plasmids and 100 nM miR-212-3p or miR-NC using Lipofectamine 2000 reagent (Invitrogen). At 48 h post-transfection, the relative luciferase activities were detected with the Dual-Luciferase Reporter Assay kit (Promega Corp.) according to the manufacturer's instructions.

2.8 Western blot analysis

Total cellular protein was extracted using ice-cold RIPA lysis buffer (Roche Diagnostics, Basel, Switzerland) and the protein concentration was measured using A BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. Then 30 μ g of protein samples were separated with 10% Tris-SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, MA, USA). Membranes were blocked with 5% non-fat milk at room temperature for 2 h and incubated with primary antibodies against NFIA (Abcam, Cambridge, MA) and GAPDH (ProteinTech, IL, USA) at 4°C overnight. After washing with TBST three times, membranes were incubated with horseradish peroxidase labeled secondary antibodies for 2 h at room temperature. The protein signal was detected by enhanced chemiluminescence detection kit (Beijing Solarbio Science & Technology Co., Ltd.).

2.9 Statistical analysis

All statistical analyses were performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). The experimental data were expressed as the mean \pm standard deviation (SD) from three independent experiments. The quantitative data were statistically analyzed using the Student's *t*-test between two groups or one-way analysis of variance followed by Tukey's test among multiple groups. The association of

miR-212-3p with NFIA expression was statistically analyzed using Spearman's correlation test. The *p* value of less than 0.05 was regarded to be a statistically significant difference.

3. Results

3.1 MiR-212-3p was down-regulated and negatively correlated with NFIA expression in BC tissues

To investigate the functional role of miR-212-3p and NFIA in BC, we first determined their expression levels in BC tissues using quantitative real-time PCR. As shown in figure 1A, the endogenous miR-212-3p expression level was significantly down-regulated in BC tissues compared with adjacent normal tissues ($p < 0.001$). In contrast, the expression level of NFIA was obviously higher in BC tissues than adjacent normal tissues (figure 1B, $p < 0.00$). We next analyzed whether there was any relationship between their expression levels in BC tissues. As expected, Spearman's correlation test showed that the expression of miR-212-3p was inversely correlated with NFIA expression in the same BC tissues (figure 1C, $p = 0.0059$, $r = -0.4760$).

3.2 Identification of NFIA as a target gene of miR-212-3p

Previous results have indicated miR-212-3p and NFIA were negatively correlated in BC tissues. Using target prediction tool TargetScan, miR-212-3p was predicted to directly bind to 3'UTR of NFIA (figure 2A). We then performed luciferase reporter assays to further validate their regulatory relationship. As shown in figure 2B, co-transfection with WT NFIA and miR-212-3p markedly decreased the luciferase activity in 293T cells ($p < 0.05$), while there was no significant change in luciferase activity after co-transfection with MUT NFIA and miR-212-3p. Moreover, mRNA and protein expression level of NFIA were examined by quantitative real-time PCR and western blot, respectively. The results showed that miR-212-3p overexpression significantly suppressed the expression of NFIA at mRNA (figure 2C, $p < 0.01$) and protein levels (figure 2D) in both T24 and J82 cells. These data suggest miR-212-3p could down-regulate NFIA expression through directly binding to its 3'-UTR.

3.3 MiR-212-3p inhibits proliferation and induced apoptosis of BC cells

Since miR-212-3p has been shown to be lower expressed in BC tissues, we thus speculated that it might be a tumor suppressor in BC. To confirm this hypothesis, T24 and J82 cells were transfected with miR-212-3p. As shown in figure 3A, miR-212-3p transfection significantly increased the expression of miR-212-3p in both T24 and J82 cells

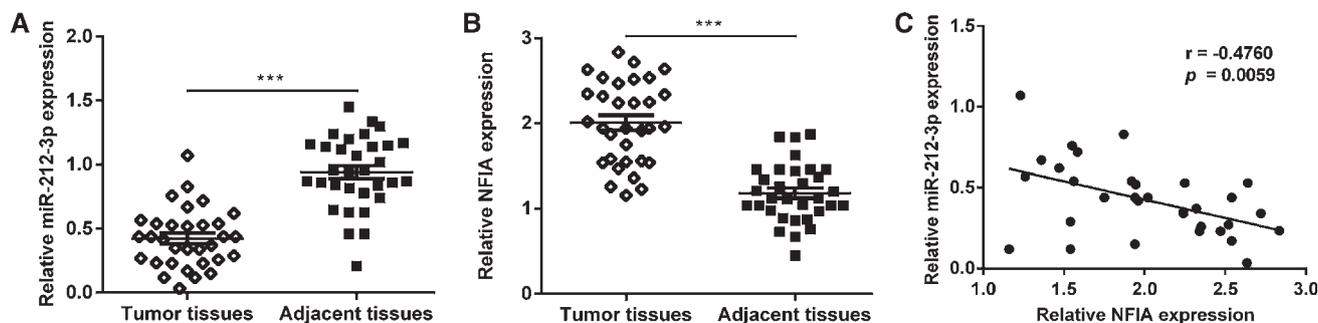


Figure 1. Endogenous expression level of miR-212-3p and NFIA is negatively correlated in BC tissues. Quantitative real-time PCR analyzed endogenous (A) miR-212-3p and (B) NFIA expression level in human BC tissues and adjacent normal tissues. (C) Negative linear correlation between miR-212-3p and NFIA in human BC tissues was determined by Spearman's correlation test. *** $p < 0.001$ vs adjacent tissues.

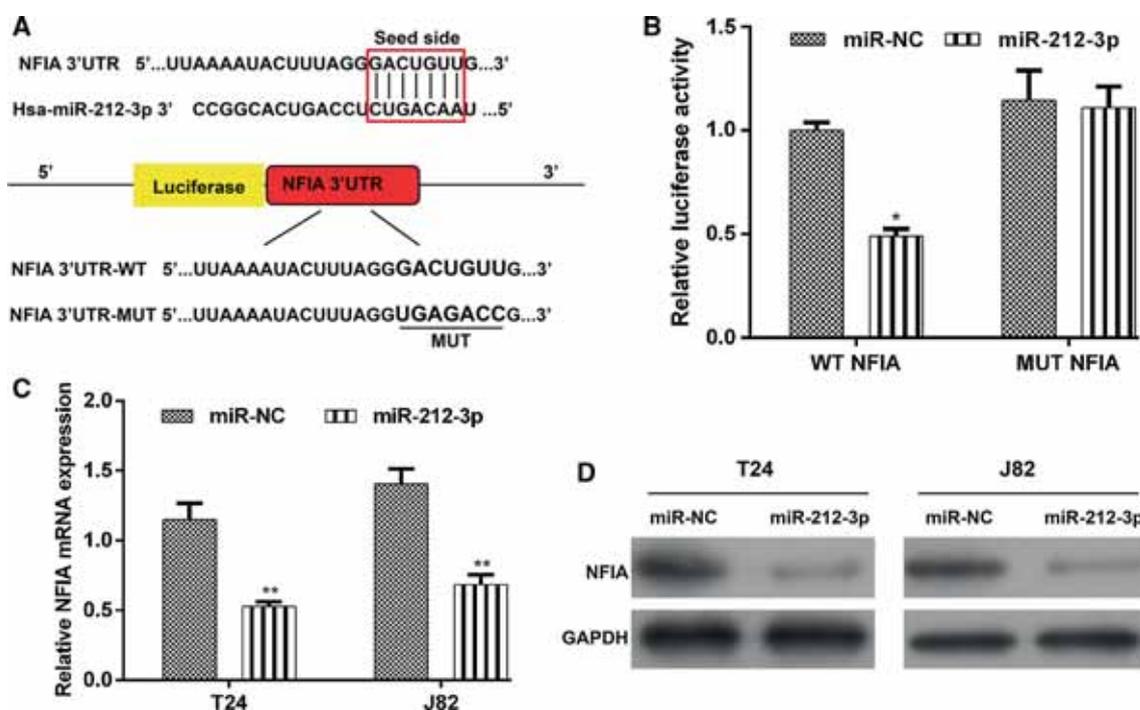


Figure 2. MiR-212-3p modulates BC cells through targeting NFIA. (A) The direct binding sites of miR-212-3p with NFIA were predicted by TargetScan. The NFIA 3'-UTRs containing the wild- or mutant-type miR-212-3p targeting sequence were inserted downstream of the luciferase reporter vector. (B) Luciferase reporter gene assay were used to detect interaction between miR-212-3p and direct binding and mutation sites of NFIA in HEK293T cells. (C) Quantitative real-time PCR and (D) western blot analysis were used to measure NFIA mRNA and protein level in BC cell with transfection of miR-212-3p or miR-NC. Data are expressed as means \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs miR-NC.

($p < 0.001$). MTT assay showed that miR-212-3p overexpression inhibited the cell proliferation at different time points, especially at day 4th and 5th in T24 and J82 cells (figure 3B, $p < 0.001$). Furthermore, flow cytometry analysis revealed that miR-212-3p overexpression remarkably increased the total number of apoptotic cells from $7.18 \pm 0.30\%$ to $18.28 \pm 0.72\%$ in T24 cells and $11.11 \pm 0.52\%$ to $22.06 \pm 0.69\%$ in J82 cells (figure 3C, $p < 0.001$). These results demonstrated that miR-212-3p negatively regulated cell growth and proliferation in BC cells.

3.4 Knockdown of NFIA imitated the effects of miR-212-3p on cell proliferation and apoptosis in BC cells

As NFIA expression was significantly up-regulated and inversely correlated with miR-212-3p, we thus performed loss-of-function assays in BC cells to investigate the biological function of NFIA. First, T24 and J82 cells were transfected with siNFIA and underwent NFIA expression determination. The results showed that the expression of

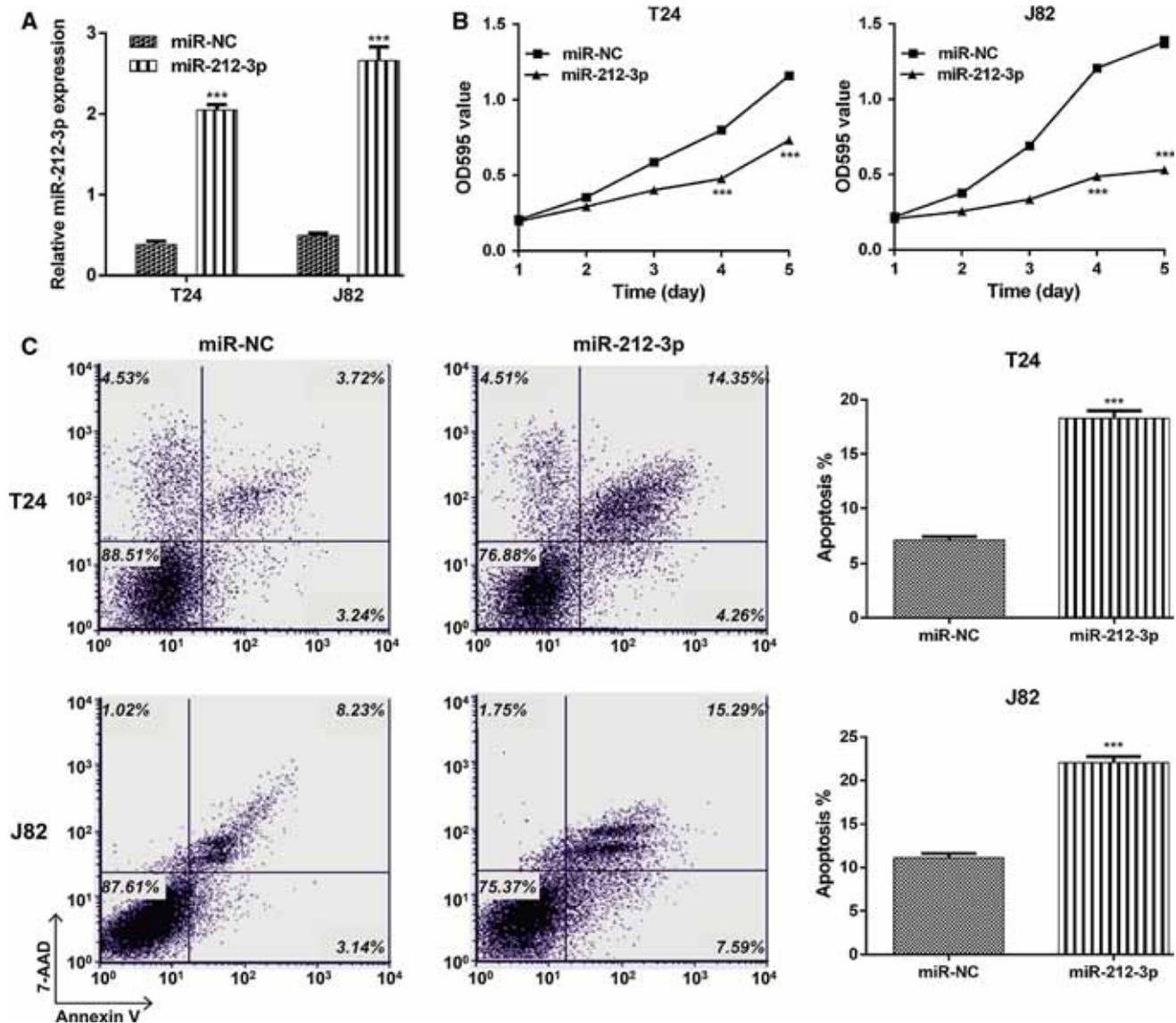


Figure 3. MiR-212-3p inhibits proliferation and induced apoptosis of BC cells. T24 and J82 cells were transfected with miR-212-3p or miR-NC, respectively. (A) Quantitative real-time PCR was used to determine the expression of miR-212-3p in T24 and J82 cells. (B) Cell proliferation was determined using the MTT assay. (C) Flow cytometry with double staining with Annexin V/7-AAD was utilized to analyze cell apoptosis in T24 and J82 cells. Data are expressed as means \pm SD of three independent experiments. ** $p < 0.01$ vs miR-NC.

NFIA was significantly down-regulated at mRNA and protein levels using quantitative real-time PCR (figure 4A, $p < 0.001$) and western blot analysis (figure 4B), respectively. Consistent with miR-212-3p overexpression, we further found NFIA knockdown significantly suppressed cell proliferation (figure 4C, $p < 0.001$) and promoted apoptosis (figure 4D, $p < 0.001$) in both T24 and J82 cells.

4. Discussion

This study shows for the first time that, miR-212-3p is under-expressed in BC tissues and that miR-212-3p depresses BC cell (T24 and J82) proliferation and induces

apoptosis. MiR-212-3p was reported to be downregulated in human prostate cancer (Ramalinga *et al.* 2015), glioblastoma (Liu *et al.* 2015), gastric cancer (Wada *et al.* 2010), hepatocellular carcinoma and derived cell lines (Tu *et al.* 2015). What's more, previous studies have shown that miR-212-3p suppresses multiple pathways including cell proliferation, invasion, migration, autophagy, angiogenesis and senescence, as well as induces apoptosis (Ramalinga *et al.* 2015). Therefore, these findings appear very similar to previous reports that miR-212-3p is also down-regulated in BC tissues and inhibit BC carcinogenesis.

Accumulating evidence highlights a critical role for miRNAs in modulating their target mRNAs (Rupaimoole and Slack 2017). According to the statistical data by Lewis

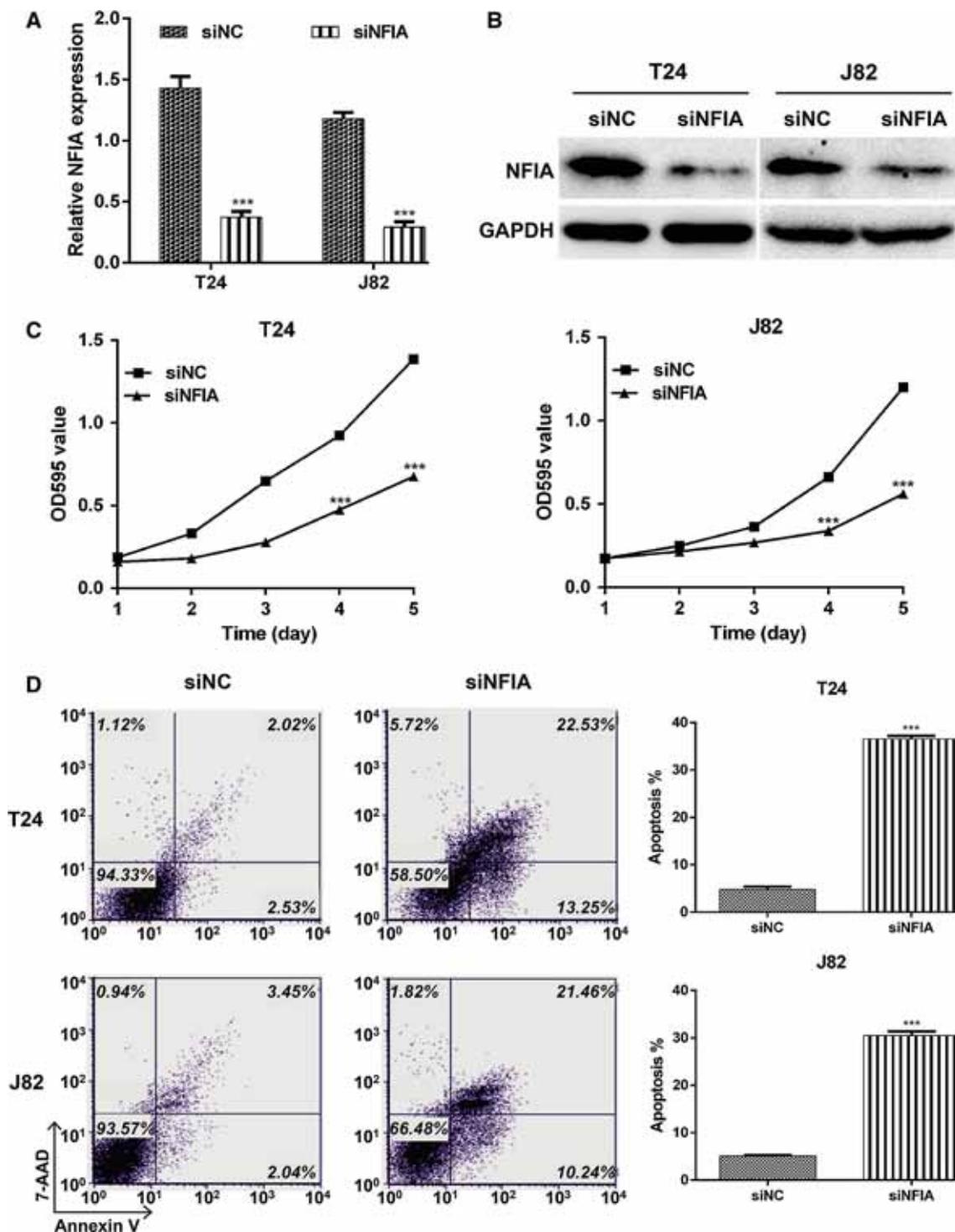


Figure 4. Downregulation of NFIA affected cell proliferation and apoptosis in BC cells. T24 and J82 cells were transfected with siNFIA or siNC, respectively. (A) Quantitative real-time PCR and (B) western blot were used to determine the expression of miR-212-3p in T24 and J82 cells. (C) Cell proliferation was determined using the MTT assay. (D) Flow cytometry with double staining with Annexin V/7-AAD was utilized to analyze cell apoptosis in T24 and J82 cells. Data are expressed as means \pm SD of three independent experiments. ** $p < 0.01$ vs siNC.

et al. (Lewis *et al.* 2005), an average of more than 200 mRNAs was predicted to be potential targets of each miRNA family members. Till now, several targets for miR-212-3p,

including SRIT1 (Ramalinga *et al.* 2015), YAP1 (Tu *et al.* 2015), HMGB1 (Wada *et al.* 2010), SGK3 (Liu *et al.* 2015), Rab1a (Hou *et al.* 2018) and FOXA1 (Xie *et al.* 2018) have

been identified. Moreover, miRNA–mRNA cross talk has been linked to the initiation and progression of human cancers (Lin and Gregory 2015). It has been reported that forced expression of miR-212-3p blocked proliferation and reduced survival of osteosarcoma cells and hepatocellular carcinoma cells via directly targeting FOXA1 (Xie *et al.* 2018). In glioblastoma, miR-212-3p functions as a tumor suppressor by targeting SGK3 (Liu *et al.* 2015). In prostate cancer, miRNA-132/212 targeting of SOX4 are thought to be responsible for suppression of TGF- β -mediated epithelial–mesenchymal transition (Fu *et al.* 2016).

NFIA is a key transcriptional modulator of brown fat genetic program (Hiraike *et al.* 2017). In recent years, much attention has been devoted to the role of NFIA in human malignancies, since it has been reported and implicated in several types of human cancers (Bernard *et al.* 2009; Zhao *et al.* 2017). Amplification and overexpression of NFIA generally occur in glioma and esophageal squamous carcinoma, and act as a novel promoter of tumor growth, proliferation and survival (Lee *et al.* 2014; Yang *et al.* 2018). In this study, using the publicly available databases TargetScan, we observed that NFIA contained a miR-212-3p seed match at the 3'UTR region. Further luciferase reporter assay revealed that the relative luciferase reporter intensity was remarkably reduced in HEK293T cells that co-transfected with WT NFIA and miR-212-3p compared with co-transfection with MUT NFIA and miR-212-3p, the result confirmed that the sequence 5'-CUGACAA-3' of miR-212-3p bound to NFIA mRNAs. We also observed that NFIA is highly expressed in BC tissues, and miR-212-3p expression levels are inversely correlated with NFIA levels. Moreover, the phenotype of BC cells with depletion of NFIA tends to imitate the BC cells that overexpressed miR-212-3p. These results indicate that miR-212-3p exerts its anti-tumor effect in BC cell through targeting NFIA. Interestingly, the members of NFIs could form homo- or heterodimers to active or inactive gene transcription according to cellular circumstances (Semenova *et al.* 2016). We suggest that NFIA is a regulator of BC proliferation and apoptosis by distinct modulation of pivotal genes that governs the biological behavior of BC.

In summary, the results of this study reveal that miR-212-3p suppresses the proliferation and induces apoptosis of BC cells by directly targeting NFIA. Our study may contribute to deep understanding of the role of miR-212-3p in human BC.

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