miR-221-induced PUMA silencing mediates immune evasion of bladder cancer cells

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Abstract. Immune evasion of cancer cells is mainly due to the impaired transduction of apoptotic signals from immune cells to cancer cells, as well as inhibition of subsequent apoptosis signal cascades within the cancer cells. Over the past few decades, the research has focused more on the impaired transduction of the apoptotic signal from immune cells to cancer cells, rather than inhibition of the intracellular signaling pathways. In this study, miR-221 inhibitor was transfected into bladder cancer cell lines 5637, J82 and T24 to repress the expression of miR-221. As a result, the repression of miR-221 on p53 upregulated modulator of apoptosis (PUMA) was abolished, resulting in increased expression of the pro-apoptotic Bax and reduced expression of the anti-apoptotic Bcl-2, which promotes apoptosis of bladder cancer cells. The expression of MMP-2, MMP-9 and VEGF-C were reduced, resulting in reduced invasiveness and infiltration capability of bladder cancer cells, thereby inhibiting the immune evasion of bladder cancer cells.

Introduction

Bladder cancer is the most common cancer of the urinary system and is showing each year increases in morbidity and mortality (1,2). During tumorigenesis and the cancer development, bladder cancer cells and the host go through three stages: immune surveillance, equilibrium and immune evasion (3). Clinical treatment of bladder cancer is often impeded by immune evasion (4).

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Previous studies have shown that impaired apoptotic signal transduction is one of the important factors promoting immune evasion by cancer cells (5). Accumulating evidence shows the apoptotic signal transduction from immune cells to bladder cancer cells is impaired, resulting in the immune evasion of bladder cancer cells. For instance, antigen B7-H1 binding to PD-1 results in functional inhibition of T cells and B cells, inhibition of body-peculiar cellular and humoral immunity, and induction of apoptosis in specific cytotoxic T lymphocytes (CTL), all of which enable immune evasion of bladder cancer cells and promote the growth of bladder tumors (6). Changes in the apoptosis receptor Fas/FasL also enable evasion of the apoptotic effect from CTL cancer cells (7). Increased expression of TGF-β-1 leads to the escape of bladder cancer cells from host immune surveillance (8), while deletion or mutation of the MHC-1 gene blocks the apoptotic response from T cells and NK cells (9).

However, immune evasion of cancer cells cannot be completely attributed to the blockage of apoptosis signal transduction from immune cells to cancer cells. It also involves the impaired apoptosis signaling transduction in the cancer cells themselves (9-11). In 2002, microRNA was found to regulate the proliferation and apoptosis of tumor cells (11). Aberrant expression of microRNA in bladder cancer cells was first reported in 2007 (12). Recent studies indicate that there are no binding sites for microRNAs in cell membrane receptor genes or their ligands in cancer cells, many microRNA binding sites are present the downstream signaling pathway effector genes. For example, upregulated miR-221 in malignant bladder tumor inhibits $p27^{kip1}$ activity, promote proliferation of bladder cancer cells, and inhibits TRAIL-mediated apoptosis signaling (13,14). Given that a microRNA can function on multiple target mRNAs, we hypothesize that miR-221 may silence target mRNAs of other genes and suppress the apoptosis of bladder cancer cells as a result, which would further enable immune evasion by bladder cancer cells. From www.targetscan.org and www.mirbase.org, we found that PUMA, a pro-apoptotic protein that promotes apoptosis might be another target of miR-221. miR-221 and miR-222 have also been found to specifically target PUMA and promote the

survival of malignant glioma cells (15). Thus, we hypothesize that increased expression of miR-221 in bladder cancer may inhibit the apoptosis of bladder cancer cells through silencing PUMA and maintain the continuous proliferation of bladder cancer cells, leading to their immune evasion.

The major consequences of immune evasion of tumor cells are invasive growth and/or metastasis of tumor cells (16). Previous studies have shown that the degradation and destruction of the extracellular matrix and basement membrane of the tumor surface is the basis for tumor invasion and metastasis (17,18). Matrix metalloproteinases (MMPs) constitute a family of enzymes that are responsible for depredating the extracellular matrix and are closely related with tumor invasion and metastasis. MMP-2 and MMP-9 are two of the most important MMP proteins. MMP-9 degrades and destroys extracellular matrix and basement membrane near the surface of tumor cells, promotes generation of new blood capillaries, tumor growth and migration. MMP-2 degrades types IV, IV, VI and X collagen (19-22). Vascular endothelial growth factor (VEGF) is the most potent known angiogenic factor and plays an important role in the invasive growth and metastasis of many human malignancies (23-25). VEGF can be classified into seven subtypes, namely VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and PIGF, among which VEGF-C is closely related to tumor invasion and metastasis. VEGF-C specifically binds to its cognate receptor on endothelial cells and facilitates mitotic proliferation of endothelial cells, enhances the permeability of blood vessels, changes gene expression in endothelial cells, and thus promotes the synthesis of MMPs (26-28). Therefore in this study, we directly measured the expression of VEGF-C, MMP-2 and MMP-9 in tumor cells to determine the invasion and metastasis state of tumor cells, to indirectly probe if tumor cells are capable of immune evasion.

Materials and methods

Cell culture. Human bladder cancer cells (T24, 5637 and J82) were obtained from the China Academia Sinica Cell Repository, Shanghai, China. T24 and 5637 cell lines were maintained in RPMI-1640, J82 was maintained in MEM. Both media were supplemented with 10% heat-inactivated fetal bovine serum without antibiotics at 37˚C in a humidified incubator with 5% CO_2. All *in vitro* experiments were performed in triplicate.

Transient transfection. The FAM-modified 2'-OMe-oligonucleotides were chemically synthesized and purified by high-performance liquid chromatography by Invitrogen. The sequence of the 2'-O-me-miR-221 inhibitor is: 5'-GAA ACC CAG CAG ACA AUG UAG CU-3'. The FAM-modified scrambled oligonucleotides are RNA duplexes with the following sequences: 5'-UUC UCC GAA CGU GUC ACG UTT/ACG UGA CAC GUU CGG AGA ATT-3'. FAM was attached to the 5' end of each oligonucleotide. When cells were grown to 50-60% confluence, oligonucleotides transfection was performed using the Lipofectamine™ 2000 transfection reagent (Invitrogen, USA) according to the manufacturer's instructions. At 6 h after transfection, the medium was replaced with fresh medium containing 10% fetal bovine serum. The experiments consisted of three groups: i) blank (without treatment), ii) negative control (transiently transfected with scrambled oligonucleotide), and iii) transfection of miR-221 inhibitor (transiently transfected with miR-221 inhibitor).

Cell viability assay. Cell viability was tested by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded into 96-well plates at a density of 10,000 cells/well in 100 μl culture medium and cultured overnight before transfection. Cells received fresh medium at 6 h. After 24 h, 20 μl MTT (5 mg/ml dimethyl thiazolyl diphenyl tetrazolium, Sigma) was added into each test well and incubated for 4 h in the humidified incubator. Formazan crystals formed by viable cells were dissolved in 150 ml dimethyl sulfoxide (Solarbio) and their absorbance values were measured at 490 nm. Wells without cells (DMSO alone) were used as a background control. The final optical density (OD) was calculated according to the formula: (final optical density = optical density of each group - optical density of DMSO group). Each test was performed daily for three consecutive days and repeated in five wells.

Quantitative real-time PCR. T24, 5637 and J82 cells were transfected for 24 h as described above. Total RNA was extracted using TRIzol (Invitrogen Life Technologies, Shanghai, China) according to the manufacturer's protocol. RNA quality was determined by running a sample with RNA loading dye (Sigma-Aldrich) on a 1% agarose gel and inspecting for distinct 18S, 28S and total RNA bands which indicate a lack of degradation. The quantity of RNA was determined by A260 measurement.

To evaluate miR-221 expression levels, quantification using the SYBR Green microRNA assay was performed using two-step RT-PCR according to the manufacturer's instructions. In the reverse transcription (RT) step, cDNA was reverse transcribed from the total RNA sample using specific miR-221 primers from the Bulge-Loop™ hsa-miR-221-5p qRT-PCR Primer Set (RiboBio, Guang Zhou, China) and the Reverse Transcription System (Takara, Dalian, China). In the polymerase chain reaction (PCR) step, PCR products were amplified from cDNA samples using the Bulge-Loop™ hsa-miR-221-5p qRT-PCR primer set and using SYBR® Premix Ex Taq™ II (TliRNase H Plus, Takara) in the ABI PRISM® 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The real-time PCR results were normalized against an internal control U6 and relative expression levels were evaluated using the 2-ΔΔCt method and then expressed as fold changes.

For qRT-PCR, 1 μg total RNA was used in the Reverse Transcription System (Takara) according to the manufacturer's instructions, and PCR was performed in the ABI PRISM® 7500 real-time PCR system (Applied Biosystems). The sequences of gene-specific primers are shown in Table I. The expression level of β-actin was used as internal control. All reactions were performed at least in triplicate.

Invasion assays. The invasion assay was performed in 24-well transwell plates. Type I collagen was coated on the upper chamber to reconstitute the basement membrane. Three groups (transfection of miR-221 inhibitor, negative control and control) of cells ($1x10^5$ per well) were seeded on the upper chamber, and the lower compartment was filled with RPMI-1640 (T24

Table I. Oligonucleotide sequences for PCR amplification.

Gene	PubMed no.	Sequence (5'-3')		Product size (bp)
Bax	NM_138764.4	F: ACCAGGGTGGTTGGGTGAGACT	R: CACCACTGTGACCTGCTCCAGA	136
Bcl-2	NM_000633.2	F: CCAGCATGCGGCCTCTGTTTGA	R: TGGGGCAGGCATGTTGACTTCAC	129
PUMA	NM_001127241.1	F: GCGGGGAGGAGGAACAGT	R: TGTGGCCCCTGGGTAAGG	177
MMP-2	NM_004994.2	F: CCTCTCCACTGCCTTCGATA	R: TGGGAGGAGTACAGTCAGCA	129
MMP-9	NM_001127891.1	F: CTGCAGTGCCCTGAGGACTA	R: ACTCCTCCCTTTCCTCCAGA	135
VEGF-C	NM_005429.2	F: GGCTGGCAACATAACAGAGAA	R: CCCCACATCTATACACACCTCC	159
β-actin	NM_001101.3	F: CATGTACGTTGCTATCCAGGC	R: CTCCTTAATGTCACGCACGAT	250

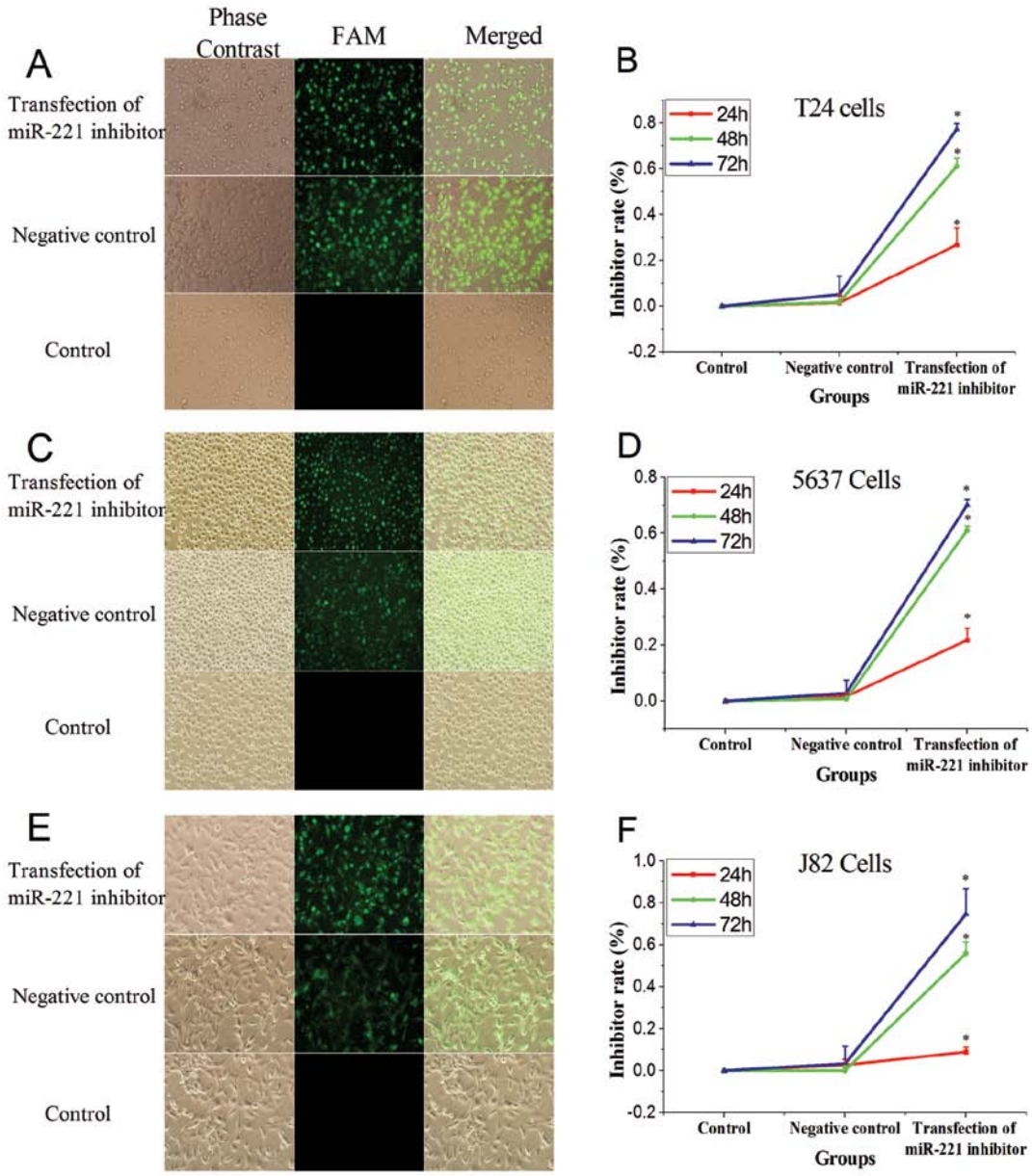

Figure 1. Transfection efficiency and cell viability. (A, C and E) Transfection efficiency of miR-221 inhibitor or scrambled oligonucleotide into T24 (A), 5637 (C) and J82 (E) cells (x200 magnification). (B, D and F) Inhibition rate of three bladder cancer cell lines after transfection for 24, 48 and 72 h. *P<0.05 vs. control group.

and 5637 cells) or MEM (J82 cells) containing 10% fetal calf serum. Cells were cultured for 24 h, fixed in 10% formaldehyde and stained with crystal violet staining, while the upper chamber cells were gently removed using cotton-tipped swabs. Five microscopic fields (x200) were randomly selected for cell counting. The experiment was repeated three times.

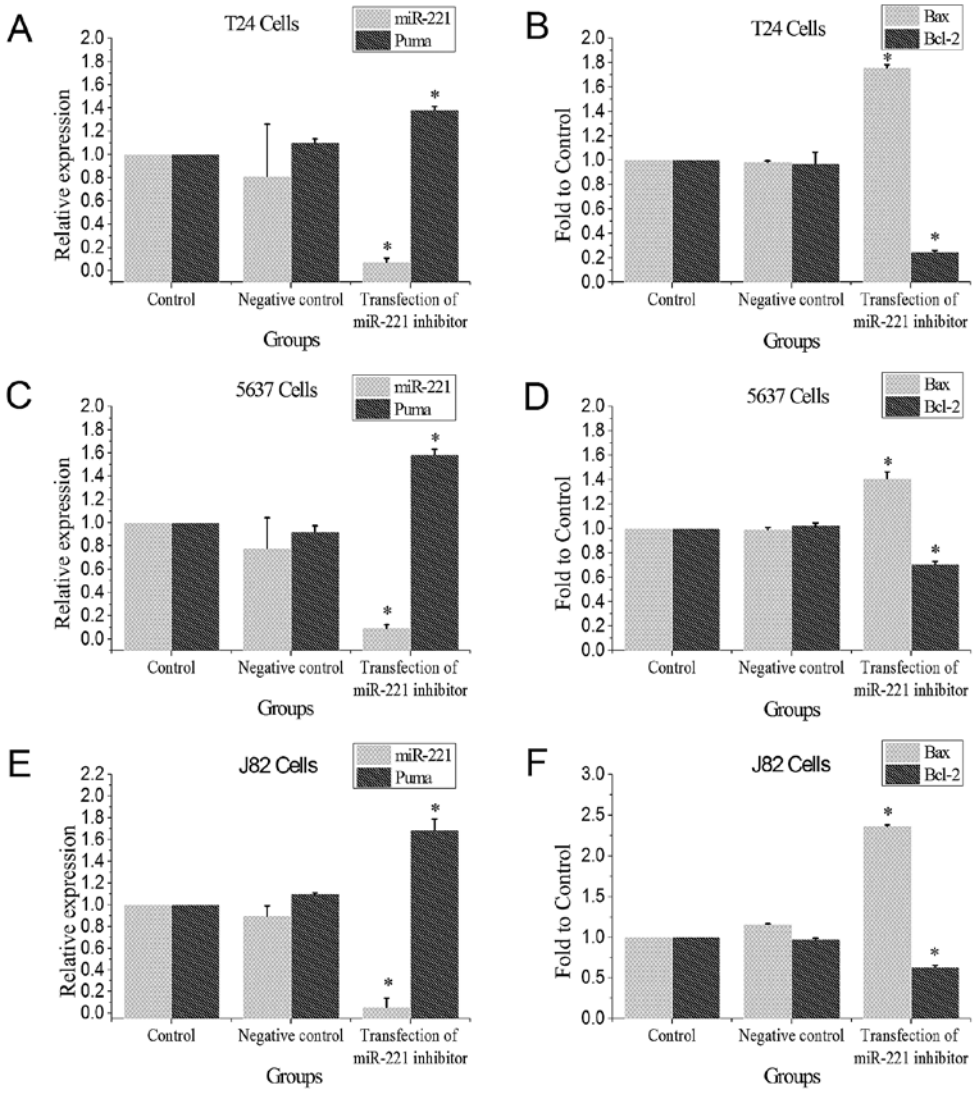

Figure 2. mRNA levels of miR-221, *PUMA*, *Bax* and *Bcl-2* in T24, 5637 and J82 cells after transfection of miR-221 inhibitor or scrambled oligonucleotide for 48 h. (A) Relative expression of miR-221 and *PUMA* of T24 cells. (B) Relative expression of *Bax* and *Bcl-2* of T24 cells. (C) Relative expression of miR-221 and *PUMA* of 5637 cells. (D) Relative expression of *Bax* and *Bcl-2* of 5637 cells. (E) Relative expression of miR-221 and *PUMA* of J82 cells. (F) Relative expression of *Bax* and *Bcl-2* of J82 cells, *P<0.05 vs. control group.

Flow cytometry. To evaluate cell apoptosis, singe cell suspensions were prepared for each experimental group, incubated with 5 μl FITC-conjugated Annexin V and 10 μl propidium iodide (PI) for 20 min at room temperature in the dark and were immediately analysed with a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). A minimum of 10,000 events were acquired for each sample.

Acridine orange/ethidium bromide (AO/EB) staining. Morphological signs of apoptosis were detected by using acridine orange-ethidium bromide (AO/EB) staining. The cells were treated as described above. AO/EB (Sigma) was freshly mixed at 0.01/100 (v/v) in dark, and one drop of the mixed solution was added to each well for 5 min. The apoptotic cells were then counted under an inverted fluorescence microscope (Eclipse TE300, Nikon, Japan). The death rate (%) was calculated as the percentage of positively stained cells, number of cells undergoing programmed cell death (PCD x 100/total number of cells. The experiments were repeated twice.

Statistical analysis. All data are presented as the average ± standard deviation (mean ± SD). All experiments were repeated three times independently, unless otherwise indicated. Statistical analysis was performed to determine the significance of the difference between groups using ANOVA (with *post hoc* Turkey's honestly significant difference test). All statistical analyses were performed using SPSS 21.0 software for Windows, P<0.05 is considered as statistically significant.

Results

miR-221 inhibitor impedes the proliferation of bladder cancer cells. Observation by fluorescence microscopy performed 5 h after transfection showed that the transfection efficiency for T24, 5637, and J82 cells were 80, 8 and 90%, respectively (Fig. 1). The miR-221 inhibitor was transfected into bladder cancer T24, 5637 and J82 cells. The cell viability was determined at 24 h, 48 and 72 h. Cell proliferation was inhibited in a time-dependent maner, but to varying degrees in the three

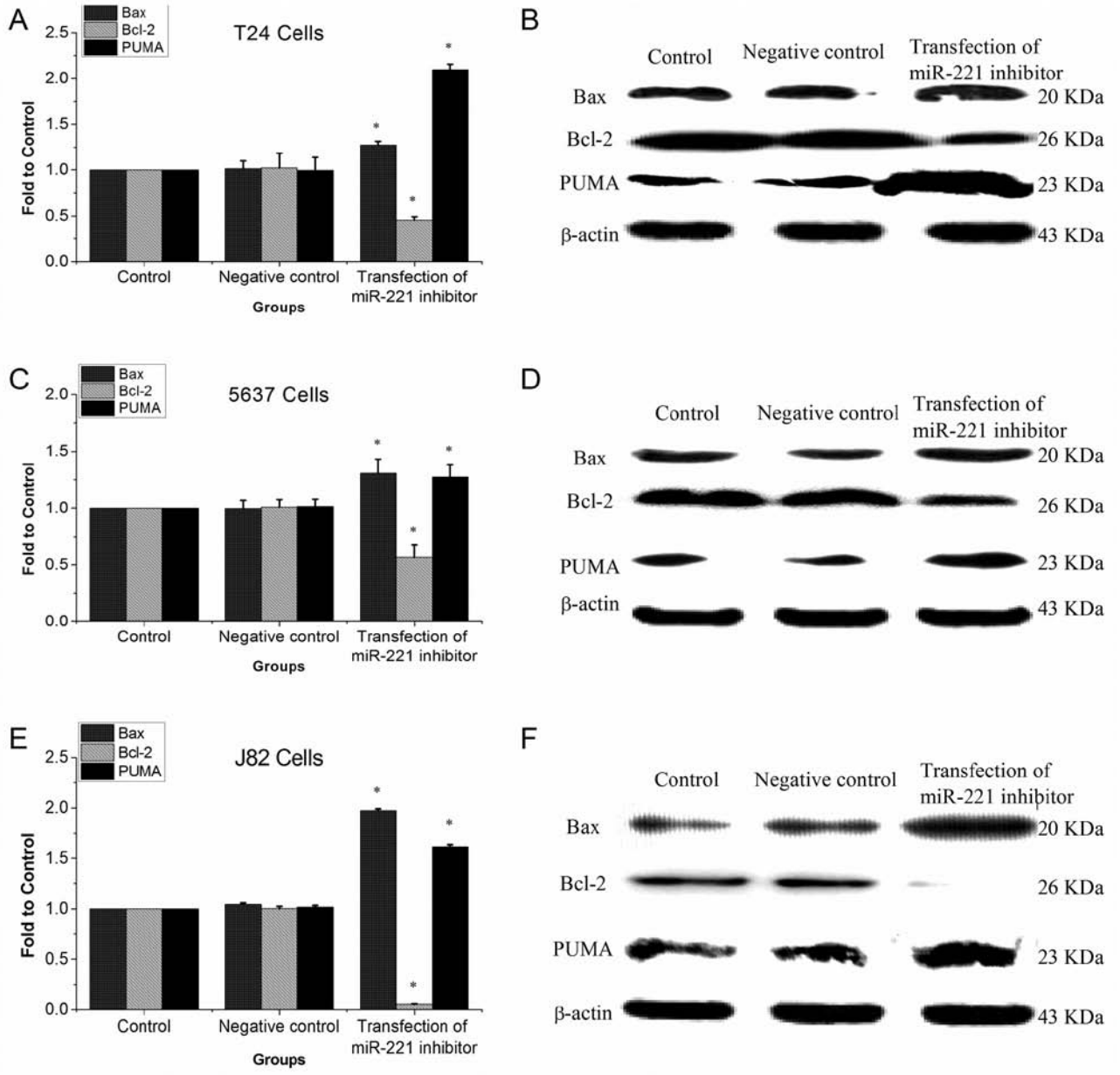

Figure 3. Western blot analysis of PUMA, Bax, and Bcl-2 in T24 (A and B), 5637 (C and D) and J82 (E and F) cells after transfection of miR-221 inhibitor or scrambled oligonucleotide for 48 h. *P<0.05 vs. control group.

tested lines. In addition, the rate of inhibition of cell proliferation increased with increasing time (P<0.05, vs. control group, Fig. 1D).

Transfection of miR-221 inhibitor reduces miR-221 expression. The T24, 5637 and J82 bladder cancer cells were transfected with miR-221 inhibitor for 48 h, miR-221 expression levels were determined by qRT-PCR. Transfection of miR-221 significantly reduced miR-221 expression when compared to the control group and the negative control group (P<0.05, Fig. 2).

miR-221 inhibitor induces mRNAs of PUMA and Bax, and reduces Bcl-2 mRNA. Results from qRT-PCR showed that after miR-221 expression was inhibited in the three bladder cancer cell lines, PUMA and Bax mRNA levels were significantly induced, while mRNA of the anti-apoptotic Bcl-2 was significantly reduced when compared to the blank group (P<0.05, Fig. 2).

miR-221 inhibitor induces PUMA and Bax protein expression and reduces Bcl-2 protein expression. After inhibiting the expression of miR-221 in three bladder cancer cell lines, proteins of PUMA and Bax were significantly devated, while Bcl-2 protein was significantly reduced when compared with control group (P<0.05, Fig. 3).

miR-221 inhibitor promotes apoptosis of bladder cancer cells. Flow cytometry results showed that both the early and late apoptosis rates in miR-221 inhibitor transfected groups were significantly higher than those in control groups (P<0.05, Fig. 4) in T24, 5637 and J82 cells. AO-EB also indicates that DNA damage in miR-221 inhibitor transfected groups was

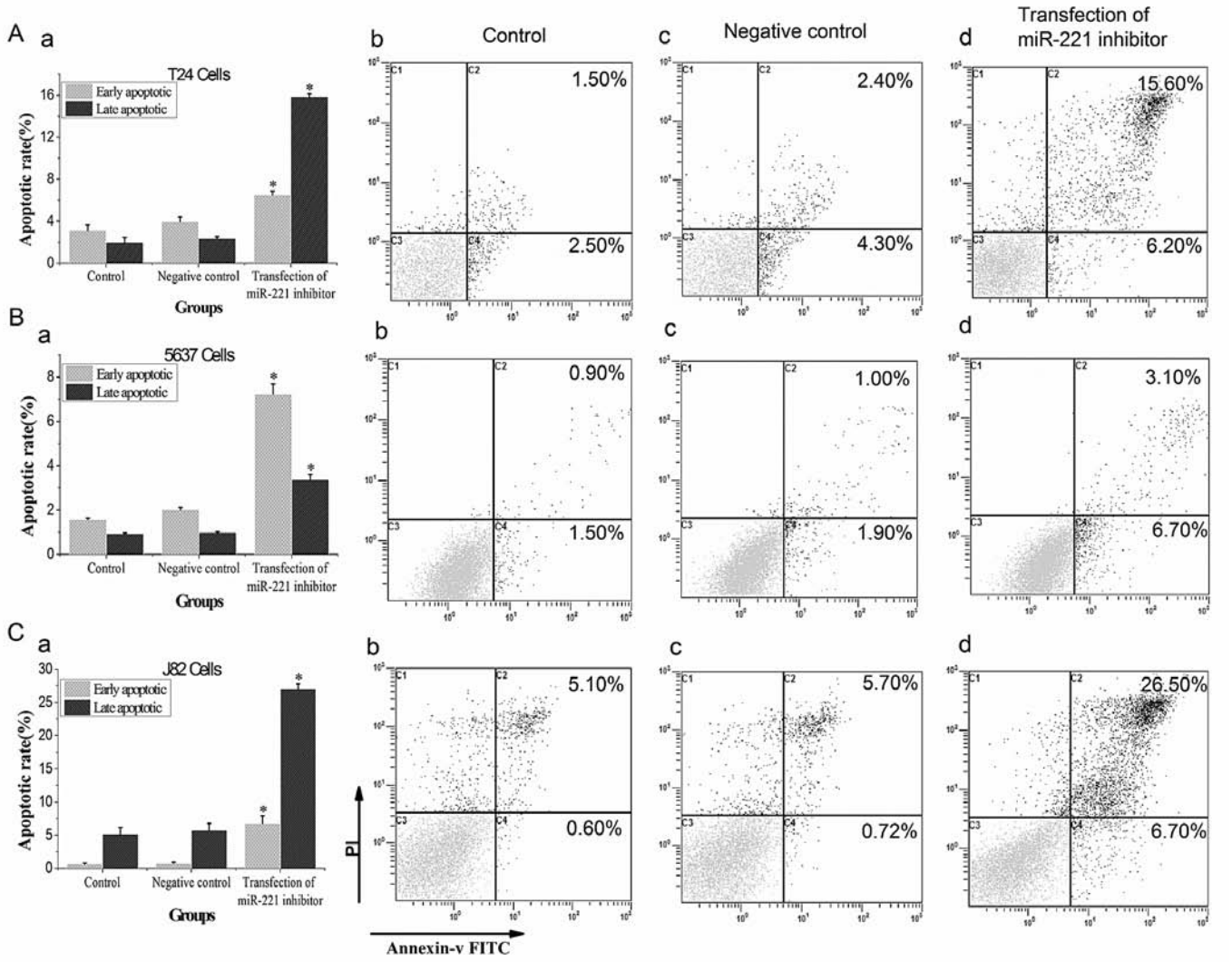

Figure 4. Flow cytometry analysis of apoptosis rate in T24 (A), 5637 (B) and J82 (C) cells after transfection of miR-221 inhibitor or scrambled oligonucleotide for 48 h. *P<0.05 vs. control group.

significantly higher than the damage in control groups in all three cell lines (Fig. 5).

miR-221 inhibitor reduces mRNA levels of MMP-9, MMP-2, and VEGF-C. Results from qRT-PCR showed that in T24, 5637 and J82 cells MMP-9, MMP-2, and VEGF-C mRNA levels were significantly lower in miR-221 inhibitor transfected groups than in control groups (P<0.05, Fig. 6).

miR-221 inhibitor reduces MMP-9, MMP-2, and VEGF-C protein expression. Western blotting showed that in T24, 5637, and J82 cells, MMP-9, MMP-2, and VEGF-C protein levels were significantly lower in miR-221 inhibitor transfected groups than in the control groups (P<0.05, Fig. 7).

miR-221 inhibitor reduces the invasiveness of bladder cancer cells. Transwell assays showed that in T24, 5637 and J82 cells, the invasiveness of bladder cancer cells was significantly reduced in miR-221 inhibitor transfected groups than in the control groups (P<0.05, Fig. 8). There was no significant difference in invasive capability between the control groups and negative control groups.

Discussion

Interactions between the immune system and tumor cells play a very important role in tumorigenesis. Failure of the immune system to recognize or kill cancer cells leads to tumor occurrence and development. Immune killing of tumor cells involves the transmission of apoptotic signals from immune cells to tumor cells, as well as the subsequent transduction of the apoptotic signal in tumor cells. However, most of the research on immune evasion of tumor cells focuses on the ability of cancer cells to block the apoptotic signal as it is transmitted from immune cells to tumor cells (29). In fact, transmission of the apoptotic signal from immune cells to tumor cells is actually not blocked completely, which means that tumor cells do receive attenuated apoptotic signals from immune cells (9-11). Therefore, abnormal intracellular transduction of apoptotic signals in tumor cells may be another important factor for immune evasion.

Apoptosis is a process of programmed autonomous cell death. It is induced by a variety of factors inside and outside the body and is controlled by strict and complex regulation of signaling networks (30-32). Currently, three pathways

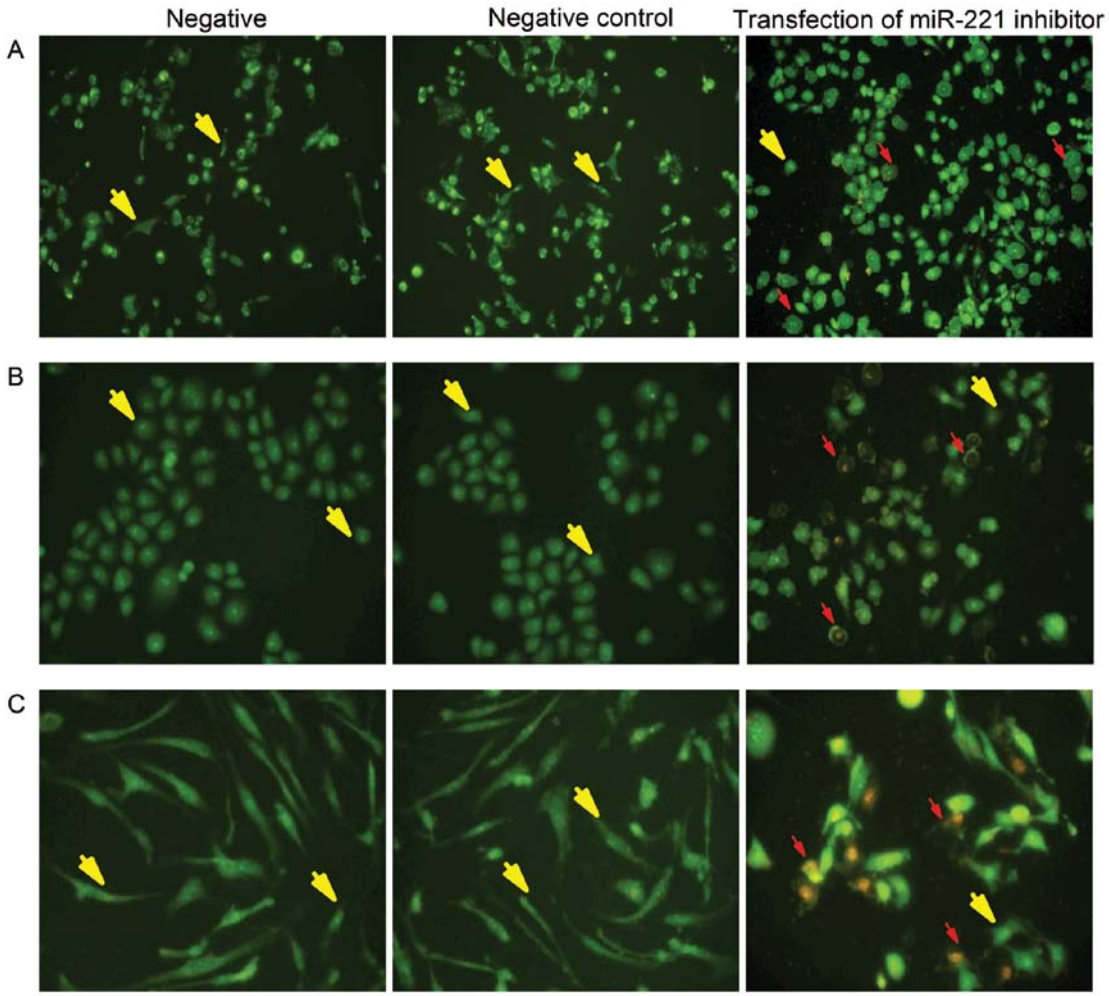

Figure 5. AO-EB staining T24 (A), 5637 (B) and J82 (C) cells. Red arrows indicate apoptotic cells and yellow arrows indicate normal cells.

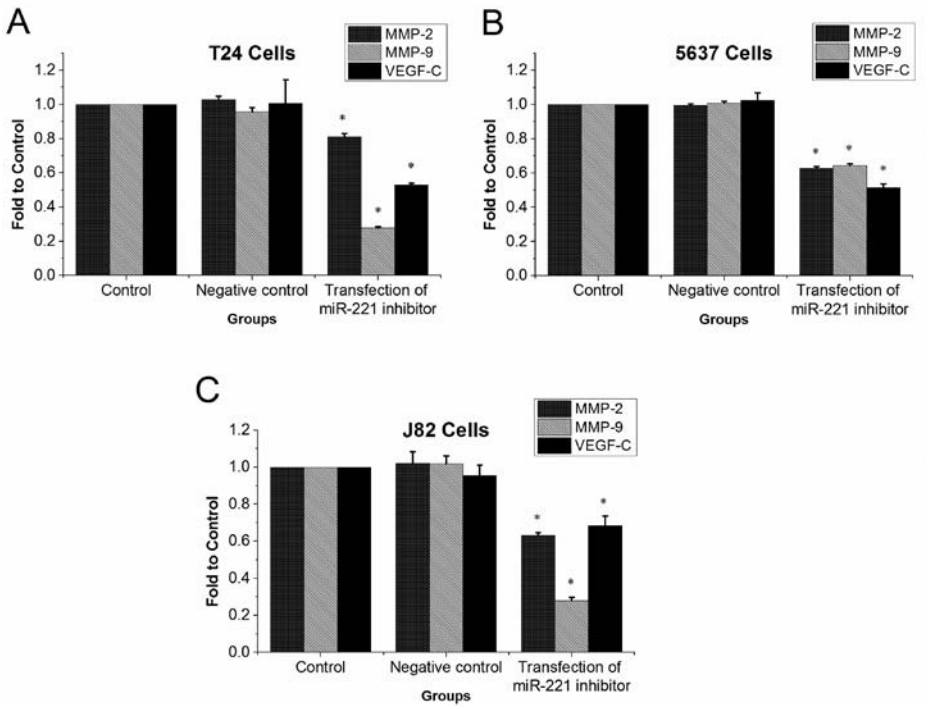

Figure 6. qRT-PCR analysis of MMP-2, MMP-9 and VEGF-C in each group of T24 (A), 5637 (B) and J82 (C) cells after transfection of miR-221 inhibitor or scrambled oligonucleotide for 48 h. *P<0.05 vs. control group.

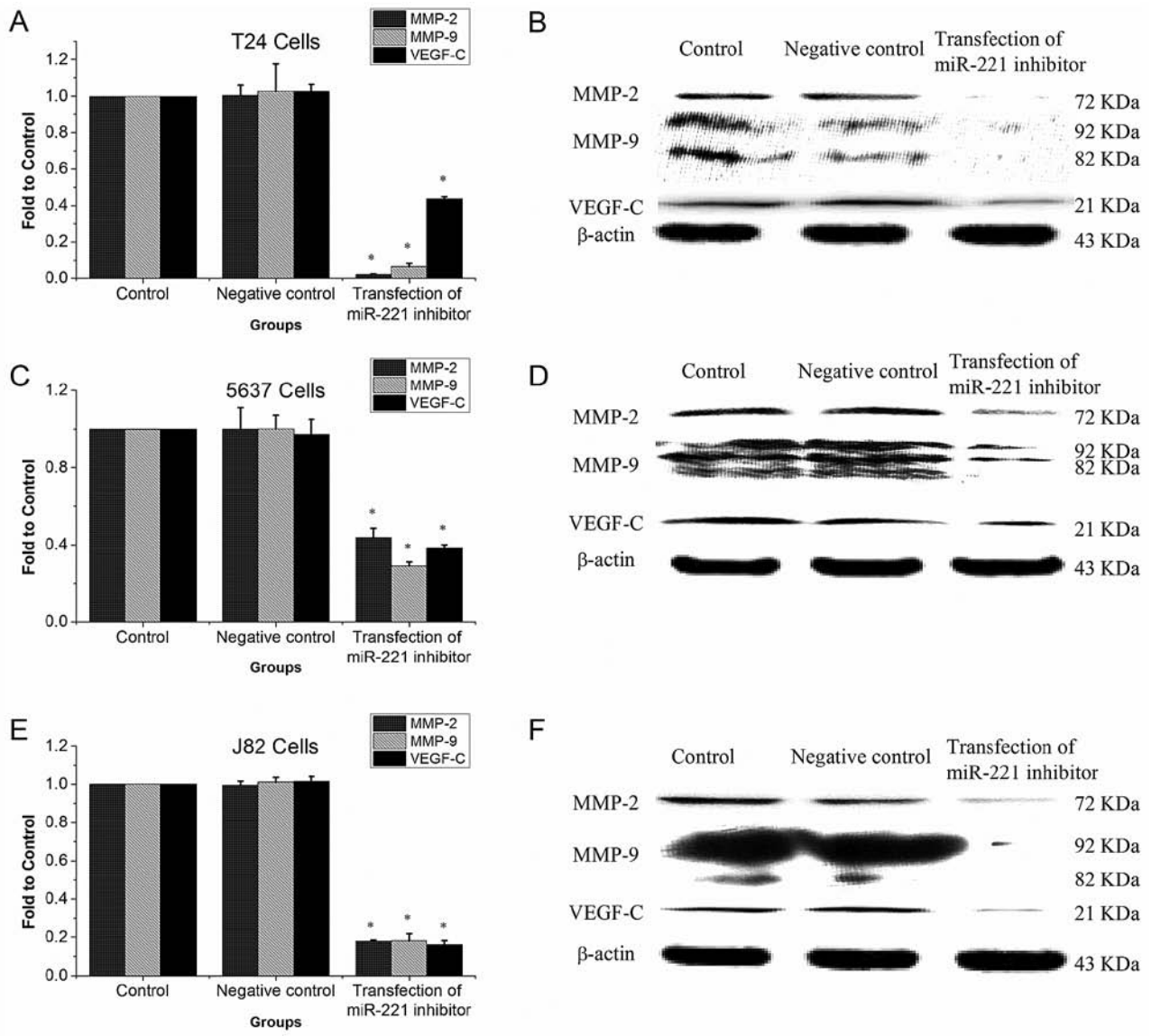

Figure 7. Western blot analysis of MMP-2, MMP-9 and VEGF-C in T24 (A and B), 5637 (C and D), and J82 (E and F) cells after transfection of miR-221 inhibitor or scrambled oligonucleotide for 48 h. *P<0.05 vs. control group.

of cell apoptosis have been characterized. The first is the intrinsic apoptotic pathway, namely the mitochondrial apoptotic pathway, in which the intracellular signal transduction involves the interaction of pro-apoptotic factors and the anti-apoptotic Bcl-2 protein family. The second apoptosis pathway is the endoplasmic reticulum signaling pathway, which does not yet have a clear mechanism. The third apoptosis pathway is the extrinsic apoptosis pathway, also termed as dead receptor pathway, which induces apoptosis by activating the corresponding ligands and initiating the death receptors on the cell surfaces (33-38). The three pathways are interrelated. For instance, the extrinsic apoptotic pathway can activate the mitochondrial apoptotic pathway by activation of Bid (39).

PUMA was first reported by Nakano in 2001 (74) as a member of the BH3-only subfamily in the Bcl-2 family. It is a pro-apoptosis factor. The pro-apoptosis function of PUMA mainly depends on its BH3 domain and the orderliness and integrity of the 43 amino acid residues at its C-terminus (34,39-41). Previous studies have shown that the pro-apoptotic mechanism of PUMA acts through various mechanisms, ultimately causing depolarization of the mitochondrial membrane potential and initiating the mitochondrial apoptosis pathway. This results in the release of cytochrome *c* and Smac/DIABLO into the cytoplasm and the activation of apoptosis (15,42-44). However, the expression of PUMA is regulated by transcription factors (p53, c-Myc and FoxO3a) as well as post-transcriptional regulation (miR-221/miR-222) (44-48).

It has been reported that miR-221 expression is upregulated in bladder cancer tissues compared to adjacent bladder tissues and that miR-221 can specifically target the 3' non-coding region of the mature PUMA mRNA and silence PUMA expression (12,44,49). In this study, miR-221 inhibitor (modified by 2' O-methylation and FAM) and a negative control sequence were transfected into bladder cancer cells. After transfection with miR-221 inhibitor for 24, 48 and 72 h, cell viability was measured using the MTT assay. Results indicated that miR-221 inhibitor significantly impedes the proliferation of bladder cancer cells. Furthermore, the inhibition of prolifera-

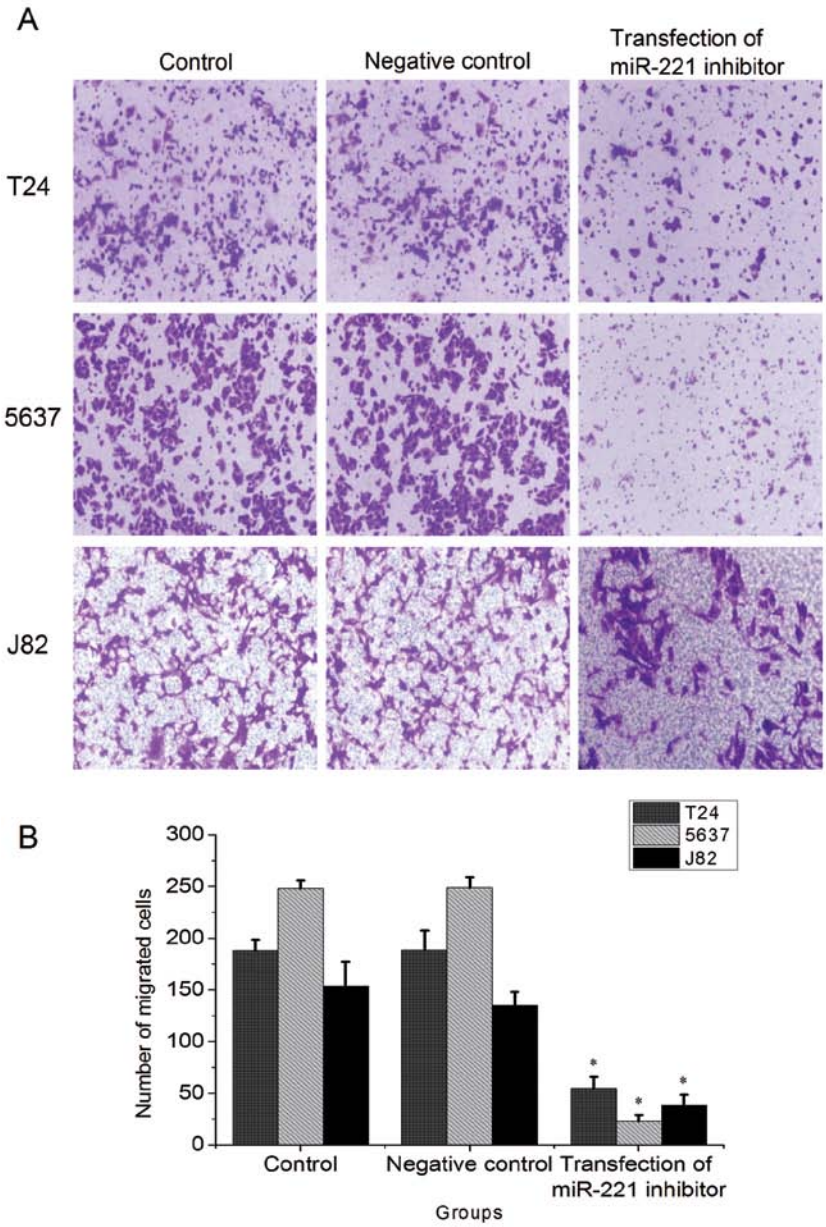

Figure 8. Downregulation of miR-221 suppresses bladder cancer cell invasion. (A) Representative image of six Transwell migration experiments is shown for T24, 5637 and J82 cells (x200). (B) Columns, mean (n=3); bars, SD. *P<0.05 vs. control group.

tion is time-dependent, indicating that a decrease in miR-221 suppresses cell proliferation. Floating cell debris after our 72-h transfection made it more difficult to meet the requirements of subsequent experiments, so we chose to transfect miR-221 inhibitor for 48 h in subsequent experiments. Taking our qRT-PCR and western blotting results together, we have shown that, 48 h after transfection of miR-221 inhibitor, miR-221 mRNA levels were significantly inhibited, resulting in an increase in PUMA and Bax mRNA and protein, but a decrease in Bcl-2 mRNA and protein. Flow cytometry and AO-EB staining assays confirmed that miR-221 inhibition promotes apoptosis in bladder cancer cells. Collectively, the overexpression of endogenous miR-221 is an important factor for the inhibition of apoptosis in bladder cancer cells.

Tumor cells capable of immune evasion are not only able to resist apoptosis, but can also release immunosuppressive factors into the extracellular environment by autocrine or paracrine secretion, which results in deep immunosuppressive regions formed locally in tumor cells. The formation of these regions is one of the important mechanisms of tumor cell immune evasion. Among these immunosuppressive factors, VEGF and MMP play important roles in tumor invasion and metastasis.

There are seven VEGF isoforms, namely VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and PIGF (50). VEGF-C plays an important role in the regulation of tumor metastasis and infiltration. Previous studies have shown that VEGF-C is a protein precursor that must be activated by converting enzyme (proprotein convertases, PC) 5 and 7 (51,52). The associated receptors of VEGF-C include VEGFR-2, VEGFR-3 and Nrp-2, among which VEGFR-3 is the receptor that promotes lymphangiogenesis and tumor

cell migration, invasion and metastasis. Study of Nrp-2 has suggested that Nrp-2 only plays a regulatory role and is not a necessary receptor during lymphangiogenesis (53-57). In human tumors, abnormal VEGFR-3 expression has been found in gastric cancer (58), lung cancer (59), colorectal cancer (60,61), head and neck cancer (62), bladder cancer (63), and breast cancer (64). Based on these previous studies, we confirmed that VEGF-C overexpression in tumor cells is the premise and one of the early events of tumor cell micrometastasis into the lymph node. Studies on immune evasion have confirmed that VEGF-C is a potent immunosuppressive factor, which not only impedes immune cells from recognizing tumor cells, but can also destroy the biological functions of immune cells (65). In this study, after transfecting miR-221 inhibitor into T24, 5637 and J82 bladder cancer cells, VEGF-C mRNA and protein expression levels decreased significantly. It remains to be further studied whether this phenomenon is due to the upregulation of pro-apoptotic genes PUMA and Bax, the downregulation of anti-apoptotic Bcl-2, or other mechanisms, but it is clear that miR-221 did not directly regulate the effects of VEGF-C. Taken together, decreased miR-221 in bladder cancer reduces the effects of VEGF-C in promoting the formation of microlymphatic vessels and capillary vessels at tumor sites, as well as the ability of VEGF-C to inhibit immune cell activity.

There are many members in the family of MMPs, among which MMP-2 and MMP-9 are closely associated with tumor invasion and development. Their main mechanism of action include: i) MMP-2 and MMP-9 degrade the extracellular matrix and basement membrane and destroy cell structure. In addition, MMP-2 has the potential capability to activate extracellular matrix structural proteins and plays an important role in the chemotaxis of inflammatory cells as well as the spontaneous stimulation of tumor cells for migration and invasion; ii) MMPs promote tumor angiogenesis. As blood vessel formation is a prerequisite for the tumor cell growth, metastasis and invasion, MMP-2 promotes angiogenesis after interacting with their corresponding substrates. At the initial stage of tumor angiogenesis in tumor nodules, MMP-2 secretion is essential for tumor cells (66-70). In this study, after transfecting miR-221 inhibitor into bladder cancer T24, 5637 and J82 cells for 48 h, we found that both the mRNA and protein levels of MMP-2 and MMP-9 decreased. Although we only detected total MMP-2 and MMP-9 protein, rather than their phosphorylated isoforms which are the activated forms, our Transwell results indicate that cancer cell invasiveness was significantly decreased after reducing miR-221. Furthermore, MMP-2 and MMP-9 are closely related to tumor cell invasion (71-73). Therefore, the overexpression of MMP-2 and MMP-9 in bladder cancer is likely an important factor for the invasion and metastasis of bladder cancer cells.

In conclusion, this study showed that the overexpression of miR-221 in bladder cancer cells inhibits the expression of the pro-apoptotic gene PUMA, thereby inhibiting the transduction of apoptotic signals. Meanwhile, the downregulation of miR-221 in bladder cancer cells reduced the expression of VEGF-C, MMP-2 and MMP-9, resulting in reduced invasion of bladder cancer cells. Therefore, miR-221-induced PUMA silencing is a key factor for the immune evasion of bladder cancer.

Acknowledgements

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