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miR-150 Modulates Cisplatin Chemosensitivity and Invasiveness of Muscle-Invasive Bladder Cancer Cells via Targeting PDCD4 *In Vitro*

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Background: Chemotherapeutic insensitivity and tumor cell invasiveness are major obstacles to effectively treating muscle-invasive bladder cancer (MIBC). Recent reports show that microRNAs (miRNAs) play an important role in the chemotherapeutic response and disease progression of MIBC. Therefore, here we investigated the role of miR-150 in MIBC cells *in vitro*.

Material/Methods: miR-150 expression was quantified by qRT-PCR in two MIBC cell lines (5637 and T24). After successful miR-150 inhibition by transfection, MTS and transwell assays were used to assess the MIBC's cisplatin sensitivity and cell invasiveness, respectively. The TargetScan database and a luciferase reporter system were used to identify whether the programmed cell death 4 protein (PDCD4) is a direct target of miR-150 in MIBC cells.

Results: miR-150 expression was found to be significantly increased in both MIBC cell lines, and treatment with a miR-150 inhibitor significantly sensitized MIBC cells to cisplatin and inhibited MIBC cell invasiveness. PDCD4 was identified as a direct target of miR-150 in MIBC cells, and increased PDCD4 expression via transfection with the pLEX-PDCD4 plasmid efficiently sensitized MIBC cells to cisplatin chemotherapy and inhibited MIBC cell invasiveness.

Conclusions: This study provides novel evidence that miR-150 functions as a tumor promoter in reducing chemosensitivity and promoting invasiveness of MIBC cells via targeting PDCD4. Thus, modulation of the miR-150-PDCD4 axis shows promise as a therapeutic strategy for MIBC.

MeSH Keywords: **Cisplatin • MicroRNAs • Urinary Bladder Neoplasms**

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Background

Bladder cancer is the fifth most common malignancy in the U.S. (with 2013 estimates reaching 72570 new cases and 15210 deaths) and remains one of the costliest diseases to manage from diagnosis to death [1]. At initial diagnosis, approximately 70% of cases are diagnosed as non-muscle-invasive bladder cancer (NMIBC) and approximately 30% as muscle-invasive bladder cancer (MIBC). Low-grade NMIBC are not immediately life-threatening, but they have a propensity for recurrence that necessitates costly lifelong surveillance [2]. In contrast, high-grade MIBC progresses rapidly to become metastatic and generates the bulk of patient mortality [3].

The standard treatment for MIBC is radical cystectomy. However, as about one-third of patients with MIBC have undetected metastasis at the time of treatment for the primary tumor and 25% of patients who undergo radical cystectomy present with lymph node involvement at the time of surgery, this standard treatment provides a mere five-year survival rate of 50% [4]. In order to improve the effects of treatment, perioperative chemotherapy has been explored since the 1980s. The updated analysis shows that cisplatin-based neoadjuvant chemotherapy significantly improves overall survival. However, as only about 50% of patients with MIBC respond to cisplatin-based chemotherapy [5,6], there is still a need to investigate the mechanism(s) that limit response to chemotherapy in order to improve its therapeutic effects.

The molecular events involved in the genesis and therapeutic response of MIBC have been intensely studied [7–9]. Despite these advances in the diagnosis and treatment of MIBC, the molecular mechanism(s) underlying MIBC carcinogenesis remain poorly understood, and specific tumor biomarkers and therapeutic targets for MIBC are still limited. To address this need, microRNAs (miRNAs) – endogenous, non-coding RNAs of about 19-25 nucleotides in length that negatively regulate gene expression through blocking translation or degrading mRNA – have been associated with almost all types of cancers and are involved in every aspect of cancer, including proliferation, differentiation, invasion, metastasis, and therapeutic response [10]. For example, Min et al. identified a miRNA expression signature that is significantly associated with breast cancer [11], and Liu et al. demonstrated that miR-10b modulates breast cancer metastasis via targeting E-cadherin [12]. With respect to bladder cancer, several dysregulated miRNAs have been identified. Namely, miR-144 and miR-16 are downregulated in bladder cancer [13,14], miR-125b suppresses bladder cancer development [15], and miRNA-101 downregulation in bladder transitional cell carcinoma (TCC) is associated with a poorer prognosis [16].

In this study, miR-150 expression was found to be significantly increased in two MIBC cell lines (5637 and T24), and treatment with a miR-150 inhibitor significantly sensitized MIBC cells to cisplatin and inhibited MIBC cell invasiveness. Using the TargetScan database and a luciferase reporter system, the programmed cell death 4 protein (PDCD4) was then identified as a direct target of miR-150 in MIBC cells. Increased PDCD4 expression efficiently sensitized MIBC cells to cisplatin chemotherapy and inhibited MIBC cell invasiveness. This study provides novel evidence that miR-150 functions as a tumor promoter in reducing chemosensitivity and promoting invasiveness of MIBC cells via targeting PDCD4. Thus, modulation of the miR-150-PDCD4 axis shows promise as a therapeutic strategy for MIBC.

Material and Methods

Cell culture

Two MIBC cell lines, 5637 and T24, were purchased from American Type Culture Collection (ATCC) and were cultured in RPMI-1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies). The non-tumorigenic bladder cell line SV-HUC-1 was also purchased from ATCC and was cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) supplemented with 10% FBS. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C.

Real-time PCR for mature miRNAs and mRNA

First, total miRNA from cultured cells was isolated and purified with a miRNA isolation system (Qiagen). cDNA was generated with the miScript II RT Kit (Qiagen) with 500 ng total miRNA and quantitative real-time PCR (qRT-PCR) was performed using the miScript SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions. Using the miRNA sequence-specific qRT-PCR primers for miR-150 and the endogenous control RNU6 (Qiagen), the qRT-PCR analysis was performed using the IQ5 System (Bio-Rad). The PCR conditions were as follows: 1 cycle of 95°C for 10 min followed by 40 cycles of 95°C for 10 s, 62°C for 20 s, and 72°C for 20 s. The gene expression threshold cycle (CT) values of the miRNAs were calculated by normalizing to the internal control RNU6, and relative quantization values were calculated.

Total RNA was extracted with a Trizol protocol, and cDNA from 2 μg total mRNA with an A260/280 purity ratio of 1.6–1.8 was synthesized with the Super-Script first-strand synthesis system (Thermo Scientific). qRT-PCR was performed according to the standard protocol on the IQ5 system (Bio-Rad) with SYBR Green detection (Applied Biosystems). The PCR conditions were as follows: 1 cycle of 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. GAPDH was

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used as an internal control, and the qRT-PCR was repeated in triplicate. The primers for GAPDH and PDCD4 were as follows: GAPDH's forward primer 5'- GAAAGCCTGCCGGTGACTAA-3' and reverse primer 5'- GCATCACCCGGAGGAGAAAT-3'; PDCD4's forward primer 5'- AACCCTGCAGAAAATGCTGG-3' and reverse primer 5'- TCCACTTCTAAGGGCGTCAC-3'.

Transfection of miR-150 inhibitor and pLEX-PDCD4 plasmids

A miR-150 inhibitor and its control were purchased from Exiqon. Cells were trypsin-digested and seeded onto six-well plates the day prior to the transfection to ensure 60–70% cell confluence on the day of transfection. The transfection was carried out using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. The miR-150 inhibitor and its control were used at a final concentration of 100 nM, and 4 μg of the pLEX-MCS control and pLEX-PDCD4 plasmids were used. At 36 h post-transfection, follow-up experiments were performed.

MTS assay

The Cell Titer 96® AQueous One Solution Cell Proliferation Assay Kit (Promega) was used to determine the sensitivity of cells to cisplatin. In brief, cells were seeded in 96-well plates at a density of 4×10^3 cells/well (0.20 ml/well) for 24 h before use. The culture medium was replaced with fresh medium containing cisplatin with different concentrations for 72 h. Then, MTS (0.02 ml/well) was added. After 2 h further incubation, the absorbance at 490 nm of each well was recorded on the Biotex ELX800. The growth rate was calculated as the ratio of the absorbance of the experimental well to that of the control well. Then, the inhibition rate for every concentration and the IC50 (the concentration of drug that results in 50% of the control value) were also calculated.

Transwell assay

The invasiveness of MIBC cells was assessed using the Cell Invasion Assay Kit (BD Biosciences) according to the manufacturer's instructions. Briefly, at 36 h post-transfection, 3×10^4 cells in 300 μl serum-free medium were added to the upper chamber pre-coated with ECMatrix™ gel. Then, 0.5 ml of 10% FBS-containing medium was added to the lower chamber as a chemoattractant. Cells were incubated for 24 h at 37°C, and then non-invading cells were removed with cotton swabs. Cells that migrated to the bottom of the membrane were fixed with pre-cold methanol and stained with 2% Giemsa solution. Stained cells were visualized by light microscopy. At least three randomly selected fields with 100× magnification were counted, and the average number was calculated.

Western blotting

Total protein was extracted using RIPA buffer (Pierce) in the presence of Protease Inhibitor Cocktail (Pierce). The protein concentration of the lysates was measured using a BCA Protein Assay Kit (Pierce). Equivalent amounts of protein were resolved and mixed with 5× Lane Marker Reducing Sample Buffer (Pierce), electrophoresed in a 10% sodium dodecyl sulfate (SDS)-acrylamide gel, and transferred onto an Immobilon-P Transfer Membrane (Millipore). The membranes were blocked with 5% non-fat milk in Tris-buffered saline and then incubated with primary antibodies (anti-PDCD4 (1:1000) and anti-β-actin (1:3000); Cell Signaling Technology) followed by a HRP-conjugated secondary antibody (1:40000; Thermo). The signal was detected using an ECL detection system (Millipore).

Luciferase reporter assay

Two single strands of PDCD4 with the wild type 3'UTR with a miR-150 binding site and two single strands of the mutant type (with seven bases deleted in the miR-150 binding site as mutant controls) were synthesized with restriction sites for SpeI and HindIII located at both ends of the oligonucleotides for further cloning. The single strand DNA sequences were as follows: the wild type 3'UTR of PDCD4 (sense: 5'-*CTAGT* TGCTGCTGTTGAGATACTGTGCTTTG GGAGTAAAAAAAGAAAGTTATTTCTTTG*A*-3'; antisense: 5'-*AGCTT* CAAAGAAATAACTTTCTTTTTTTACTCCCAAAGCACAGTATCTCA ACAGCAGCA*A*-3') and the mutant type 3'UTR of PDCD4 (sense: 5'-*CTAGT* TGCTGCTGTTGAGATACTGTGCT------- TAAAAAA AGAAAGTTATTTCTTTG*A*-3'; antisense: 5'- CAAAGAAATAACTTTCTTTTTTTA-------AGCACAGTATC TCAACAGCAGCA*A*-3'). The corresponding sense and antisense strands were annealed and subsequently cloned into a pMir-Report plasmid downstream to a firefly luciferase reporter gene. Cells were seeded onto 96-well plates and co-transfected with a pMir-Report luciferase vector, a pRL-TK Renilla luciferase vector, and a miR-150 mimic (Qiagen). After 48 h, luciferase activity was determined using a Dual-Luciferase Reporter Assay System (Promega), in which Renilla luciferase activity was used as an internal control. The firefly luciferase activity was calculated as the mean ± SD after being normalized to Renilla luciferase activity.

Statistical analysis

Quantitative results were expressed as the mean ±SD. Statistical analysis was conducted with the Statistical Package for Social Science (SPSS for Windows Version 11.0, IBM). The comparative CT method ($2^{-\Delta\Delta}$Ct method) was used to calculate relative quantities of gene expression. Student's *t*-test was used to evaluate statistical significance. *P*-values of less than 0.05 or 0.01 were set as the thresholds for statistical significance.

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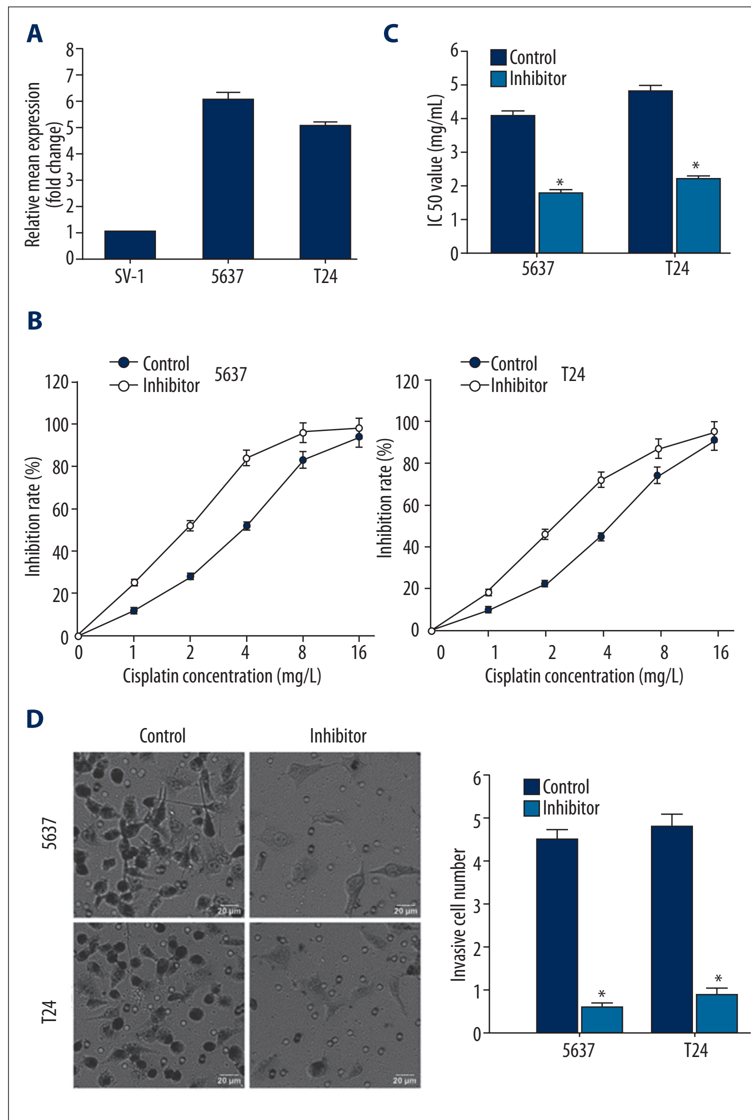

Figure 1. miR-150 inhibition enhances chemosensitivity to Cisplatin and suppresses invasiveness of MIBC cells. (**A**) miR-150 expression in MIBC cell lines tested by qRT-PCR. (**B**) Dose-inhibition rate curves plotted from three independent MTS assays showing the effect of miR-150 inhibition on MIBC cells' chemosensitivity to cisplatin. (**C**) IC50 values affected by miR-150 inhibition. (**D**) Transwell assay performed on the control cell lines and miR-150-inhibited cell lines. * *p*<0.01.

Results

miR-150 reduces chemosensitivity and promotes invasiveness of MIBC cells

To investigate the role of miR-150 in MIBC, we first evaluated miR-150 expression in two MIBC cell lines (5637 and T24) against a non-tumorigenic bladder cell line SV-HUC-1 by miRNA-specific qRT-PCR. Compared to SV-HUC-1 cells, the 5637 and T24 cell lines displayed significantly higher miR-150 expression (Figure 1A). Then, we inhibited the function of miR-150 using a miR-150 specific inhibitor in the 5637 and T24 cells and recorded the change in chemosensitivity to cisplatin and invasiveness by MTS assay and transwell assay, respectively. As shown Figure 1B and 1C, the miR-150 inhibitor significantly increased the sensitivity of 5637 and T24 cells to cisplatin, with an IC50 value decrease from 4.12±0.18 mg/l to 1.82±0.13 mg/l and from 4.84±0.23 mg/l to 2.21±0.08 mg/l, respectively. The miR-150 inhibitor also efficiently suppressed the invasiveness of both 5637 cells (control group, 45±3.34 cells/field; miR-150 inhibitor, 6±1.00 cells/field) and T24 cells (control group, 48±3.45 cells/field; miR-150 inhibitor, 9±2.12 cells/field) (Figure 1D).

miR-150 directly targets PDCD4 in MIBC cells

To investigate the association between miR-150 and cisplatin chemosensitivity and invasion potential in MIBC cells, we sought miR-150's target gene using the public database TargetScan (*http://www.targetscan.org*), and PDCD4, which possesses a critically conserved binding site for miR-150, was selected for further molecular and functional confirmation (Figure 2A).

To determine whether miR-150 expression correlates with PDCD4 expression, the mRNA and protein expression levels

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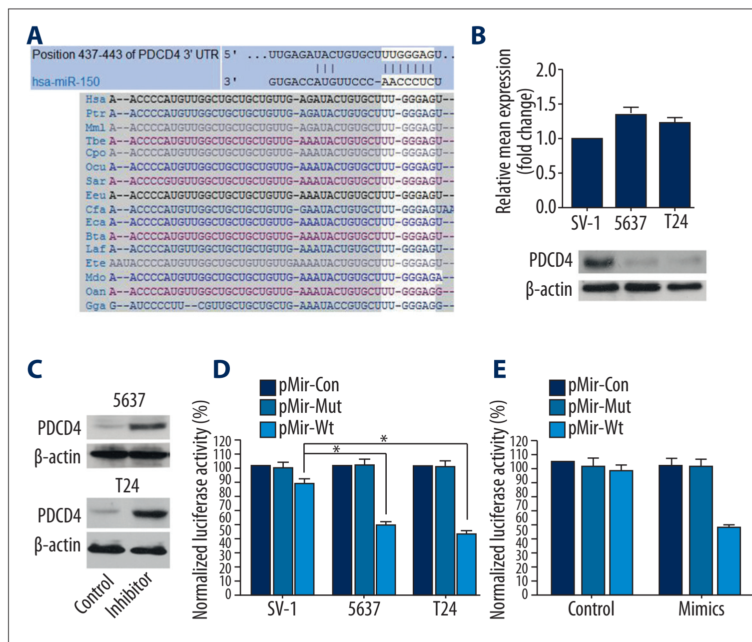

Figure 2. miR-150 targets PDCD4 in MIBC cells. (**A**) Schematic of the broadly-conserved miR-150 target site in the human PDCD4 3'UTR. (**B**) Inverse relationship between miR-150 expression and PDCD4 protein levels. (**C**) miR-150 inhibitor increases PDCD4 protein expression in MIBC cells. (**D, E**) miR-150 suppresses the luciferase activity linked to the 3'UTR of PDCD4 with a Renilla luciferase reporter used for normalization. Data were obtained from three independent experiments, and the mean of the results from (**D**) pMir-control-transfected SV-HUC-1 cells and (**E**) pMir-control-transfected HEK293T cells were set at 100%. * $p<0.01$.

of PDCD4 were detected in the SV-HUC-1, 5637, and T24 cell lines. There was no significant differences in PDCD4 expression at the mRNA level among the cell lines, but the PDCD4 protein levels in the 5637 and T24 cell lines (which possess endogenously higher expressions of miR-150) were significantly lower than that in the SV-HUC-1 cell line (Figure 2B). As expected, the PDCD4 protein levels in the 5637 and T24 cells significantly increased after miR-150 inhibition (Figure 2C).

To assess whether PDCD4 is a direct target of miR-150, the luciferase reporter vector with the PDCD4 3'-UTR including the putative target site for miR-150 downstream of the luciferase gene (pMir-PDCD4-Wt) and a mutant version with a 7-bp deletion in the binding site (pMir-PDCD4-Mut) were constructed. The luciferase activity of pMir-PDCD4-Wt in the 5637 and T24 cells was 31.6% and 38.8% lower, respectively, compared to that in the SV-HUC-1 cells. However, there was no significant difference in luciferase activity for pMir-PDCD4-Mut (Figure 2D). Moreover, the luciferase reporter assay performed in Human Embryonic Kidney 293 (HEK293T) cells showed that a miR-150 mimic (i.e., a non-natural, double-stranded miRNA-like RNA fragment that mimics endogenous miR-150 by gene-specific translational inhibition [17]) efficiently suppressed luciferase activity of pMir-PDCD4-Wt by about 43.4%, but the mutant version abrogated the repressive ability of the miR-150 mimic (Figure 2E). These results demonstrate the specificity of miR-150's targeting of PDCD4.

PDCD4 enhances chemosensitivity and inhibits invasiveness of MIBC cells

As demonstrated above, PDCD4 is a direct target of miR-150, and its expression is downregulated in MIBC cells. To investigate whether PDCD4 has a biological role in MIBC, we restored PDCD4 expression by transfecting the pLEX-PDCD4 vector in the 5637 and T24 MIBC cell lines. Post-transfection, PDCD4 protein expression significantly increased (Figure 3A). Ectopic expression of PDCD4 significantly enhanced the effect of cisplatin on the 5637 and T24 MIBC cells with an IC50 drop from 3.83±0.24 mg/l to 1.53±0.09 mg/l and from 4.52±0.26 mg/l to 1.84±0.11 mg/l (Figure 3B and 3C). Furthermore, PDCD4 also significantly inhibited the invasiveness of both 5637 (control group, 44±3.75 cells/field; miR-150 inhibitor, 5±1.34 cells/field) and T24 (control group, 52±5.24 cells/field; miR-150 inhibitor, 7±2.02 cells/field) cells (Figure 3D). These results indicate that restoring PDCD4 expression shows similar effects to miR-150 inhibition in MIBC cells.

Discussion

Bladder cancer is caused by a series of genetic and epigenetic alterations [18]. Despite recent progress in diagnosis and treatment, the molecular mechanisms of bladder carcinogenesis and progression remain poorly understood, and specific tumor biomarkers and therapeutic targets for bladder cancer are still limited, especially for MIBC. Recently, some studies have reported that the aberrant expression of some miRNAs displays a strong relationship with bladder cancer progression. In this study, we demonstrated that miR-150 expression is significantly increased in the MIBC cell lines 5637 and T24 as compared to normal human bladder epithelial SV-HUC-1 cells. Furthermore, miR-150 inhibition significantly enhanced chemosensitivity to cisplatin and suppressed the invasiveness of 5637 and T24 cells.

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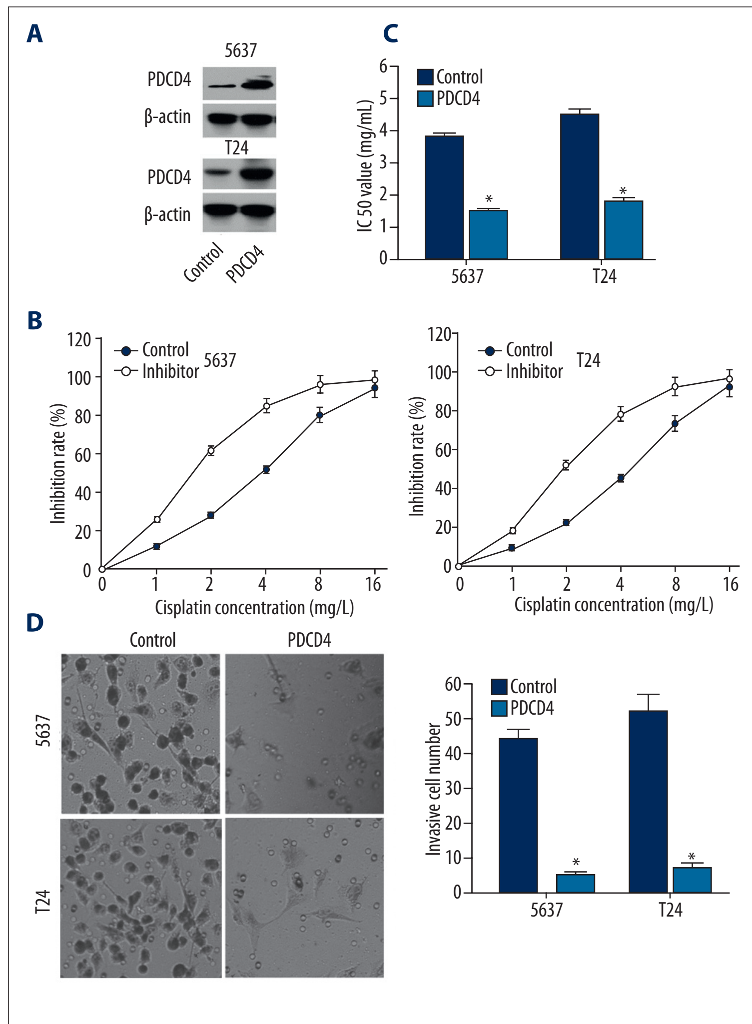

Figure 3. PDCD4 enhances chemosensitivity to Cisplatin and suppresses invasiveness of MIBC cells. (**A**) PDCD4 expression increased after pLEX-PDCD4 transfection. (**B**) Dose-inhibition rate curves were plotted from three independent MTS assays, showing that restoring PDCD4 expression significantly decreased cell viability upon cisplatin treatment. (**C**) IC50 values decreased by restoring PDCD4 expression. (**D**) Transwell assay performed on the control cell lines and PDCD4-restored cell lines. * $p<0.01$.

The role of miR-150 in cancer continues to be extensively studied on account of its complex biological function, acting as a tumor promoter or suppressor across various cancer types. For example, due to post-transcriptional regulation by miR-150, levels of the pro-apoptotic protein P2X7 receptor are lower in cancer epithelial cells than in normal cells, and treatment with a miR-150 inhibitor significantly increases P2X7 expression in cancer cells [19]. miR-150 overexpression has also been observed in breast cancer cells, and miR-150 targets the P2X7 receptor to promote growth and clonogenicity while reducing apoptosis in breast cancer cells [20]. In gastric cancer cells, miR-150 exerts its oncogenic role by targeting the pro-apoptotic gene EGR2 to promote tumor cell proliferation [21]. In lung cancer, miR-150 promotes proliferation by targeting p53 [22], migration by targeting SRC kinase signaling inhibitor 1 [23], and survival of lung cancer cells [24]. In contrast, miR-150 displays a tumor suppressive role in malignant lymphoma [25], and colorectal cancer patients whose tumors have lower miR-150 expression display a shorter survival horizon and a worse response to adjuvant chemotherapy [26]. In addition to the direct action of miR-150 on cancer cells, microvesicle-mediated transfer of miR-150 from monocytes to endothelial cells promotes angiogenesis in a tumor-implanted mouse model [27], which may upregulate VEGF expression to promote tumorigenesis [28]. Thus, the role of miR-150 may be cancer type-dependent, and further studies across various cancer types are still needed.

The function of miRNA primarily relies on its target gene(s). To investigate the association between miR-150 and cisplatin chemosensitivity and invasion potential in MIBC cells, we sought miR-150's target gene using the public database TargetScan (*http://www.targetscan.org*), and PDCD4, which possesses a critically conserved binding site for miR-150, was selected for further molecular and functional confirmation. From experimental analysis, we found that miR-150 directly targets PDCD4, which is downregulated in MIBC cells with endogenous miR-150 overexpression. These findings suggest a tumor suppressive role of PDCD4 in MIBC. PDCD4 has been found to play key roles in a number of cellular processes, including cell growth

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and invasion via inhibiting the transcription factor AP-1 and suppressing translation of the eukaryotic initiation factor (elF) 4A. Furthermore, PDCD4 expression is significantly downregulated in several human cancers [29], and a growing number of recent reports have pointed to PDCD4's role as a tumor suppressor gene involved in suppressing cell transformation, tumorigenesis, and invasion [29,30]. For example, ectopic PDCD4 expression significantly inhibits cell proliferation, migration, and invasion via elevating PTEN and inhibiting Akt activity in ovarian cancer [31] and blocks cell growth and survival in nasopharyngeal carcinoma via modulating miR-184-mediated direct suppression of c-Myc and Bcl-2 [32].

With respect to PDCD4's effect on the chemotherapeutic response, we found that ectopic PDCD4 expression enhanced chemosensitivity to cisplatin and inhibited invasiveness in MIBC cells. Loss of PDCD4 contributes to enhanced chemoresistance in glioblastoma multiforme through de-repression of Bcl-xL translation [33]. In rectal cancer, patients with higher PDCD4 expression were more sensitive to neoadjuvant chemoradiotherapy and displayed improved five-year disease-free survival and five-year overall survival rates [34].

There are several limitations to this study. First, the focus of the current study is solely on MIBC cells – future studies should explore the role of the miR-150/PDCD4 axis in other bladder cancer cell lines. Second, here we showed the miR-150/PDCD4 axis modulated the response of MIBC cells to chemotherapy, but we did not specifically assess the precise cause(s) of MIBC cell death. Whether apoptosis, autophagy, or other cause(s) of MIBC cell death are responsible for the observed effect must be explored in our later studies. Third, we did not assess other biological effects of the miR-150/PDCD4 axis on MIBC, such as cell migration and cell cycle phase, so these biological effects should be investigated in future studies. Fourth, PDCD4 was the only target protein candidate of miR-150 validated here, but other target proteins of miR-150 may exist and should be scanned for in future studies. Finally, we did not investigate miR-150 levels after restoration of PDCD4 expression; future studies should investigate this issue in order to determine whether a feedback loop exists between miR-150 and PDCD4.

Conclusions

This study provides novel evidence that miR-150 functions as a tumor promoter in reducing chemosensitivity and promoting invasiveness of MIBC cells via targeting PDCD4. Modulation of the miR-150-PDCD4 axis shows promise as a therapeutic strategy for MIBC. Because PDCD4 shows promise as an interventional target for MIBC, the detailed mechanism(s) underlying PDCD4's role in MIBC will be explored in our future studies.

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