MiR-210-3p inhibits the tumor growth and metastasis of bladder cancer via targeting fibroblast growth factor receptor-like 1

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Abstract: Current evidence indicates that microRNAs are widely down-regulated in various tumors including colorectal carcinoma, liver cancer and lung cancer, and function as tumor suppressors through inhibiting cancer cell growth, invasion and migration. Here, we demonstrated that miR-210-3p level was significantly reduced in the bladder cancer compared to paratumor tissues, and attempt to reveal the regulatory role of miR-210-3p in bladder cancer progression. Exogenous overexpression of miR-210-3p inhibited the proliferation, migration and invasion of bladder cancer cells in vitro. In addition, the nude mouse xenograft model showed that miR-210-3p over-expressing inhibited bladder cancer growth and liver metastasis whereas silencing miR-210-3p caused an opposite outcome, which is mainly regulated by targeting fibroblast growth factor receptor-like 1 (FGFRL1). We also demonstrated that the expression of FGFRL1 in bladder cancer specimens were negatively correlated with miR-210-3p level, and FGFRL1 overexpression rescued the cell proliferation and invasion inhibited by ectopic expression of miR-210-3p. Moreover, knockdown of FGFRL1 was able to mimic the cell growth and metastasis effects induced by miR-210-3p over-expressing in bladder cancer cells. Together, these results indicate that miR-210-3p plays an important role in the regulation of bladder cancer growth and metastasis in vitro and in vivo through targeting FGFRL1.

Keywords: Bladder cancer, miR-210-3p, FGFRL1, metastasis

Introduction

Bladder cancer is one of the most common cause of cancer-related death, and the incidence of bladder cancer is expected to increase worldwide [1]. Despite improvements in bladder cancer therapeutic strategies, the long-term survival of patients with bladder cancer following surgical resection remains unsatisfactory as a result of recurrence and metastasis [2]. Additionally, the molecular mechanisms underlying bladder cancer metastasis have not been fully elucidated. A better understanding of the events responsible for bladder cancer metastasis is critical important for disease treatment and prognosis improvement [3].

microRNAs, which are small non-coding RNAs, post-transcriptionally regulate their downstream genes expression. Importantly, its complicated regulatory network consists of several microRNAs not only regulates the expression of multiple genes, but also allows the one gene regulate by the combination of several microRNAs [4]. Previous studies demonstrate that several microRNA expression is associated with a variety of human cancers progression. For example, microRNAs regulate the expression of cancer related genes, and function as a tumor suppressor gene or an oncogene gene, playing an functional role in the progression and treatment of cancer [5]. In the present study, we found that miR-210-3p was markedly down-expression in bladder cancer, as compared with the paired adjacent non-tumor tissues. It has been proved that miR-210 was indicated to affect various tumor biological functions, including tumor proliferation, survival and metastasis in, breast cancer, prostate cancer and gastric carcinoma by promoting or suppressing series of target genes [6]. However, the roles of miR-210-3p in bladder cancer progression and
the effects of miR-210-3p on bladder cancer tumorigenesis are still needed to be investigated [7].

microRNA-210 as one of the miRNAs, the level of it is significantly different during the process of epithelial cells differentiation [8]. Few research reported that miR-210 expression is down-regulated during tumor cells epithelial-mesenchymal transition (EMT), the aberrant activation of which triggers cancer cells metastasis [9]. The family of the fibroblast growth factor receptors (FGFRs) comprises five trans-membrane receptors that regulate the growth, apoptosis, differentiation, and migration of most cell types [10]. The classical two receptors, FGFR1 and FGFR4, function by binding to fibroblast growth factor (FGFs) and heparin. This interaction accelerates the phosphorylation of selected residues in the intracellular part of the polypeptides, followed by the activation of various signaling cascades, such as the phospholipase Cγ pathway, RAS-MAP kinase signaling pathway, and PI3-kinase-AKT pathway [11]. In addition FGFR1 and FGFR4, fibroblast growth factor receptor-like 1 (FGFRL1) is a newly described member of the FGFR family that is expressed in embryonic bone development and adult pancreas. FGFRL1 exhibits an ectodomain closely resembling the canonical FGFR family members, thereby retaining the ability to bind FGF ligands with varying affinity [12]. However, the significance of FGFRL1 in bladder cancer progression has not been elucidated.

In this study, we verified that miR-210-3p is down-regulated in bladder cancer samples both in cell lines and the clinical specimens. With miR-210-3p overexpression, the cell proliferation, invasion and soft agar colony formation ability were decreased significantly, and tumor growth in vivo was suppressed. Furthermore, miR-210-3p overexpressing significantly inhibited the metastasis of bladder cancer cells in vivo whereas silencing endogenous miR-210-3p caused an opposite outcome. In addition, we demonstrated that FGFRL1, one of miR-210-3p target genes, plays a very important role in bladder cancer cells growth and metastasis. Together, our results provide new evidence that miR-210-3p overexpression inhibits the progression of bladder cancer and might represent a novel therapeutic target for bladder cancer treatment.

Materials and methods

-human bladder cancer specimens and cell lines culture

Human bladder cancer samples and para-tumor tissue were obtained from the 1st Affiliated Hospital of Zhengzhou University and were classified according to the WHO standard. Informed consents were obtained from all patients with bladder cancer before surgery by the regional ethics committee. The bladder cancer cell line T24, SW780, HT1376, HT1197 and human uroepithelial cells SV-HUC-1 were purchased from Cell Resource Center (Beijing, China). All of the cell lines were maintained at 37°C in an atmosphere of 5% CO₂ in Dulbecco's Modified Eagle Medium (Gibco, USA) or RPMI-1640 supplemented with 10% FBS (Gibco, USA).

miRNA mimic, inhibitor and shRNA transfection

The miR-210-3p mimic and the negative control duplex (named as NC) which was non-homologous to all human gene sequences were used for transient gain of function study. The miR-210-3p inhibitor oligo (named as miR-210-3p inhibitor) and inhibitor negative control oligo (named as inhibitor NC) were used for transient loss of function study. A small interfering RNA duplex (shRNA) targeting human FGFRL1 mRNA was used for shRNA study (named as shFGFRL1). All the RNA duplexes and RNA oligos were synthesized by Gene Pharma (Shanghai, China). The Lipofectamine 2000 reagent (Invitrogen, USA) was used for transient transfection following the manufacturer’s instructions.

MTT assay

1 × 10⁴ T24 or SW780 cells were seeded into 96 well plates, and cultured for 1, 2, or 3 days. 100 µl MTT (Sigma) was added to well and then cells were incubated at 37°C for 4 hours. The cell supernatant was removed, 200 ul of DMSO was added and shaking it well plates for 15 minutes. Finally, the optical density (OD) value was measured at 490 nm. The effect of miR-210-3p on T24 or SW780 cells viability was assessed as the relativity of cell viability compared with control cells, which were assigned 100% viability.
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Wound-healing migration assay

1 × 10^5 T24 or SW780 cells were seeded into 6-well plates with complete culture medium. The confluent monolayer of both cells was scratched with a 200 μl pipette tip, and cell migration into the wound closure was visualized by microscopy, and scored by measuring the size of the initial wound and comparing it to the size of the wound after 6, 12, 18, 24, 30, 36, 42 and 48 h, respectively.

Transwell invasion assay

1 × 10^3 T24 or SW780 cells were seeded into the upper chambers of Transwell with 8 um pore size coated with Matrigel, and then chambers were placed into 24 well culture plates. After 6 hours, the invaded cells in lower chambers were fixed and stained with 0.1% crystal violet. The invaded T24 or SW780 cells were photographed and counted by microscope [13].

The soft agar colony formation assay

1 × 10^3 T24 or SW780 cells were seeded in complete medium mixed with soft agar, and cultured in the incubator for two weeks. Cell colonies were stained with 0.1% crystal violet, and then analyzed the number of colonies [14].

Western blot

For protein preparation, the cells were collected and split for 15 minutes with cell lysis buffer on ice, the supernatant was denatured in 5 × loading buffer containing SDS in boiling water for 10 minutes. PVDF membrane blocking and antibody dilution used 5% skimmed milk powder dissolved in TBST. The following antibodies were used: MMP-2 (1:1000, Cell Signaling Technology), MMP-9 (1:1000, Cell Signaling Technology), TIMP-1 (1:1000, Proteintech), GAPDH (1:1000, Cell Signaling Technology), FGFRL1 (1:1000, Cell Signaling Technology), goat anti-rabbit IgG-HRP (1:5000, Santa Cruz). Target proteins were detected by the ECL system (Millipore, Germany) by Immobilon western chemiluminescent HRP substrate (Millipore) and visualized by the ChemiDoc XRS system (Bio-Rad, USA).

qRT-PCR

Total RNA in bladder cancer cells or clinical tissues was extracted with Trizol. 1 ug of RNA was reverse-transcribed to cDNA using ReverTra Ace qPCR RT Kit (FSQ-101, TOYOBO). qRT-PCR was performed to detect the mRNA level of miR-210-3p or other genes using AceQ qPCR SYBR Green Master Mix (Q111, Vazyme). We used the reference gene, UBC when detecting the expression level of miR-210-3p in paratumor and bladder cancer tissues. The formula of relative expression value was as follows: 2 (-ΔΔCt). The primer sequences for GAPDH were 5'-TAATCTTCGCTTAACTT-3' and 5'-AGCCTT-CATACATCTCAA-3'; FGFRL1 were 5'-CACGTCCT-TTCCAGTCAAG-3' and 5'-GGCAGGACCCGACAA-CTTCTG-3'.

Luciferase reporter assay

The luciferase reporter analysis was performed to detect the direct binding of miR-210-3p to FGFRL1. The 3'-UTR of human FGFRL1 was cloned into the p-MIR-reporter vector (Ambion, USA) and confirmed by sequencing to form a wild type luciferase reporter vector (FGFRL1 WT 3'-UTR). To verify the direct binding specificity, the sequences of FGFRL1 3'-UTR that bound to the miR-210-3p were mutated, and it was cloned into p-MIR-reporter vector to form a mutated luciferase reporter vector (FGFRL1 MUT 3'-UTR). For the luciferase reporter assays, HEK 293T cells were seeded in 96-well plates and then co-transfected with luciferase reporter vectors with miR-210-3p mimic or NC using Lipofectamine 2000. After transfection for 48 h, the relative luciferase activity was detected using a dual luciferase reporter assay system (Promega) [15].

In vivo tumorigenicity assays and immunohistochemistry

Nude mice were bred and housed in AAALAC-accredited specific pathogen-free rodent facilities at the 1st Affiliated Hospital of Zhengzhou University. Mice were housed in sterilized, ventilated microisolator cages and supplied with autoclaved commercial chow and sterile water.

All mouse experiments were conducted in accordance with standard operating procedures approved by the University Committee on the Use and Care of Animals at Zhengzhou University. Tumorigenicity was determined by subcutaneously injecting control or miR-210-3p over-expressing or down-expressing cells into the flanks of male nude mice (1 × 10^6 cells
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Figure 1. miR-210-3p expression is down-regulated in bladder cancer. A. microRNA meta-analysis in YM500v2 (http://ngs.ym.edu.tw/ym500v2/index.php) was performed to detect differently expressed miRNA of bladder cancer and control noncancerous solid tissues. The hierarchical clustering of miRNAs that were differentially expressed among bladder cancer patients (n = 31) and normal controls (n = 19). At most top 16 as shown. B. Total RNA was extracted from para-tumor tissues and bladder cancer tissues, and then reversely transcribed to cDNA. miR-210-3p level was assayed by qRT-PCR. There were 23 cases of bladder cancer tissues and 12 corresponding para-tumor tissues. N stands for total numbers of patients. C. Representative CISH staining of miR-210-3p in para-tumor and bladder cancer tissues. D. qRT-PCR analysis was performed to measure the miR-210-3p expression level in six different bladder cancer cell lines. PCR values were normalized to the levels of UBC. Data are presented as the mean ± SD from three independent measurements.
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per site). The tumor sizes were measured once a week with a caliper, and calculated as tumor volume = 0.5 × Length × Width². The tumor tissues of mice were fixed in formalin and processed for paraffin embedding. Sectioned samples were de-paraffined in xylene and rehydrated in graded alcohol. Antigen retrieval was done according to the manufacturer’s protocol (MVS-0100, Maxvision), and then the endogenous peroxidase was inactivated with 3% hydrogen peroxide methanol solution, blocked with animal non-immune serum (SP KIT-B, Maxvision) and incubated with primary antibodies overnight at 4°C, and then incubated with secondary antibodies for 15 minutes. Slides were stained using the detection kit, cell nucleus was stained with Ki67.

In vivo metastasis development assay

In lung metastasis assays, 1 × 10⁶ T24 or SW-780 cells were injected into BALB/c mice via the lateral tail veins. After 4 weeks, the mice were killed under anesthesia. The lungs were collected and fixed in 10% PFA. For lung morphology evaluation, Hematoxylin and Eosin (HE) staining was performed on sections from embedded lung samples. Animal experiments were approval by the Animal Care and Use Committee of the 1st Affiliated Hospital of Zhengzhou University.

Statistical analysis

Results are presented as the Mean ± SD for at least three repeated individual experiments for each group, the student’s t-test was performed to compare the difference of two groups. P-value < 0.05 was considered significant.

Results

The expression of miR-210-3p is down-regulation in bladder cancer

Firstly, the online database YM500 (http://ngs.ym.edu.tw/ym500/) was analyzed to compare the miRNA expression in bladder cancer and normal solid tissues. This database compared the miRNA expression profiles between 19 normal solid tissues and 31 primary bladder cancer. For these miRNAs, we found miR-210-3p was significantly down-regulated in bladder cancer, with compared to normal tissues (Figure 1A). In order to investigate the expression difference of miR-210-3p in bladder cancer, we detected miR-210-3p levels in 23 cases of clinical bladder cancer tissues paired with adjacent normal tissues by quantitative real-time PCR (qRT-PCR). The results indicated that the expression levels of miR-210-3p in tumor tissues were dramatically down-regulated compared with paired normal tissues (Figure 1B). Furthermore, we conducted the chromogenic in situ hybridization (CISH) staining of miR-210-3p in the bladder cancer. Our studies show that miR-210-3p expression level was much lower in bladder cancer compared to the para-tumors (Figure 1C). Finally, we detected the level of miR-210-3p in several bladder cancer cell lines, including T24, SW780, HT1376 and HT1197 in comparison with human uroepithelial cells SV-HUC-1 cells by qRT-PCR. Similarly, the expression levels of miR-210-3p in bladder cancer cell lines were also markedly low in contrast with SV-HUC-1 cells (Figure 1D). These results demonstrated that miR-210-3p levels were negatively correlated with malignancy of bladder cancer.

miR-210-3p overexpression suppresses cell growth in vitro and in vivo

In order to investigate the role of miR-210-3p in tumor growth, we transfected T24 and SW780 cells with miR-210-3p mimic to figure out the actual effects of miR-210-3p on cell proliferation. qRT-PCR assay suggested the level of miR-210-3p was significantly up-regulated in bladder cancer cells after the transfection of miR-210-3p mimic (Figure 2A). MTT assay suggested that the overexpression of miR-210-3p remarkably inhibited the proliferation of bladder cancer cells at different time points (Figure 2B). Consistently, miR-210-3p overexpression obviously inhibited the anchorage-independent growth of tumor cells in contrast with cells transfected with the negative control duplex (named as NC) in soft agar growth assay (Figure 2C). These results suggested that miR-210-3p acts as a tumor suppressor for bladder cancer in vitro. We next tested the involvement of miR-210-3p in tumor growth in vivo. One million miR-210-3p overexpressing or control cells were implanted to the flanks of nude mice subcutaneously. As shown in Figure 2D, miR-210-3p overexpression significantly inhibited tumor growth, and the volume of tumor derived from T24 and SW780 with miR-210-3p overexpression was significantly smaller than
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Figure 2. miR-210-3p overexpression significantly inhibits cell growth in vitro and in vivo. A. Cells were transfected with miR-210-3p mimic or NC. Total RNA was isolated and miR-210-3p expression levels were measured by qRT-PCR. B. miR-210-3p transfected bladder cancer cells were seeded in 96-well culture plates and cultured for 1, 2 or 3 days. MTT assays were conducted following the manufacturer’s protocol and the optical density (OD) value was measured at 490 nm. The relative viabilities of T24 and SW780 cells treated with miR-210-3p were significantly lower than negative control duplex (named as NC) treated cells. C. Colony-formation assay. The colony formation rates of miR-210-3p transfected bladder cancer cells were obviously lower in contrast with NC transfected cells. And colonies were counted in the whole field (right). **P < 0.01. D. One million indicated cells were implanted into the flanks of nude mice and the volume of tumor was measured once a week. The tumor volumes implied that the growth of tumors in miR-210-3p group was significantly slower than NC group. E. Xenografted tumors were embedded in paraffin and performed to IHC. Lower Ki-67 expressions was also detected in miR-210-3p treated tumors. Error bars represent the S.D. from three nude mice. **P < 0.01, compared to NC group.
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Figure 3. miR-210-3p overexpression inhibits bladder cancer cell metastasis. A. Cell morphology was captured with optical microscope after T24 and SW780 cells were transfected with miR-210-3p mimic or NC for three weeks. B. T24 and SW780 cells were transfected with miR-210-3p mimic or NC, western blot was carried out to detect the expression levels of MMP-2, MMP-9 and TIMP-1. C. T24 and SW780 were transfected with miR-210-3p mimic or NC, and then the migration ability of the cells was investigated with the wound healing assay. D. The invasion ability of the cells was investigated with the Transwell invasion assay according to the manufacturer’s protocol. Quantitative analysis of the total invasive cells from three independent experiments was shown on the right panel. E. Representative hematoxylin and eosin staining of lung tissue collected from mice injected with control cells and miR-210-3p over-expressing cells. Arrowheads indicate tumors. Bar: 100 µm.

Tumor derived from control group. miR-210-3p levels in the collected tumors were also measured by qRT-PCR, and showed that miR-210-3p was overexpressed in tumors of T24 and SW780 with miR-210-3p overexpression (Supplementary Figure 1), confirming miR-210-3p inhibited bladder cancer growth. In addition, the IHC staining also showed that the Ki-67 indexes of tumors in the miR-210-3p overexpressed group were lower than those in the control group (Figure 2E). All these results supported that miR-210-3p could suppress the
growth of bladder cancer cells in vitro and in vivo.

miR-210-3p inhibits cell invasion and induces epithelial phenotype in vitro

Given the evidence of its association with both bladder cancer cells growth in vitro and in vivo, we then assessed the functional role of miR-210-3p in bladder cancer biology. We observed no obvious cell morphology change in the third week after miR-210-3p overexpression (Figure 3A). To determine whether miR-210-3p induced matrix metalloproteinases (MMPs) expression, we analyzed the expression of the MMP-2, MMP-9 and TIMP-1. Western blot assay showed miR-210-3p overexpression decreased MMP-2/9 expression, and increased TIMP-1 expression in T24 and SW780 cell lines (Figure 3B). MMPs always accompany alteration of cellular invasion and migration. Therefore, we tested the effect of miR-210-3p on migration and invasion of bladder cancer cells. miR-210-3p overexpression dramatically inhibited the cell migration of T24 and SW780 in wound healing assay (Figure 3C). Likewise, the overexpression of miR-210-3p remarkably inhibited the invasion ability of bladder cancer cells in vitro (Figure 3D). Lastly, we next tested the involvement of miR-210-3p in the dissemination of cancer cells in vivo. miR-210-3p overexpression or control cells were injected into the tail veins of nude mice as previously described [16]. Histological analysis of lung sections show that mice injected with control cells have larger tumors and a significantly higher number of lung lesions per mouse relative to miR-210-3p overexpression cells (Figure 3E). According to all the evidence mentioned above, we proposed that the over-expression of miR-210-3p might result in the inhibition of metastasis in bladder cancer cells.

Down-regulation of miR-210-3p promotes the growth and metastasis in bladder cancer cells

We used the inhibitor of miR-210-3p to simulate the down-regulation of miR-210-3p in T24 and SW780 cells. The levels of miR-210-3p after the transfection of inhibitor were measured by qRT-PCR (Figure 4A). The outcomes of MTT (Figure 4B) and colony formation (Figure 4C) assays showed that the down-regulation of miR-210-3p could significantly increase the proliferation in bladder cancer cells. In addition, contrary to the effect of miR-210-3p up-regulation, the down-regulation of miR-210-3p effectively promoted the migration and invasion in T24 and SW780 cells (Figure 4D and 4E). Finally, we transfected T24 and SW780 cells with the inhibitor of miR-210-3p, and subcutaneously implanted into BALB/c mice to figure out the actual effects of miR-210-3p on cell growth in vivo. It showed that the down-expression of miR-210-3p could dramatically accelerate the growth of tumors in vivo (Figure 4F). Finally, we tested the role of miR-210-3p in the metastasis of bladder cancer cells in vivo. miR-210-3p down-expression cells were injected into the tail veins of nude mice. Histological analysis of lung sections show that mice injected with miR-210-3p down-expression cells have larger tumors and a significantly higher number of lung lesions per mouse (Figure 4G). All the outcomes implied that the down-regulation of miR-210-3p could accelerate the growth of bladder cancer cells and induce metastasis in vitro and in vivo.

miR-210-3p directly binds to the 3’UTR of FGFR1

microRNAs generally regulate target gene expression by directly binding to its 3’UTR region. Targetscan (http://www.targetscan.org) was used to predict the target genes of miR-210-3p, and inspired us that FGFR1 might be the target of miR-210-3p (Figure 5A). For the purpose of verifying the 3’-UTR of FGFR1 as a direct target of miR-210-3p, the fragment of FGFR1 3’-UTR with either of the predicted wild type miR-210-3p binding sites (WT) was separately inserted into the downstream of the firefly luciferase in the p-MIR-reporter vector, and vector with either of the corresponding mutated binding sites (MUT) were also constructed. The relative luciferase activity in HEK 293 T cells co-transfected with miR-210-3p mimic and WT vector was sharply inhibited, while in those cells simultaneously transfected with miR-210-3p mimic and MUT vector, the luciferase activity was unaffected (Figure 5B). The results supported that miR-210-3p could directly inhibit the expression of FGFR1 by binding to the 3’-UTR of its mRNA. Furthermore, qRT-PCR (Figure 5C) and western blot (Figure 5D) assay suggested FGFR1 was higher in bladder cancer cell lines compared to SV-HUC-1 cells. We also analyzed the correlation between miR-210-3p levels and FGFR1 levels in bladder
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A. Relative mRNA expression of miR-210-3p.

B. Relative cell growth (% of control): T24 and SW780.

C. Inhibitor NC, inhibitor miR-210-3p, SW780.

D. T24 and SW780.

E. Inhibitor NC, inhibitor miR-210-3p.

F. Inhibitor NC, inhibitor miR-210-3p.

G. T24 and SW780.
Figure 4. Down-regulation of miR-210-3p promotes the growth and metastasis in bladder cancer cells. A. T24 and SW780 cells were transfected with miR-210-3p inhibitor or inhibitor NC. Total RNA was isolated and miR-210-3p expression levels were measured by qRT-PCR. B. MTT assay. The relative viabilities of T24 and SW780 cells transfected with miR-210-3p inhibitor were significantly higher than inhibitor NC transfected cells. C. Colony-formation assay. The colony formation rates of bladder cancer cells treated with miR-210-3p Inhibitor were higher compared with those treated with Inhibitor NC. D. Wound healing analysis of cell migration ability in vitro. miR-210-3p inhibitor promoted the progression of migration in bladder cancer cells. E. Cell invasion ability was measured by the Transwell matrigel invasion assay, the image (left) and quantitative analysis of the total invasive cells (right) were shown. F. Tumor xenograft model. The tumor volumes implied that the growth of tumors in miR-210-3p group was significantly larger than NC group. G. Representative hematoxylin and eosin staining of lung tissue collected from mice injected with control cells and miR-210-3p down-expressing cells. Arrowheads indicate tumors. Scale bar: 100 µm.
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Figure 5. FGFRL1 knockdown mimics the effects of miR-200c overexpression on cell growth and metastasis of bladder cancer cells. A. Predicted miR-210-3p target sequences in 3'UTR of FGFRL1. FGFRL1 is a direct and functional target of miR-210-3p. B. Dual-luciferase reporter assay. The relative luciferase activity in 293 T cells co-transfected with miR-200c mimic and wild vector (WT) was sharply inhibited, while in those cells simultaneously transfected with miR-210-3p mimic and mutant vector (MUT) the luciferase activity was unaffected. Error bars represent the S.D. from three independent experiments. *P < 0.05, compared to cells transfected with NC. C. Total RNA was extracted from several bladder cancer cells, and FGFRL1 expression level was measured by qRT-PCR. D. The expression level of FGFRL1 was detected by western blot in different bladder cancer cell lines. E. The expression of FGFRL1 was measured by IHC in clinical bladder cancer tissues. F. The correlation analysis of miR-210-3p and FGFRL1 expressions. G. Cell proliferation activity was measured by MTT assay (left panel) as described. Anchorage-independent growth ability was measured by the soft agar colony formation assay (right panel). H. Cell migration (left panel) invasion (right panel) ability was measured by the wound healing and Transwell Matrigel invasion assay as described.
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cancer specimens. In our study, we discovered that FGFRL1 was widely over-expressed in bladder cancer (Figure 5E), and there was a negative correlation between miR-210-3p levels and FGFRL1 mRNA levels (Figure 5F) illustrated by qRT-PCR assays. However, little is known about the role of FGFRL1 in bladder cancer progression, and whether FGFRL1 knockdown could mimic the effects of miR-210-3p on bladder cancer cells. Short hairpin RNA (shRNA) was applied to knock down the expression of FGFRL1 in T24 and SW780 cell line stably (Supplementary Figure 2). To define the role of FGFRL1 in bladder cancer, the effect of FGFRL1 knockdown on tumor cell growth (Figure 5G) and metastasis (Figure 5H) were examined. Stable knockdown of FGFRL1 in T24 and SW780 inhibited cell proliferation, migration and invasion, suggesting a probably oncogenic effect of FGFRL1 on bladder cancer growth, and FGFRL1 knockdown has similar effects on bladder cancer cells as miR-210-3p overexpression.

FGFRL1 plays a crucial role in miR-210-3p induced growth and metastasis inhibition of bladder cancer cells

In order to explore whether miR-210-3p inhibited bladder cancer proliferation and invasion through inhibiting FGFRL1, we constructed a cell line overexpressing miR-210-3p and FGFRL1 in T24 cells. qRT-PCR and western blot assay showed FGFRL1 was up-regulated and was not inhibited by miR-210-3p over-expressing (Figure 6A). MTT assay (Figure 6B) and soft agar growth assay (Figure 6C) revealed that double overexpression of miR-210-3p and FGFRL1 promoted bladder cancer cell proliferation compared to miR-210-3p overexpression alone. Wound healing (Figure 6D) and Transwell invasion (Figure 6E) assay revealed that double overexpression of miR-210-3p and FGFRL1 promoted bladder cancer cell invasion compared to miR-210-3p overexpression alone. All these results confirmed miR-210-3p inhibiting bladder cancer cell growth and metastasis in vivo by down-regulating FGFRL1 and the phenotype caused by miR-210-3p overexpression could be rescues by FGFRL1 overexpression. To further confirm the function of FGFRL1 in miR-210-3p induced growth inhibition, we transfected T24 cells with shFGFRL1, which specifically knock down the expression of FGFRL1 in T24 cells transfected with miR-210-3p inhibitor. It showed that the down-regulation of FGFRL1 could offset the acceleration of bladder cancer growth (Figure 7A) and colony formation (Figure 7B) induced by miR-210-3p down-expression. We also performed the wound healing and Transwell invasion assays to evaluate the effect of inhibition of FGFRL1 in T24 cells. It appeared that down-expression of FGFRL1 could reverse the promotion of cell migration (Figure 7C) and invasion (Figure 7D) caused by miR-210-3p inhibitor. Taken together, these in vitro results demonstrate an important role of FGFRL1 in miR-210-3p induced growth and metastasis inhibition of bladder cancer cells.

Discussion

Bladder cancer is one of the most severe forms of human malignant cancer and is the leading cause of cancer-related death worldwide. Although treatments options for bladder cancer have improved in recent years, the overall prognosis for patients with bladder cancer remains very poor. Therefore, in order to identify biomarkers and therapeutic targets in bladder cancer, investigates into the molecular mechanisms of bladder cancer progression are urgent. Recently, the roles of microRNAs (miRNAs) in several cancers draw attention, which brings about that the differential expression level and/or function of miRNAs become aberrant in the pathogenesis of cancer [17]. MicroRNAs are an endogenous group of small (18-25 nucleotides), non-coding RNA molecules, which play key roles including post-transcriptional regulators by binding to the 3′-untranslated region (3′UTR) of target gene. Recent results demonstrate that miRNAs modulate numerous tumor cellular processes, including cells differentiation, proliferation, survival and metastasis. Thus, miRNAs are considered to be potential regulators in the development and progression of cancers.

miR-210 has been implicated in a number of human cancers [18]. In pancreatic ductal adenocarcinoma, it has been observed that miR-210 exerts tumor suppressive effects, by modulating the tumor cells growth, apoptosis, epithelial-to-mesenchymal transition (EMT) and chemo-resistance of pancreatic ductal adenocarcinoma cells [19]. Similarly, previous studies in lung cancer demonstrated that miR-210 had an inhibitory effect on the cancer cells metastasis to bone tissue. It has also been
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Figure 6. The effect of miR-210-3p on bladder cancer can be rescued by FGFRL1 overexpression. A. The expression level of FGFRL1 in indicated cells was detected by qRT-PCR (left panel). Western blot was performed to assay the expression level of FGFRL1 (right panel). B. Cell proliferation activity was measured by MTT assay as described. C. Anchorage-independent growth ability was measured by the soft agar colony formation assay as described. The image (left) and quantitative analysis of colonies (right) were shown. **P < 0.05 compared to NC. ##P < 0.01 compared to miR-210-3p. Error bars, Mean ± SM. D. Cell migration ability was measured by the wound healing assay as described. The image (left) and quantitative analysis of the total invasive cells (right) were shown. **P < 0.05 compared to NC. ##P < 0.01 compared to miR-210-3p. E. Cell invasion was analyzed by the Transwell Matrigel invasion assay. The image (top) and quantitative analysis of the total invasive cells (low) were shown. **P < 0.05 compared to NC. ##P < 0.01 compared to miR-210-3p.
MiR-210-3p inhibits bladder cancer tumor growth and metastasis

Figure 7. The effect of miR-210-3p on bladder cancer cells growth and migration can be blocked by FGFRL1 knock-down. A. The expression level of FGFRL1 in indicated cells was detected by qRT-PCR (left panel). Western blot was performed to assay the expression level of FGFRL1 (right panel). B. Cell proliferation activity was measured by MTT assay as described. C. Anchorage-independent growth ability was measured by the soft agar colony formation assay as described. The image (left) and quantitative analysis of colonies (right) were shown. **P < 0.05 compared to inhibitor NC. ##P < 0.01 compared to inhibitor miR-210-3p. Error bars, Mean ± SM. D. Cell migration ability was measured by the wound healing assay as described. The image (left) and quantitative analysis of the total invasive cells (right) were shown. **P < 0.05 compared to inhibitor NC. ##P < 0.01 compared to inhibitor miR-210-3p. E. Cell invasion was analyzed by the Transwell Matrigel invasion assay. The image (top) and quantitative analysis of the total invasive cells (low) were shown. **P < 0.05 compared to inhibitor NC. ##P < 0.01 compared to inhibitor miR-210-3p.
proved in glioblastoma that miR-210 promotes the glioma-initiating cells growth and self-renewal [20]. Furthermore, miR-210 is up-regulated in chemo-resistant osteosarcoma and promotes resistance of osteosarcoma cells to cisplatin through suppressing of the transcription factor Twist. In additional, the levels of miR-210 expression was elevated in steatotic chronic hepatitis C when compared to control liver tissue, and miR-210 in liver tissue has been found to significantly increase in a fibrosis progression-dependent manner [21]. Collectively, these data indicate an oncogenic role of miR-210, though its clinical significance and potential roles in bladder cancer remain unknown. In the present study, significant down-expression of miR-210-3p was confirmed in bladder cancer tissues. All these results from our experiments indicate that miR-210-3p may serve an oncogenic role in bladder cancer and as a potential biomarker for the diagnosis and prognostic prediction of bladder cancer.
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**Supplementary Figure 1.** Xenografted tumors were isolated. Total RNA was collected and miR-210-3p expression levels were measured by qRT-PCR.

**Supplementary Figure 2.** To knock down FGFRL1, shFGFRL1 and shControl was transfected T24 and SW780 cell lines. The FGFRL1 expressing level was detected by qRT-PCR.