miR-192 suppresses the tumorigenicity of prostate cancer cells by targeting and inhibiting nin one binding protein

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Abstract. Nin one binding (NOB1) protein has been identified as an oncogene in various human cancers, including prostate cancer. MicroRNAs (miRs) have also been recognized as novel regulatory molecules of gene expression. The present study aimed to discover potential miRs that target NOB1 and regulate NOB1 expression in prostate cancer. miR-192, which is an important regulator of numerous cancers, was found to be significantly downregulated in prostate cancer cells. Moreover, we noted that miR-192 overexpression markedly inhibited the proliferation, colony-forming ability, and migratory capacity of the prostate cancer cells. miR-192 overexpression also induced cell cycle arrest in the G1 phase, as shown by flow cytometry. Bioinformatics analysis results revealed that NOB1 was a possible candidate target gene of miR-192. This discovery was further verified through dual-luciferase reporter assay, RT-qPCR, and western blot analysis. We suggest that miR-192 directly regulates the mRNA and protein expression of NOB1. Furthermore, miR-192 inhibited the expression of p38 mitogen-activated protein kinase. The results of our study indicated that miR-192 negatively regulated NOB1 expression and impaired the tumorigenicity of prostate cancer cells. Therefore, we suggest that targeting miR-192 and NOB1 is a novel strategy which will assist in the development of new therapeutics that will be used in the future to prevent and treat prostate cancer.

Introduction

Prostate cancer is one of the most common type of malignant tumor which affects males and is the second leading cause of cancer-related mortality among the male gender (1). However, currently available treatments are unable to completely cure advanced prostate cancer, as prostate cancer cells exhibit a high rate of proliferation, as well as high invasion capacity and metastatic ability (2). The underlying mechanism of the tumorigenesis of prostate cancer is not yet fully understood, and as such the development of efficient therapies is impeded. Therefore, the molecular mechanism of prostate cancer must be studied in order to develop novel approaches and agents to prevent and treat prostate cancer.

Prostate cancer tissues have been shown to express NOB1 in the nucleus, and this expression has been correlated with lymph node metastasis (11). NOB1 expression has been suggested to function as a potential prognostic marker of prostate cancer (12,13). Gene silencing of NOB1 has previously been shown to suppress the malignant transformation of prostate cancer cells (14). Therefore, we posit that targeting NOB1 constitutes a promising approach to preventing and treating prostate cancer.

It has previously been noted that a group of short, non-coding RNAs termed microRNAs (miRs) act as novel regulatory molecules of gene expression (15). miRs are known to negatively regulate the expression of target genes at a post-transcriptional level by binding the 3'-untranslated region (3'-UTR) of mRNA (15,16). Thus, miRs play an important role in the diagnosis, prognosis and treatment of various diseases (17,18). miRs have also been implicated in the pathogenesis of prostate...
cancer; these molecules serve as a novel target in the treatment of prostate cancer (19).

The present study aimed to discover potential miRs that target NOB1 and regulate NOB1 expression in prostate cancer. miR-192 functions as a tumor suppressor in the development and progression of certain human cancers, for example colon (20) and bladder (21) cancers. However, the role of miR-192 in prostate cancer had not previously been examined, to the best of our knowledge. In the present study, we revealed that miR-192 expression was significantly decreased in prostate cancer cells. miR-192 overexpression markedly impaired the tumorigenicity of prostate cancer cells. NOB1 was predicted as a candidate target gene of miR-192 through bioinformatics analysis; this result was confirmed through a dual-luciferase reporter assay. We further demonstrated that miR-192 directly regulated NOB1 expression, and noted that miR-192 overexpression also inhibited the expression of p38 mitogen-activated protein kinase (MAPK) by suppressing NOB1. Thus, we suggest that miR-192 negatively regulated NOB1 expression in prostate cancer. Targeting miR-192 and NOB1 may thus constitute a novel strategy with which to develop new therapeutics for prostate cancer.

Materials and methods

Cell cultures. The human prostate cancer lines PC-3 and DU145 were both purchased from the The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Human prostate epithelial RWPE-1 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). PC-3 and DU145 cells were grown in RPMI-1640 medium with 10% fetal bovine serum (FBS) and 1% streptomycin-penicillin (all from Invitrogen, Carlsbad, CA, USA). The RWPE-1 cells were grown in keratinocyte serum-free media containing 0.5% streptomycin-penicillin (both from Invitrogen). All cells were cultured in a CO2 incubator (HeraCell 2401; Thermo Fisher Scientific, Waltham, MA, USA) containing 5% CO2 at 37°C.

Cell transfection. The cells were grown in a 6-well tissue-culture plate at a density of 1×106 cells/well. After the cells reached 60-80% confluence, the miR-192 mimics and the non-specific miR control (miR NC) synthesized by the Shanghai GenePharma Co., Ltd. (Shanghai, China) were transfected into the cells using Lipofectamine 2000 (Invitrogen) at a final concentration of 50 nM, in accordance with the manufacturer’s recommendations. After 48 h of transfection, gene expression was detected through reverse-transcription quantitative polymerase chain reaction (RT-qPCR) and western blot analysis.

RT-qPCR analysis. Total RNA was obtained from the cells using an miRNasy mini kit (Qiagen, Düsseldorf, Germany) in accordance with the manufacturer’s instructions. In RT-qPCR mRNA analysis, total RNA was reverse transcribed using M-MLV reverse transcriptase (Clontech Laboratories, Palo Alto, CA, USA). Complementary DNA was synthesized using an miScript reverse transcription kit (Qiagen). qPCR experiments were conducted using an ABI 7500 Real-Time PCR system (Applied Biosystems Life Technologies, Carlsbad, CA, USA) with a SYBR Premix Ex Taq™ II commercial kit (Takara Bio, Dalian, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 snRNA were used as the internal control for relative gene expression quantification using the 2−ΔΔCt method.

Western blot analysis. Proteins were extracted from each sample of the cells and quantified using a bicinchoninic acid kit (Peyotime Institute of Biotechnology, Haimen, China). Equivalent amounts of protein (25 μg) were loaded onto 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membranes (Milltenyi Biotec, Auburn, CA, USA). After the membranes were blocked with 2.5% skim milk in Tris-buffered saline (TBS) at 37°C for 1 h, the membranes were blotted with primary antibodies, namely anti-NOB1 (sc-160594), anti-p38 MAPK (sc-6176), and anti-GAPDH (sc-48166) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C overnight. After the membranes were washed with TBS and Tween-20, horseradish peroxidase-conjugated secondary antibodies (1:2,000; sc-2768; Santa Cruz Biotechnology, Inc.) were applied for 1 h at room temperature. The membranes were incubated for 1 h and subjected to an enhanced chemiluminescence detection system (Amersham Biosciences, Little Chalfont, UK). The blots were developed using an enhanced chemiluminescence detection kit (Amersham Biosciences). The gray value of each protein band was quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Cell proliferation assay. Human prostate cancer cells were seeded into a 96-well plate at a density of 1×103 cells/well and cultured for 24 h. The cells were transfected with 50 nM of miR-192 mimics or miR NC for 48 h. Cell proliferation was detected using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. In brief, 5 mg/ml MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was added at 20 μl/well to the cell cultures. After 4 h of incubation, the medium was discarded, and the formazan product was dissolved with dimethyl sulfoxide (200 μl/well). The optical density of each well was detected at 490 nm using an enzyme-linked immunosorbent assay reader (ELx808; BioTek Instruments, Inc., Winooski, VT, USA).

Colony formation assay. Human prostate cancer cells were cultured in a 6-well plate and transfected with the miR-192 mimics or miR NC. After transfection for 48 h, the cells were re-plated into a 6-well plate in growth medium containing 0.3% noble agar at 200 cells/well to form natural colonies. After 14 days, the cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, and stained with Giemsa (Sigma-Aldrich). The total number of colonies was counted under a microscope (Leica AF6000; Leica, Solms, Germany), and the results were then averaged.

Cell cycle analysis. In the present study, cell cycle distribution (G1, S or G2/M) was detected through flow cytometry. Human prostate cancer cells were transfected with the miR-192 mimics or miR NC for 48 h, harvested, washed, and then fixed with 70% ethanol. Propidium iodide (PI) (100 μg/ml; Sigma-Aldrich) in PBS containing 10 μg/ml of RNase A was added to the cells and incubated for 30 min in a dark place at 37°C. The percentage of cells in each cell cycle phase was
measured using a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Cell migration assay. In the present study, human prostate cancer cells were transfected with miR-192 mimics or miR NC for 48 h, and the in vitro migration ability was determined using a Transwell chamber (Corning Incorporated, Corning, NY, USA). In brief, 1.0x10^4 cells re-suspended in 200 µl serum-free medium were seeded into the upper chamber. Subsequently, 500 µl medium containing 10% FBS was placed in the lower chamber. After the cells were incubated at 37˚C for 14 h, the cells that remained on the upper chamber were removed using a cotton swab. The cells that had migrated to the lower chamber were subsequently fixed with 10% methanol for 30 sec and stained with 0.1% crystal violet (Abcam, Cambridge, UK) for 30 min. The number of cells was then counted under the microscope, and the data obtained in five random fields were averaged.

Dual-luciferase reporter assay. The 3'-UTR of NOB1 containing the predicted binding sites of miR-192 was subcloned into the pGL3 luciferase promoter vector (Promega Corp., Madison, WI, USA). The relevant mutant containing the mutated binding sites of miR-192 was also constructed. The 3'-UTR recombinants of pGL3-NOB1 (100 ng) were cotransfected into the PC-3 cells with miR-192 mimics or miR NC control using Lipofectamine2000 (Invitrogen) in accordance with the manufacturer's instructions to detect the luciferase reporter activity. pGL3 luciferase reporter vectors contained either the 3'-UTR of wild-type NOB1 or its relevant mutant and miR-192 mimics or non-specific miR control. After the cells were transfected for 48 h, the cells were harvested and lysed. Relative luciferase activity was evaluated using the dual-luciferase reporter system detection method (Promega Corp.) in accordance with standard protocols and the manufacturer's instructions.

Statistical analysis. Data are expressed as the means ± standard deviation. Statistical significance was calculated through one-way ANOVA, followed by the Bonferroni post hoc test using SPSS version 11.5 (SPSS, Inc., Chicago, IL, USA). A p-value <0.05 was considered to indicate a statistically significant difference between groups.

Results

Expression of miR-192 in prostate cancer cell lines. We evaluated the expression level of miR-192 in PC-3 and DU145 cells through RT-qPCR analysis to determine the potential role of miR-192 in prostate cancer. We noted that miR-192 expression was significantly decreased in the prostate cancer cell lines PC-3 and DU145 compared with that in the prostate epithelial RWPE-1 cells (Fig. 1A). These results indicate that miR-192 is involved in the tumorigenicity of prostate cancer. To explore the potential role of miR-192 in prostate cancer, chemosynthetic miR-192 mimics were used to overexpress miR-192 in the PC-3 and DU145 cells. The results showed that transfection with the miR-192 mimics significantly promoted the expression level of miR-192 in PC-3 (Fig. 1B) and DU145 (Fig. 1C) cells.

Overexpression of miR-192 inhibits the growth of prostate cancer cells. To investigate the effect of miR-192 overexpression on prostate cancer cell proliferation, the PC-3 and DU145 cells transfected with the miR-192 mimics were subjected to MTT assay. miR-192 mimics significantly inhibited the proliferation of PC-3 (Fig. 2A) and DU145 (Fig. 2B) cells compared with the untreated or miR NC-transfected cells. These data indicate that miR-192 plays an important role in regulating the growth of prostate cancer cells.

Overexpression of miR-192 suppresses the colony-forming capacity of prostate cancer cells. To detect whether miR-192 plays an important role in the colony-forming ability of prostate cancer cells, we subsequently performed a colony-forming experiment using PC-3 and DU145 cells which had been transfected with miR-192 mimics. The results showed that miR-192 mimics significantly inhibited the colony formation of the PC-3 (Fig. 3A) and DU145 (Fig. 3B) cells.

Overexpression of miR-192 impairs cell cycle progression. In order to further elucidate the role which miR-192 plays in the tumorigenicity of prostate cancer cells, we detected the effect of miR-192 mimics on cell cycle progression. PC-3 and DU145 cells transfected with miR-192 for 48 h were subjected to a flow cytometry assay. The results indicated that miR-192 overexpression significantly increased the number
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of cells in the G1 phase whereas miR-192 mimic transfection markedly decreased the proportion of PC-3 (Fig. 4A) and DU145 (Fig. 4B) cells in the S-phase.

Overexpression of miR-192 reduces the migration capacity of prostate cancer cells. To further detect whether miR-192 had an impact on the cell migration of prostate cancer cells, a Transwell migration assay was carried out. We found that miR-192 overexpression considerably reduced the number of PC-3 (Fig. 5A) and DU145 (Fig. 5B) cells that migrated to the lower chamber, implying that miR-192 overexpression reduced the migration capacity of prostate cancer cells.

NOBI is a direct target gene of miR-192. Taking into consideration that miRs regulate cellular processes through their target genes, we identified the potential target gene of miR-192 through bioinformatics analysis. Notably, we found that NOBI, an oncogene, contained the predicted miR-192 targeting site in the 3’-UTR of NOBI (Fig. 6A). To verify the direct association between miR-192 and the 3’-UTR of NOBI, pGL3
luciferase reporter vectors were constructed with the 3'-UTR of the wild-type NOB1 or its relevant mutant. The miR-192 mimics were cotransfected with the reporter vectors into the PC-3 cells. Transfection with miR-192 mimics in the 3'-UTR of wild-type NOB1 significantly reduced relative luciferase activity, whereas transfection with miR-192 mimics had no obvious effect on the corresponding mutant (Fig. 6B). These results showed that NOB1 is a direct target gene of miR-192.

**Figure 6.** Identification of the miR-192 target sites in the 3'‑UTR of NOB1 binding (NOB1). (A) Schematic of the predicted miR-192 targeting site in the 3'-UTR of NOB1. (B) Dual-luciferase reporter assay was utilized to confirm the direct interaction between miR-192 and the 3'-UTR of NOB1. The PC-3 cells were cotransfected with pGL3 luciferase reporter vectors containing either the 3'-UTR of wild-type NOB1 or its relevant mutant and miR-192 mimics or non-specific miR control (miR NC). After transfection was performed for 48 h, the cells were harvested and lysed. The relative luciferase activity was determined using the dual-luciferase assay detection method. **p<0.01 vs. non-treatment or miR NC.

Overexpression of miR-192 suppresses p38 MAPK expression. To further explore the underlying mechanism of miR-192 in prostate cancer, we detected the effect of miR-192 mimics on p38 MAPK. The results demonstrate that transfection with miR-192 mimic significantly reduced the protein expression level of p38 MAPK in PC-3 cells (Fig. 8A) compared with the miR-NC-transfected group. Similar results were observed in DU145 cells (Fig. 8B).

**Discussion**

Previous studies have suggested that abnormally expressed miRs play an important role in the development and progression of cancers (22,23). miR-192 has been reported to act as a tumor suppressor in numerous human cancers (20,21). However, the role of miR-192 in prostate cancers had not previously been documented: to the best of our knowledge, this study is the first to describe the functional significance of miR-192 in prostate cancers. We demonstrated that miR-192 expression was significantly decreased in prostate cancer cell lines. Our results further elucidated that miR-192 overexpression markedly impaired the tumorigenicity of prostate cancer cells. Moreover, miR-192 overexpression induced cell cycle arrest in the G1 phase. Thus, we suggest that miR-192 serves as a tumor suppressor in prostate cancers.

The NOB1 protein was initially reported to play an essential role in both 20S proteasome maturation and protein degradation (6). NOB1 was noted to be extensively involved in various human cancers (4,7,8). NOB1 knockdown has been noted to markedly decrease the colony forming- and proliferative abilities of ovarian cancer cells and induce
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cell cycle arrest in the G0/G1 phase (4). Likewise, the gene silencing ability of NOB1 inhibits the cell growth and tumor formation ability of human hepatocellular carcinoma cells (7).

In breast cancer cells, NOB1 knockdown also suppresses the proliferation and growth of cancer cells (8). Studies have also reported that NOB1 was highly expressed in papillary thyroid carcinoma tissues, and silencing NOB1 enhances the antitumor effect of doxorubicin (9,24). NOB1 has also been implicated in the development and progression of non-small-cell lung cancer; it has been suggested that high NOB1 expression acts as a potential indicator of poor prognosis (10,13,25,26). In other types of human cancers, including gliomas (3), osteosarcoma (27), colorectal cancer (28,29), and renal cancer (30), NOB1 has also been found to function as an oncogene. In line with these previous studies, NOB1 has also been studied as an oncogene in prostate cancers. We noted that NOB1 is positively and highly

Figure 7. miR-192 regulates NOB1 expression. RT-qPCR was performed to examine the effect of the miR-192 mimic transfection on mRNA expression of NOB1 in (A) PC-3 and (B) DU145 cells. These cells were transfected with miR-192 mimics or non-specific miR control (miR NC) for 48 h. *p<0.05 vs. non-treatment or miR NC. Western blot analysis was performed to detect the effect of miR-192 mimic transfection on the protein expression of NOB1 in (C) PC-3 and (D) DU145 cells. Relative protein was quantitatively analyzed using Image-Pro Plus 6.0 and normalized to GAPDH. **p<0.01 vs. non-treatment or miR NC.

Figure 8. miR-192 regulates p38 mitogen-activated protein kinase (MAPK) expression. Western blot analysis was performed to analyze the effect of miR-192 mimic transfection on the protein expression of p38 MAPK in (A) PC-3 and (B) DU145 cells. Relative protein was quantitatively analyzed using Image-Pro Plus 6.0 and normalized to GAPDH. *p<0.05 vs. non-treatment or non-specific miR control (miR NC).
expressed in prostate cancer tissues, as has also previously noted (12). More recently, it has been reported that high NOB1 expression in prostate cancer tissues is significantly correlated with distant metastasis and Gleason score (31). These studies also pointed out that NOB1 serves as a potential prognostic indicator of prostate cancer. Furthermore, the gene silencing of NOB1 was found to significantly suppress the tumorigenicity of prostate cancer cells (14). In accordance with these findings, in the present study we found that the NOB1 downregulation induced by miR-192 overexpression also considerably inhibited the tumorigenicity of prostate cancer cells. Our results further confirmed the important role which NOB1 plays in regulating prostate cancer; thus, we suggest that NOB1 serves as a promising molecular target which will assist in the treatment of prostate cancer.

The role which miR-192 plays in several types of human cancer has been widely investigated (20-21,32-34). The tumorigenic or tumor-suppressive function of miR-192 has also been reported in various human cancers. Frequent, high miR-192 expression in gastric cancer is likely involved in the progression of gastric cancer (32). miR-192 overexpression inhibits the proliferation and migration rates in neuroblastoma cells by modulating dicer 1, ribonuclease type III (Dicer1) expression (33). Geng et al (20) have reported that miR-192 overexpression inhibits the metastasis of colon cancer cells. miR-192 overexpression also decreases cell proliferation and increases cell apoptosis in lung cancer cells by regulating retinoblastoma 1 expression (34). Furthermore, overexpression of miR-192 inhibits the growth of human bladder cancer cells (21). However, whether miR-192 is involved in the development of prostate cancer remains unknown. In the present study, we demonstrated that miR-192 is downregulated in prostate cancer cells, and we thus suggest that it serves as an tumor suppressor in prostate cancer. We then further elucidated that NOB1 is a direct target gene of miR-192 and that miR-192 overexpression inhibited the mRNA and protein expression of NOB1 in prostate cancer cells. Our results indicate that downregulated miR-192 expression in prostate cancer cells accounts for the overexpressed NOB1 which promotes the development and progression of prostate cancer.

miRs are known to be novel regulators of gene expression (15,16). Several studies have evaluated the possibility of decreasing NOB1 expression using specific miRs. For example, it has previously been reported that miR-326 directly targets and regulates NOB1, which inhibited the cell growth of glioma cells associated with the MAPK pathway (35). Moreover, miR-326 has been found to act as a tumor suppressor in colorectal cancer by modulating NOB1 expression (36). In a study on clear cell renal carcinoma, it was noted that miR-646 negatively modulates the expression of NOB1 and suppresses the proliferation and migration of cancer cells; it has been suggested that the MAPK pathway is involved in these processes (37). In the present study, we demonstrated that miR-192 was a novel regulator of NOB1 expression. We also demonstrated that overexpression of miR-192 inhibited the expression of NOB1 and p38 MAPK, which affected the migratory and invasive activity on tumor cells, as was also previously shown (38). The results of our present study are consistent with the reports of Che et al (11), who revealed that silencing of NOB1 inhibited p38 MAPK expression.

In conclusion, our data in the present study demonstrated that miR-192 was downregulated in prostate cancer cells, and also that the restoration of miR-192 inhibited the proliferation, colony-forming capacity, and migratory ability of prostate cancer cells. We also noted that miR-192 overexpression induced cell cycle arrest in the G1 phase. The tumor-suppressive role of miR-192 was likely mediated by regulating NOB1, an oncogene in prostate cancer. Therefore, we suggest that miR-192 serves as a promising therapeutic target to treat prostate cancer.

References