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lncRNA-RMRP promotes proliferation, migration and invasion of bladder cancer via miR-206

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Abstract. – OBJECTIVE: The incidence of bladder cancer (BC) is common in the world, but its detail mechanisms for occurrence and development remain unclear. Recently, long non-coding RNAs (lncRNAs) have been observed to play an important role in many different diseases. In this research, we mainly explored the role of the RNA component of mitochondrial RNA processing endoribonuclease (lncRNA-RMRP) in bladder cancer.

MATERIALS AND METHODS: We used qRT-PCR to detect the expression of lncRNA-RMRP in bladder cancer patients and tumor cells, and the clinical significance was also analyzed. The methyl thiazolyl tetrazolium (MTT) assay was used to detect the cell proliferation, and we used transwell to detect the migration and invasion, after the lncRNA RMRP was inhibited. Western-blot was used to measure the relative protein expression level in bladder cancer cells after transfection with siRNA-NC or siRNA-RMRP.

RESULTS: We found that the lncRNA RMRP was highly expressed in bladder cancer tissue, compared with adjacent tissue. We also found that the expression of RMRP was closely related with the size, lymph node metastasis and survival time of patients. What's more, RMRP could promote the proliferation, migration and invasion of BC cell lines via regulating miR-206 as a sponge.

CONCLUSIONS: According to the results, we found that lncRNA RMRP was closely related to the progression of bladder cancer, which could be a potential target for treating BC patients.

Key Words:
Bladder cancer, miR-206, lncRNA-RMRP, Migration, Invasion, Proliferation.

Introduction

Bladder cancer (BC) is the most common malignant tumor in the urinary system and it is the fourth of male malignant tumors in European and American countries[1]. Recently, it has increased its morbidity and rejuvenation. BC risk factors are smoking and long-term exposure to industrial chemical products, which are related to the changes in the gene level of patients; moreover, the variety of apparent heredity is involved in the development of tumors[2]. The occurrence and development of bladder cancer is a chronic process with multi-factors and multi-steps[3]. Thus, investigating the detail mechanisms involved in BC bladder cancer will provide a novel strategy for its prevention and treatment. Long noncoding RNAs (lncRNAs) are new members in the oncology field, containing 200 nucleotide (nt)-long RNA molecules and stably existing in the plasma and urine, with disease and tissue specificity and with no protein-coding potential[4-6]. At the present, they are being studied to suggest a potentially pivotal role in tumor occurrence and inhibition pathways[7,8]. The lncRNAs are able to identify complementary sequences, and this particular role is important to RNA in the post-transcription, which involves cutting, editing, transport and translation[9,10]. Wang et al[11] found that lncRNA-HULC can be used as molecular bait, or can be called miRNA sponge, which can influence the expression of target protein RPKACB by regulating the expression of miR-372 in bladder cancer. Moreover, Yuan et al[12] suggested that TGF-beta promoted the expression of lncRNA-ATB in hepatocytes, which could play the function of microRNA sponge. It could be specific to the combination of miR-200 family, thus inhibiting miR-200s as the function of anticancer microRNA, improving significantly the expression of CDK9/2, which is target gene of miR-200s, and promoting the migration of bladder cancer[12]. The RNA component of mitochondrial RNA processing endoribonuclease (RMRP), a lncRNA, was first discovered in cartilage-hair hypoplasia

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(CHH), an autosomal recessive inherited disease[13]. Recently, some studies also found that lncRNA-RMRP played a very important role in tumor. Shao et al[14] demonstrated that lncRNA-RMRP play a crucial role in the occurrence and progression of gastric cancer by acting as a miR-206 sponge. However, whether the lncRNA-RMRP executed its function in bladder cancer remains largely unclear. In this work, we aimed to detect the expression of lncRNA RMRP in the bladder cancer and explored the possible mechanism of lncRNA RMRP affecting the malignant activity of bladder cancer.

Patients and Methods

Patients

91 cases of bladder cancer (BC) tissue and adjacent tissue were collected from Beijing Rehabilitation Hospital Affiliated to Capital Medical University in Beijing City (Beijing, China) from May 2016 to June 2017. All of the patients were well informed and informed consents were also signed. The tissues were divided into equal size and frozen in liquid nitrogen after surgery. The experiment protocol was approved by the Institutional Review Board of the Beijing Rehabilitation Hospital Affiliated to Capital Medical University (Beijing, China).

Cell Culture

Normal urothelial cell line SV-HUC-1 and bladder cancer cells BIU-87, T24 were purchased from the Institute of Cell Research, Chinese Academy of Sciences (Shanghai, China). The SV-HUC-1 and T24 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) plus 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA). The BIU-87 was cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) Medium plus 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA). All cell lines were placed at 37°C with a humidified atmosphere of 5% CO_2 in incubator.

RNA Extraction and Real-Time Quantitative PCR Assays

Total RNA from tissue samples and transfected cells was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. Reverse transcription polymerase chain reaction (RT-PCR) was performed using ABI PRISM 7000 Fluorescent Quantitative PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The GAPDH was used as a reference gene. The PCR primers for RMRP, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: RMRP, 5′-ACTCCAAAGTCCGCCAAGA-3′ and 5′-TGC GTAACTAGAGGGAGCTGAC-3′; GAPDH, 5′-ACCCACTCCTCCACCTTTGAC-3′ and 5′-TGTTGCTGTAG CCAAATTCGTT-3′. All experiments were repeated at least three times.

CCK8 Assays

The Cell Counting Kit-8 (Beyotime Inst. Biotech, Shanghai, China) was used to determine the cell proliferation, according to the manufacturer's instructions. Three replicate wells were set in each group. CCK8 assay was done as follows: the 96-well flat-bottomed plate was used to seed 6×10^3 cells/well, and grown at 37°C for 24 h, then transfected with corresponding vector. Finally, the absorbance was determined at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The data were collected for 3 days. All experiments were repeated at least three times.

Transwell Assay

The transwell insert (8 μm, Corning, Corning, NY, USA) was used to determine the invasion BC cells. After 24 h transfection, 5×10^4 cells were first starved in 200 ml serum free medium and then seeded in the dishes. The lower chamber was filled with 500 ml of completed medium. We incubated the cells for 48 h at 37°C, which had migrated to the bottom surface of the filter membrane; next, they were stained with 0.5% crystal violet solution and photographed. At last, the absorbance was determined at a wavelength of 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The whole experiments were repeated at least three times.

Luciferase Assays

Wt-RMRP/mut-RMRP sequences were amplified and cloned into the downstream of the stop codon of the firefly luciferase in basic vector (Promega, Madison, WI, USA).

Total RNA was extracted from HEK293 cells, which was reverse transcribed into cDNA. The potential binding sites of pmiR-RMRP-WT and mutant sequence pmiR-RMRP-MT were synthesized into pmiR-GLO (Promega, Madison, WI, USA). After that, miR-206 mimics and miR-206 negative control (NC) were co-transfected into

HEK293 cells with pmiR-GLO for 24 h. Renilla expression vector was transfected into each group to serve as a normalized control. 48 h after transfection, firefly and Renilla luciferase activities were measured using Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Western Blot Assays

Protein was extracted by RIPA lysis buffer containing phenylmethylsulfonyl fluoride (PMSF) (Beyotime Biotechnology, Shanghai, China). The concentration of protein was detected by the standard bovine serum albumin (BSA) protein quantitation assay. 60 μg of samples were added to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% denaturing gel. The protein was transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) after electrophoresis, and it was blocked in the 5% non-fat milk for one half of an hour at room temperature. TBST (Boster, China) was used to wash these membranes, and then the membranes were put into the primary antibody at 4°C overnight. We used the second antibody to incubate these membranes, respectively. Immunoblots were visualized by ECL chemiluminescent detection system. The protein bands were quantified by densitometry analysis in Quantity One software (Bio-Rad, Hercules, CA, USA), which were analyzed by GraphPad Prism software (GraphPad, La Jolla, CA, USA).

Statistical Analysis

All statistical analyses were performed with Statistical Product and Service Solutions (SPSS) 20.0 software (SPSS Inc., Armonk, NY, USA). GraphPad Prism 7.0 (GraphPad, La Jolla, CA, USA) software was used to draw graphs. The data was expressed as the mean±SD, and each assay was applied at least three independent experiments or replicates. Multiple comparisons between groups were performed using S-N-K method. Student's *t*-test, one-way analysis of variance (ANOVA) and the rank-sum test were used by actual conditions. p-value<0.05 was regarded as statistically significant.

Results

LncRNA RMRP was Highly Expressed in Heatmap and Patients with BC

To explore the differentially expressed lncRNA of bladder cancer, we used the Affymetrix GeneChip Human Gene 2.0 ST Array to scanning fluorescence.

Using small sample statistical learning and secondary test of random sampling, we found that RMRP was highly expressed in heatmap of bladder cancer (Figure 1A). Furthermore, we used qRT-PCR to detect the expression of RMRP in BC tissues and adjacent tissues (Figure 1B). The results showed that RMRP was high expressed in BC tissues compared with adjacent tissues.

The Clinical Characteristic of RMRP

In order to explore the clinical significance expression of RMRP in BC, we investigated the clinical characteristic of RMRP. We measured the expression level of RMRP from the clinic pathological information of the patients. The results revealed that RMRP was significantly higher in tumor size > 3 cm compared with the tumor size < 3 cm (Figure 2A). Meanwhile, it was dramatically higher in lymph node metastasis compared with non-lymph node metastasis (Figure 2B). Moreover, we analyzed the relationship between the expression of RMRP and the survival time of BC patients. This result indicated that the BC patients with low RMRP expression showed a better prognosis when compared with high level of RMRP (Figure 2C). Collectively, our findings suggested that the expression of RMRP was negatively correlated with the survival time of patients with BC. The lncRNA RMRP was an important prognostic index for BC patients.

Suppression of RMRP may Reduce Proliferation, Migration and Invasion in BC Cell Lines

To further study the role of lncRNA RMRP in BC, we continued to research the function of RMRP in cell proliferation, migration and invasion. Previously, we already found that lncRNA RMRP was closely related with tumor size and lymph node metastasis of BC patients. According to the above results, we investigated whether RMRP could influence the proliferation, migration and invasion of BC cell lines. Firstly, we used the qRT-PCR to check the expression of RMRM in BC cell lines. It was said that RMRP was higher expressed in BIU-87 and T24, compared with SV-HUC-1 (Figure 3A). And we transfected siRNAs into BIU-87 and T24, whose expression of RMRM were down-knocked compared with control group (Figure 3B). CCK-8 was used to detect the proliferation of BIU-87 and T24. We found that silencing the expression of RMRP in BIU-

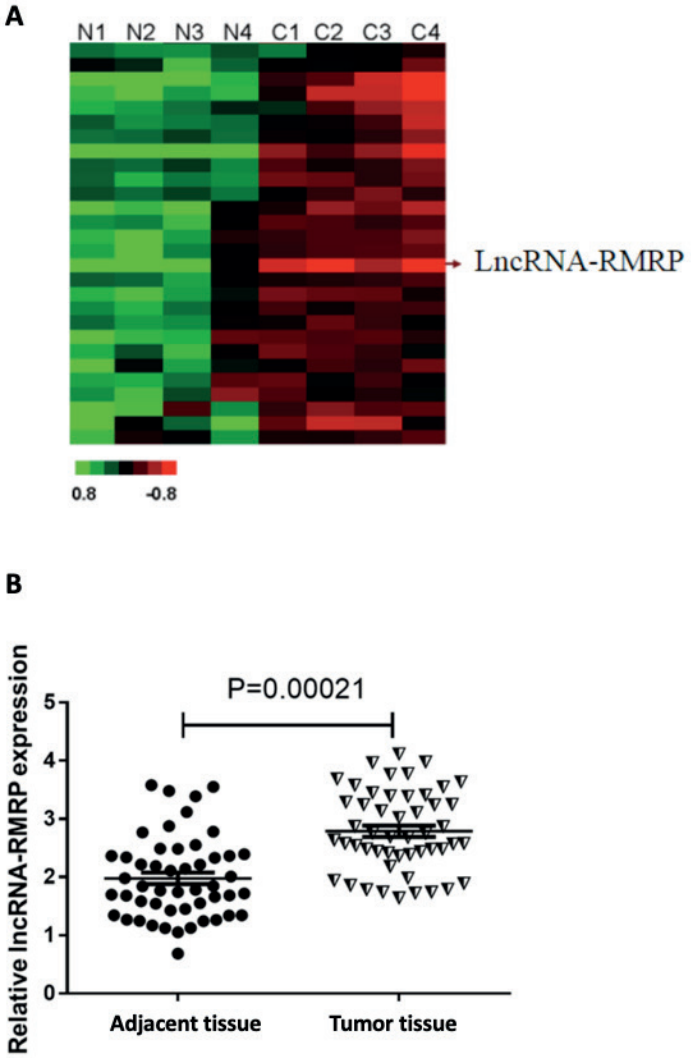

Figure 1. LncRNA RMRP was highly expressed in heatmap and patients of BC. ***(A)*** LncRNA-RMRP was highly expressed in heatmap of bladder cancer. ***(B)*** The expression of RMRP in the BC tissue and adjacent tissue was detected by qRT-PCR assay. *** $p<0.001$.

87 and T24 would lead to the decrease of proliferation of BIU-87 and T24 (Figure 3C-D). Then, we indicated that the migratory and invasion capacity of BIU-87 and T24 cells transfected with siRNA-RMRP were significantly down-regulated in comparison with the control group (Figure 3E-F). These results revealed that the expression of RMRP had a close relation with the proliferation, migration and invasion in BC cell lines.

Overexpression of RMRP Improved the Abilities of Proliferation, Migration and Invasion in BC Cell Lines

To further validate the oncogenic roles of RMRP in BC, we constructed BC cell lines with the plasmid of lncRNA RMRP stable over-expression. We found that RMRP was overexpressed in BIU-87 and T24 within overexpression vector (Figure 4A). Subsequently, we checked the ability of proliferation, migration and invasion in overexpressed BC cell lines (Figure 4B-E). The results indicated that the RMRP overexpressed in BIU-87 and T24 were higher ability of proliferation, migration and invasion. In conclusion, the expression of lncRNA RMRP was associated with the progression of BC *in vitro*.

LncRNA RMRP Could Directly Binds with miRNA-206 in BC Cells

Accumulated reports demonstrated that lncRNA play important role in regulating gene expression by acting as miRNA sponges. Shao et al[14] indica-

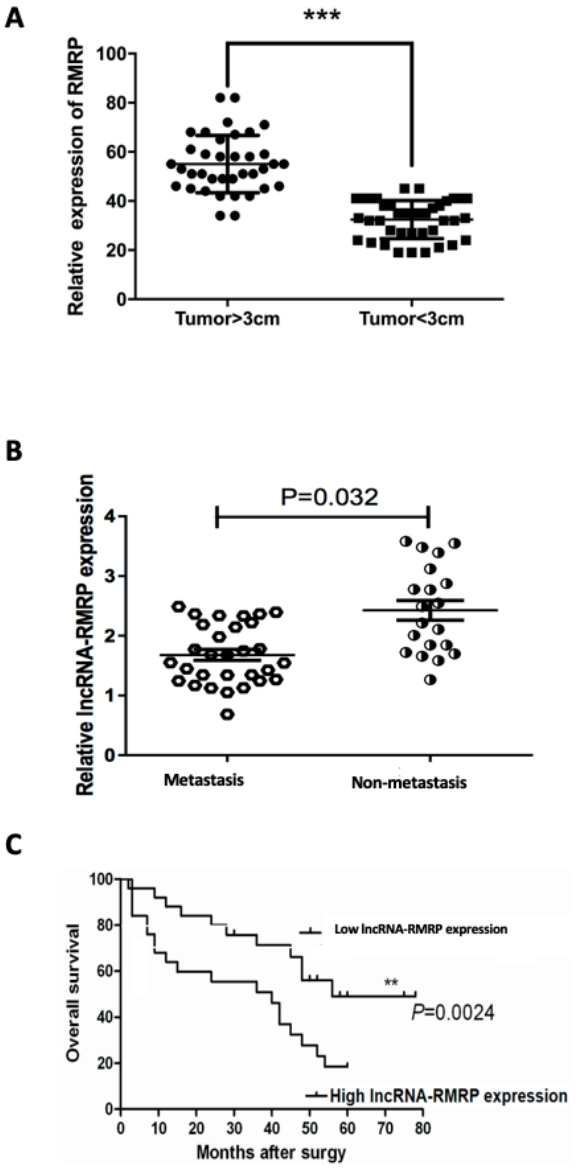

Figure 2. The clinical characteristic of RMRP. ***(A)*** The expression of RMRP in BC tissue was detected according to the lymph node metastasis in tumor. * $p<0.05$. ***(B)*** The expression of RMRP in BC tissue was detected according to the tumor size. *** $p<0.001$. ***(C)*** Association between patient's survival time and expression of RMRP. ** $p<0.01$.

ted that RMRP promotes carcinogenesis by acting as a miR-206 sponge in gastric cancer. To explore whether RMRP could bind with miR-206, we used luciferase reporter assay to work. We synthesized the potential binding sequence into pmiR-GLO vector, which was pmiR-RMRP-WT. And the mutant binding sequence was also synthesized into pmiR-GLO vector, which was pmiR-MEG3-MT (Figure 5A). After miR-206 mimics and miR-206 NC were transfected into BIU-87 cells for 24 h, we checked the relative luciferase activity. We demonstrated that the luciferase activity of cells transfected with pmiR-RMRP-WT was significantly decreased, compared with pmiR-GLO vector. And the luciferase activity in pmiR-RMRP-MT was increased, compared with transfected with pmiR-RMRP-WT ($p<0.01$) (Figure 5B). To further investigate the potential association and oncogenic functions between RMRP and miR-206, we used 20 serum specimens of BC patients to study the correlation between the expression of RMRP and miR-206. The data indicated that the expression of miR-206 was negatively correlated with the expression of RMRP (Figure 5C). Then, we overexpressed RMEP and found that significantly suppressed the expression of miR-206; meanwhile, we inhibited the expression of RMRP and the expression of miR-206 was increased (Figure 5D-E). In summary, lncRNA RMRP was a target gene of miR-206, which acted as an endogenous "sponge" and promoted proliferation, migration and invasion in BC cell lines.

Discussion

Bladder cancer is the most common genitourinary deadliest tumor worldwide[15-17]. There are no remarkable symptoms for BC patients at the early stage so that the treatments for bladder cancers are less effective[18-20]. Hence, searching new prognostic and therapeutic targets are crucial to develop the clinical strategies and outcomes of bladder cancer. Growing evidence shows how long non-coding RNAs (lncRNAs) involving development, diagnosis, and prognosis in tumor have been discovered. The lncRNAs are important emerging members of non-coding RNA family, which are longer than 200 nucleotides[21]. An increasing number of evidences have demonstrated that lncRNAs modulate gene expression at different processing levels, including chromatin modification, transcription and posttranscriptional regulation[22]. Liang et al[23] indicated that lncRNA H19 modulated the expression of multiple genes involved in EMT by acting as a competing endogenous RNA, which may build up the missing link between the regulatory miRNA network and EMT progression. Zhou et al[24] found that H19 and miR-141 could compete with each other and affect of their target genes in gastric cancer, providing important clues for understanding the key roles of lncRNA-miRNA functional network in

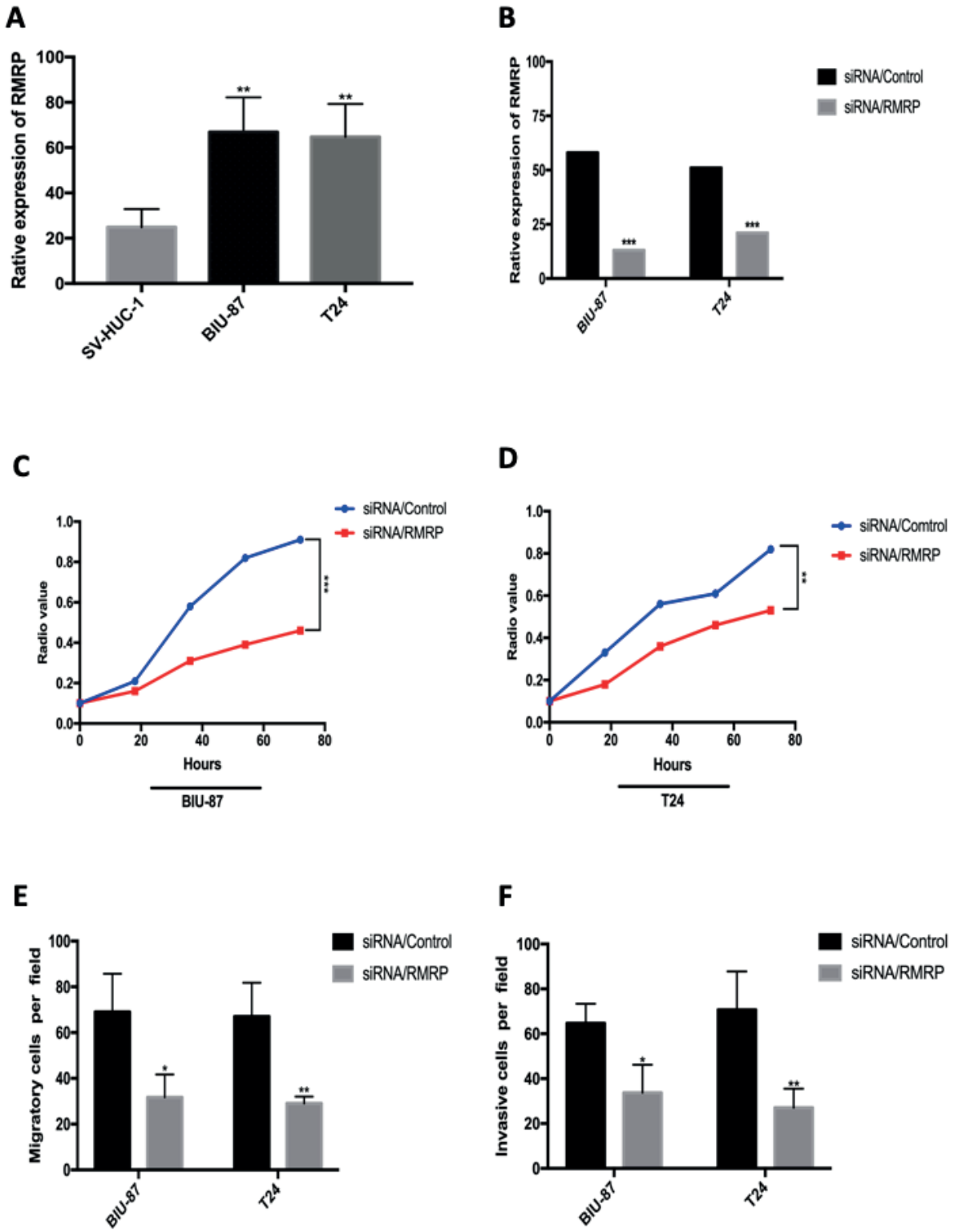

Figure 3. Suppression of RMRP may reduce of proliferation, migration and invasion in BC cell lines. (A) qRT-PCR was used to measure the expression level of RMRP in the treated BIU-87 and T24 cells. ** $p<0.01$. (B) The expression of RMRP was suppressed in the BC cell lines. *** $p<0.001$. (C) (D) CCK8 assays were used to detect the proliferation ability of BC cell after RMRP was suppressed. *** $p<0.001$, ** $p<0.01$. (E) (F) Transwell assay were used to detect the migration and invasion of BC cell after RMRP was suppressed. ** $p<0.01$, * $p<0.05$.

cancer. Zhan et al[25] showed that DANCR plays a critical regulatory role in bladder cancer cell and may serve as a potential diagnostic biomarker and therapeutic target of BC.

These studies proved that lncRNAs were important in regulating the progression of tumor and other diseases. In addition, all the reports indicated that lncRNAs can target a series of miRNA,

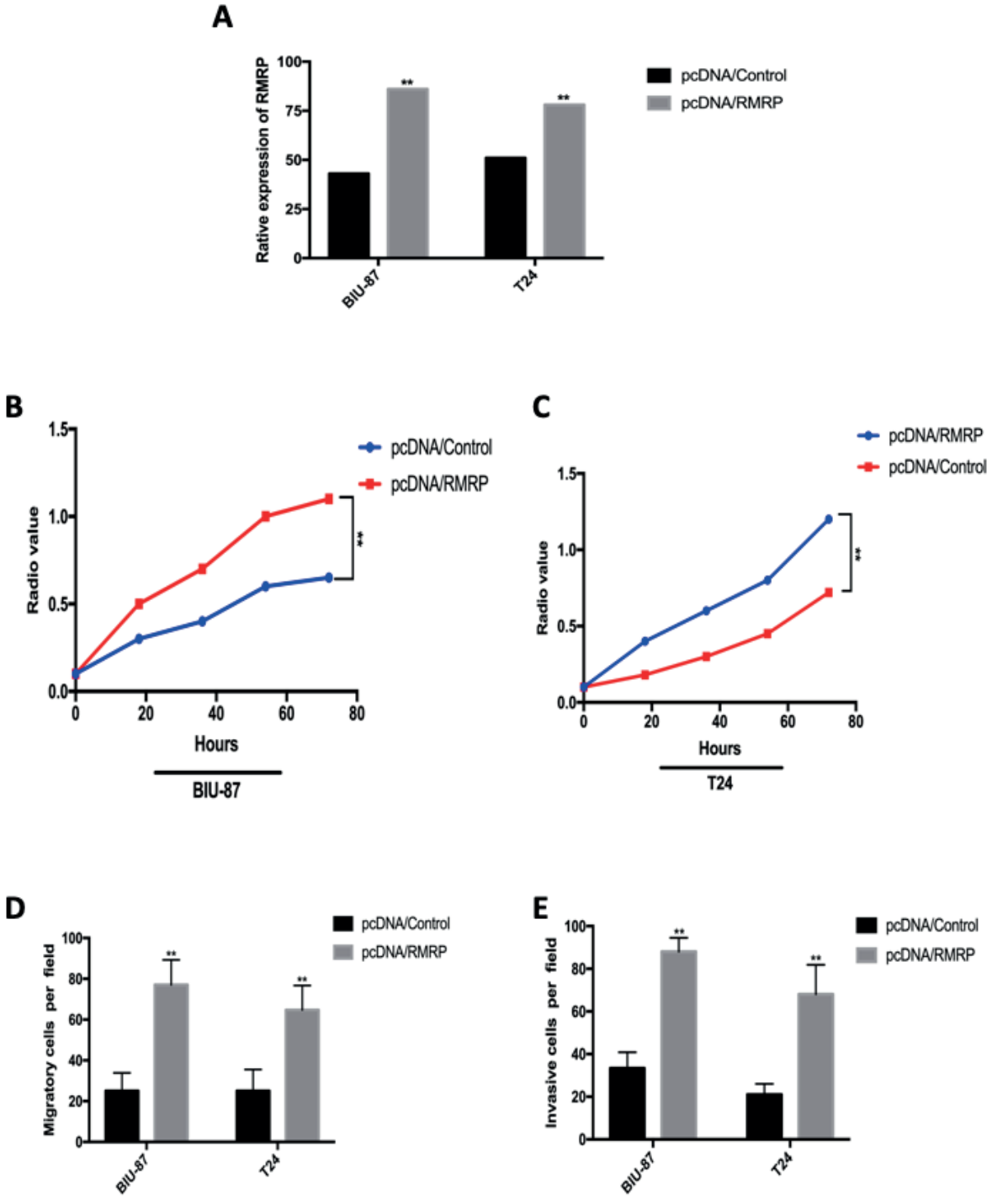

Figure 4 Overexpression of RMRP improved the abilities of proliferation, migration and invasion in BC cell lines. ***(A)*** The expression of RMRP was overexpressed in the BC cell lines. ** $p<0.01$. ***(B-C)*** CCK8 assays were used to detect the proliferation ability of BC cell after RMRP was overexpressed. ** $p<0.01$. ***(D-E)*** Transwell assay was used to detect the migration and invasion of BC cell after RMRP was overexpressed. ** $p<0.01$.

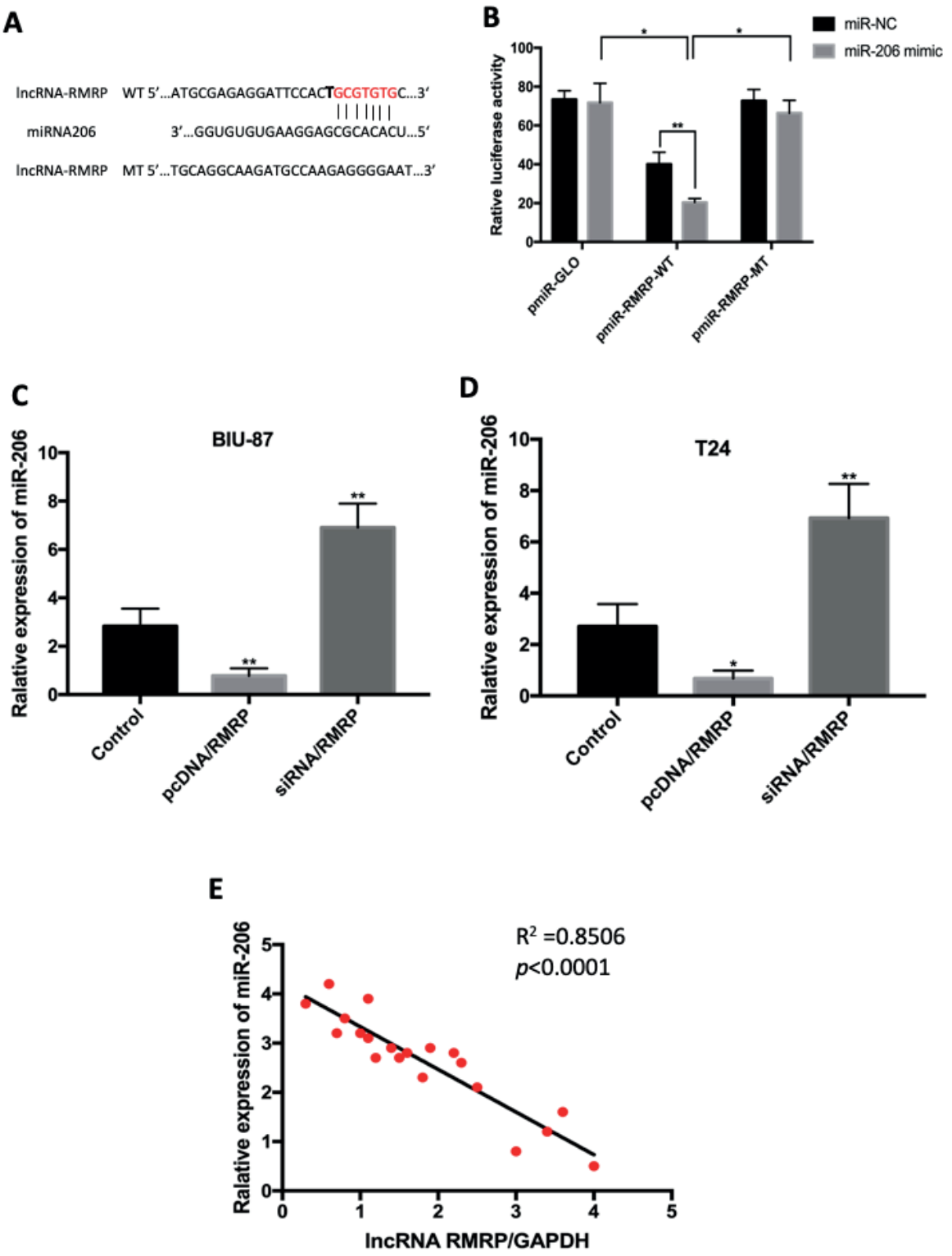

Figure 5. LncRNA RMRP could directly binds with miRNA-206 in BC cells. ***(A)*** Bioinformatics analysis predicted binding sites between RMRP and miRNA-206. ***(B)*** The luciferase reporter assay. Co-transfection with miR-206 and RMRP WT significantly increased the luciferase activity of BIU-87 cells compared with others. $^{**}p<0.01$, $^{*}p<0.05$. ***(C-D)*** Up-regulation of LncRNA RMRP significantly reduced the expression of miRNA-206, while knocked-down of LncRNA RMRP significantly increased the expression of miRNA-206 in BC cells. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$. ***(E)*** The LncRNA RMRP expression level was negatively correlated with miRNA-206 expression in BC patients. $R^2=0.8506$, $^{****}p<0.0001$.

through the combination with miRNA and inhibiting the negative regulation function of miRNA. Thus, it could promote the expression of oncogene and lead to the development of tumor.

LncRNA RMRP was first discovered in cartilage-hair hypoplasia (CHH), an autosomal recessive inherited disease[13]. In gastric cancer, RMRP also plays a crucial role in the occurrence and progression[14]. However, the clinical significance and biological function of RMRP in bladder cancer remain unknown.

To the best of our knowledge, this is the first report of RMRP being participated in the progression of bladder cancer. In our study, we found that RMRP was up-regulated in heatmap of bladder cancer. Then, we demonstrated the expression level of RMRP was significantly higher in bladder cancer tissues compared with adjacent tissues, and the increased RMRP expression was closely correlated with tumor size, lymph node metastasis in BC patients. Furthermore, BC patients with high expression of RMRP have a poor survival rate. Mechanistically, the RMRP was significantly overexpressed in bladder cancer cell lines, compared with normal urothelial cell line. Furthermore, our data demonstrated that knockdown or upregulation of the expression of RMRP would inhibit or improve malignant phenotypes (proliferation, migration, and invasion) of bladder cancer cells. These findings indicate that lncRNA RMRP may be a novel therapeutic strategy to treat human bladder cancer.

As reported by Shao et al[14], RMRP promoted carcinogenesis by acting as a miR-206 sponge in gastric cancer. Thus, we studied whether miR-206 was correlated with the expression of lncRNA RMRP. In this research, we validated RMRP and miR-206 and they had a negative correlation. Finally, we transfected lncRNA RMRP inhibitor, which significantly up-regulated the expression of miR-206 and transfected with RMRP promoter significantly decreasing the expression of miR-206. Taken together, these results indicate that RMRP acts as a miR-206 sponge regulating the progression of BC.

Conclusions

We firstly found that lncRNA-RMRP plays a crucial role in the occurrence and progression of bladder cancer *in vivo* and *in vitro*. Thus, RMRP may be a potential target for treatment and predicting the prognosis of bladder cancer.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgement

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