European Review for Medical and Pharmacological Sciences 2018; 22: 3363-3369

Effects of paclitaxel combined with miR-448 on growth and proliferation of bladder cancer EJ cells

Y. WANG, L.-J. LI, M.-X. QIU, B.-S. GONG

Department of Urology, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, Chengdu, Sichuan, China

Abstract. – OBJECTIVE: Bladder cancer is one of the most common malignant tumors of the urinary system characterized by a high recurrence rate after treatment. Paclitaxel is a new type of anti-neoplastic agent used for treatment by inhibiting the proliferation of bladder cancer cells. This study aimed to explore the mechanism of paclitaxel on the inhibition of bladder cancer cell proliferation by applying paclitaxel combined with miR-448 on bladder cancer cells.

MATERIALS AND METHODS: Bladder cancer EJ cells were divided into 5 groups, including control group, paclitaxel group, negative control (NC) group, microRNA-448 (miR-448) mimic group, and paclitaxel combined with miR-448 mimic group. Cell apoptosis was tested by flow cytometry. Cell proliferation was determined by cell counting kit 8 (CCK8) assay. The regulation of miR-448 on B cell lymphoma 2 (Bcl-2) gene was assessed by dual luciferase reporter assay. Bcl-2 protein expression was detected by Western blot.

RESULTS: The apoptotic rate of cells was significantly increased, while the cell proliferation ability was significantly reduced in paclitaxel group and miR-448 mimic group compared with control ($p<0.05$). Cell apoptotic rate in paclitaxel combined with miR-448 mimic group was markedly higher than that of paclitaxel group ($p<0.05$). MiR-448 can bind to the 3'UTR region of Bcl-2 mRNA and regulate the expression of Bcl-2. The expression of Bcl-2 protein in paclitaxel group and miR-448 mimic group was significantly lower than that in control group and higher than paclitaxel combined with miR-448 mimic group ($p<0.05$).

CONCLUSIONS: Paclitaxel combined with miR-448 promoted EJ cell apoptosis and inhibited cell proliferation by suppressing Bcl-2 gene expression.

Key Words

Paclitaxel, miR-448, Bcl-2, Bladder cancer, Proliferation.

Introduction

Bladder cancer (BC) is a common malignant tumor in the urinary system that mostly occurred in male[1]. The incidence of BC ranks the ninth among all types of malignancies globally, of which the seventh in male and seventeenth in female[2-4]. Its incidence ranks eighth in all types of tumors and first in urinary tract tumors in China[5]. Its incidence in recent years showed a rising trend. BC is a multi-factorial disease with complex pathological changes. Among them, smoking and occupational exposure are the main risk factors[6]. Surgical resection is the preferred treatment for BC, whereas the recurrence rate is still high, which brings great difficulties for clinical treatment. Paclitaxel is a diterpene alkaloid compound with anticancer activity extracted from cedar, which is used in the treatment of many tumors, such as breast cancer, esophageal cancer, lung cancer, and BC[7]. It was found that paclitaxel acts on the cell spindle, blocks eukaryotic cells in the G2/M phase, and induces apoptosis, while its mechanism has not been fully elucidated[8]. It was showed that tumor growth is often accompanied by an abnormal expression of microRNAs in tumor tissues[9]. MicroRNAs are a class of conserved small non-coding RNAs that are approximately 18-24 bases in length. Mature miRNAs regulate target gene expression by incompletely pairing with the 3'UTR region of the target gene mRNA. It was reported that miRNAs mainly regulate the expression of target genes in cells by directly inducing mRNA degradation or inhibiting the translation of target mRNAs, which are involved in the process of cell growth, proliferation, differentiation, metabolism, and apoptosis, so as to affect tumor formation and activity[10-12]. This study aimed to explore the me-

Corresponding Author: Lijun Li, MD; e-mail: hehehuangzhan@163.com

chanism of paclitaxel on the inhibition of bladder cancer cell proliferation by applying paclitaxel combined with miR-448 on bladder cancer cells.

Materials and Methods

Main Reagents and Instruments

Roswell Park Memorial Institute-1640 (RPMI 1640) medium, fetal bovine serum (FBS), and Trypsin were purchased from Gibco (Grand Island, New York, USA). Paclitaxel was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell counting kit 8 (CCK8) was purchased from Dojindo Molecular Technologies (Rockville, MD, USA). Reverse transcription kit and SYBR green fluorescent dye were purchased from Toyobo Life Science (Osaka, Japan). Annexin V-FITC/PI cell apoptosis detection kit was purchased from Yeasen Biotechnology Co., Ltd. (Shanghai, China). β-actin and B cell lymphoma 2 (Bcl-2) primary antibodies were purchased from Abcam Biotechnology (Cambridge, MA, USA). The secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The CO_2 incubator was purchased from Pierce (Rockford, IL, USA). Real-time PCR amplifier (Mode: VIIA7, ABI, Foster City, CA, USA). Flow cytometry was purchased from (BD Biosciences, San Jose, CA, USA).

Cell Line and Tumor Tissue

Human BC cell line EJ was purchased from Shanghai Wuli Biological Co., Ltd. (Shanghai, China). The cells were cultured in RPMI-1640 medium containing 10% FBS and maintained at 37°C and 5% CO_2.

Cell Grouping and Treatment

EJ cells were divided into 5 groups, including control group, paclitaxel group (100 nmol/l), negative control (NC) group, miR-448 mimic group, and paclitaxel combined with miR-448 mimic group. MiR-448 mimic or negative control was transfected to the cells using lipo-2000 according to the manual. The cells in paclitaxel group and paclitaxel combined with miR-448 mimic group were treated by 100 nmol/l paclitaxel for 48 h.

Dual Luciferase Reporter Gene Assay

The wild-type and mute-type of 3'UTR region of Bcl-2 mRNA were amplified by the primers. The mute base was located in the target region of miR-448. After digestion and connection, the segment was inserted into pMIR plasmid to construct Bcl-2 luciferase reporter gene plasmid. Next, the above-mentioned plasmid, PTK loading plasmid, and miR-448 mimic were transfected to EJ cells. After 48 h, the cells were tested on dual luciferase reporter gene system (Promega, Madison, WI, USA).

Real-Time PCR

Total RNA was extracted by the TRIzol method from the cells and reverse transcribed to cDNA. The PCR reaction system contained 1 μl cDNA, 1 μ PCR primers, 10 μl SYBR® qPCR Mix, and 8 μl water. The PCR reaction was performed at 95°C pre-degeneration for 5 min, followed by 40 cycles of 95°C degeneration for 15 s, 60°C annealing for 15 s, and 72°C elongation for 45 s. The results were calculated using the $2^{-\Delta\Delta Ct}$ method. The primers used were listed in Table I. The details of the RT-PCR processes were conducted according to the previous study[13,14].

Western Blot

EJ cells were collected and added with radioimmunoprecipitation assay (RIPA) containing cocktail. Total protein was extracted and quantified using bicinchoninic acid (BCA) method. The protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (NC) membrane. After blocked in 5% skim milk, the membrane was incubated in primary antibody (1:1000) at 4°C overnight. Then, the membrane was incubated in secondary antibody (1:10000) at room temperature for 1 h. At last, the membrane

Table I. Primer sequences.

Gene	Forward	Reverse
β-actin	GAGGGAAATCGTGCGTGAC	CTGGAAGGTGGACAGTGAG
Bcl-2	CCTCTCGAGAAGGATGGCGCACGCTGG	CCGGATTGGGATTCCATCAGGCTTGACT
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
miR-448	TTATTGCGATGTGTTCCTTATG	ATGCATGCCACGGGCATATACACT

was analyzed on BOX Chemi XR 5 gel imaging system (Syngene, Cambridge, UK) to calculate the protein expression intensity. The details of the Western blot processes were conducted according to the previous study[15].

Flow Cytometry

The cells were collected and centrifuged at 1000 r/min for 5 min. Then, the cells were added with 100 µl blocking buffer at room temperature for 10 min and centrifuged at 1000 r/min for 5 min. Next, the cells were added with 500 µl Ca^{2+} buffer and centrifuged. Then, the cells were added with 100 µl Annexin V labeling fluid and propidium iodide (PI) dye for 10 min. At last, the cells were analyzed by flow cytometry.

CCK8 Assay

EJ cells were seeded in 96-well plate at 0.5×10^4 cells/well. Cell proliferation ability was tested by CCK8 kit at 12 h, 24 h, 36 h, 48 h, 60 h, 72 h, 84 h, and 96 h. At last, the cells were tested on a micro-plate reader at 450 nm to obtain the absorbance value as the previous study described[16].

Statistical Analysis

All data were presented as mean ± SEM and analyzed on SPSS 19.0 software (IBM Corp., IBM SPSS Statistics for Windows, Armonk, NY, USA). The measurement data were compared by ANOVA or Dunnett's *t*-test. $p<0.05$ was considered as statistical significant.

Results

MiR-448 Expression in EJ Cells

Real-time PCR was used to test miR-448 expression in EJ cells. As shown in Figure 1, the miR-448 level was significantly higher in miR-448 mimic group and paclitaxel combined with miR-448 mimic group compared with NC group, control, and paclitaxel group ($p<0.05$).

Bcl-2 Protein Expression in EJ Cells

Western blot was applied to test Bcl-2 expression in EJ cells. As shown in Figure 2, the Bcl-2 level was significantly lower in paclitaxel group compared with control. In addition, Bcl-2 expression in the miR-448 mimic group was significantly reduced compared with NC group. Furthermore, it was markedly lower in paclitaxel combined with miR-448 mimic group compared with NC group.

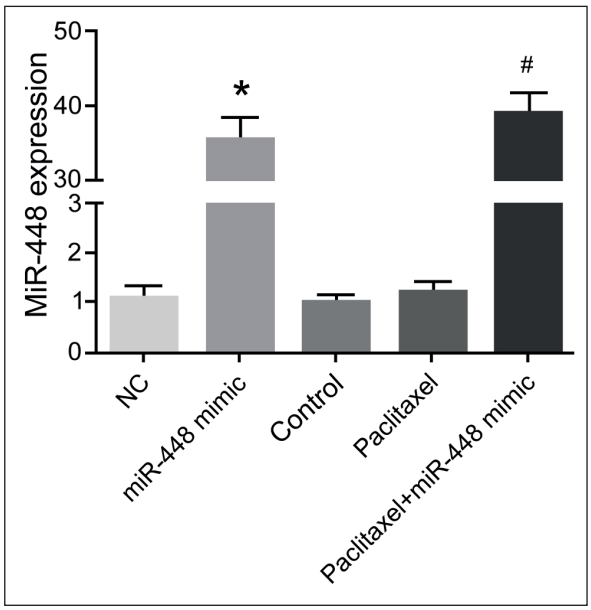

Figure 1. MiR-448 expression in EJ cells. *$p<0.05$, miR-448 mimic group compared with NC group. #$p<0.05$, paclitaxel combined with miR-448 mimic group compared with control and paclitaxel group.

Bcl-2 is the Target Gene of miR-448

TargetScan and miRanda exhibited that Bcl-2 is predicted as one of the target genes of miR-448 (Figure 3). Thus, we constructed wild-type and mute-type of 3'UTR plasmid of Bcl-2 mRNA and tested the result using dual luciferase reporter gene system. As shown in Figure 4, the luciferase activity in cells transfected by Bcl-2 wild-type plasmid was suppressed by miR-448, whereas the luciferase activity in cells transfected by Bcl-2 mute-type plasmid showed no significant difference.

MiR-448 Regulated Bcl-2 Gene Expression

EJ cells were divided into four groups, including NC group, miR-448 mimic group, NC inhibitor group, and miR-448 inhibitor group.

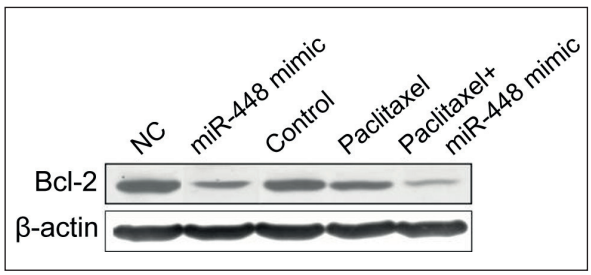

Figure 2. Bcl-2 protein expression in EJ cells.

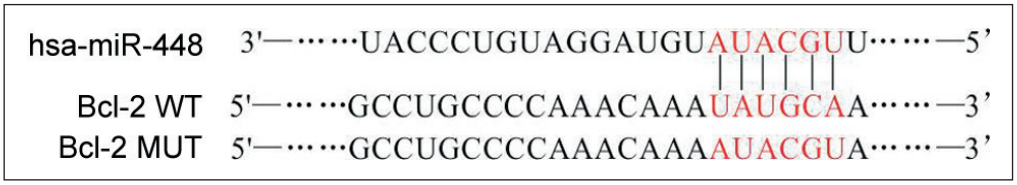

Figure 3. MiR-448 targeted to the 3'UTR region of Bcl-2 mRNA.

Bcl-2 mRNA and protein levels were detected after 48 h. As shown in Figure 5, Bcl-2 mRNA and protein expressions were declined in the miR-448 mimic group compared with NC group. On the contrary, Bcl-2 mRNA and protein levels markedly elevated in miR-448 inhibitor group compared with NC inhibitor group.

The Impact of Paclitaxel Combined with miR-448 on EJ Cell Apoptosis

Flow cytometry was adopted to test EJ cell apoptosis (Figure 6). The apoptotic rate of cells was significantly increased in paclitaxel group, miR-448 mimic group, and paclitaxel combined with miR-448 mimic group compared with NC group ($p<0.05$). Cell apoptotic rate in paclitaxel combined with miR-448 mimic group was markedly higher than that of paclitaxel group ($p<0.05$).

The Influence of Paclitaxel Combined with miR-448 on EJ Cell Proliferation

The CCK8 assay was selected to assess EJ cell proliferation (Figure 7). EJ cell proliferation was significantly attenuated in paclitaxel group, miR-448 mimic group, and paclitaxel combined with miR-448 mimic group compared with NC group ($p<0.05$). Cell proliferation in paclitaxel combined with miR-448 mimic group was declined compared with paclitaxel group ($p<0.05$).

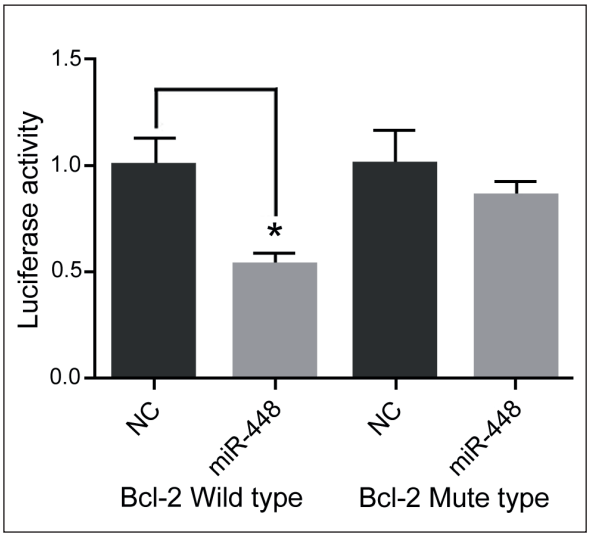

Figure 4. Dual luciferase reporter gene assay. *$p<0.05$. **A**, Bcl-2 wild-type. **B**, Bcl-2 mute type.

Discussion

Paclitaxel is an important anticancer drug which has been approved by the US FDA for the treatment of breast cancer, gastric cancer, and bladder cancer[17,18]. It was found that paclitaxel inhibits about 70% of the tumor cell DNA synthesis and induces 90% cell apoptosis *in vitro*[8]. In recent years, several studies showed that paclitaxel-induced tumor cell apoptosis may be related to the regulation of a variety of apoptosis-related gene expressions, such as Bcl-2 family, p53, p21, and Caspase family[19]. MiRNA binds to the 3'UTR region of target mRNA to regulate the expression of the target gene and affect cell function.

Targetscan analysis showed that there is a complementary pairing binding site between miR-448 and the 3'UTR region of Bcl-2 mRNA. Dual luciferase reporter assay demonstrated that miR-448 binds to the 3'UTR region of Bcl-2 mRNA and inhibits its expression. Cell transfection experiments indicated that miR-448 changes Bcl-2 gene expression, suggesting that miR-448 regulates the expression of Bcl-2 gene in cells. It is revealed that Bcl-2 gene may be a target gene of miR-448. Bcl-2 gene is an oncogene with the function of inhibiting apoptosis[20]. Apoptosis, also known as programmed cell death, is the process by which the body removes unwanted cells and plays an important role in cell growth, cell differentiation, development, and aging. Paclitaxel specifically binds to intracellular tubulin, affecting cell mitosis, arresting cells in the G2/M phase, and promoting apoptosis. It was found that paclitaxel can promote

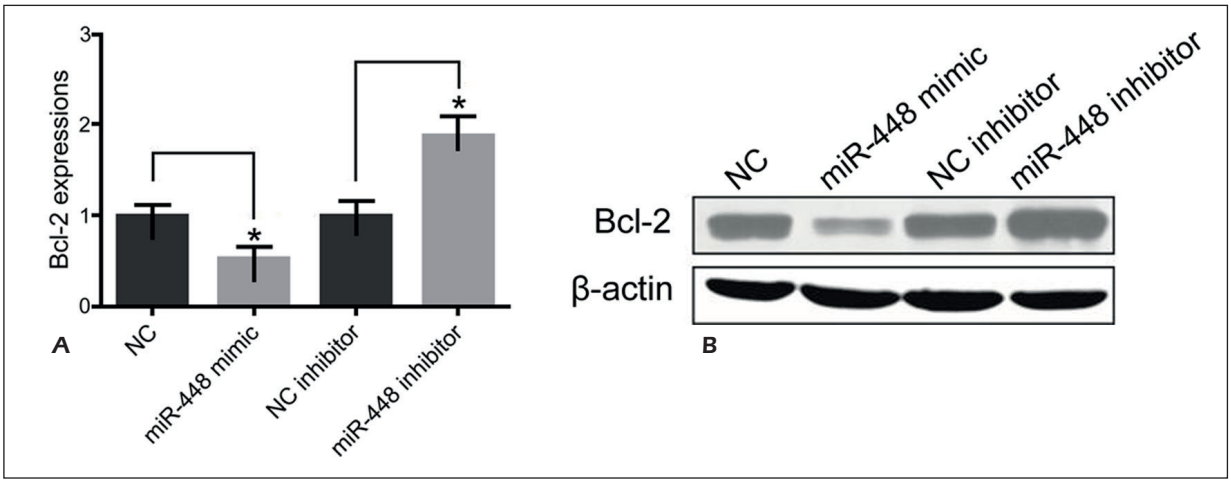

Figure 5. Bcl-2 mRNA and protein expressions in EJ cells transfected by miR-448. *p<0.05. **A**, Bcl-2 mRNA expression. **B**, Bcl-2 protein expression.

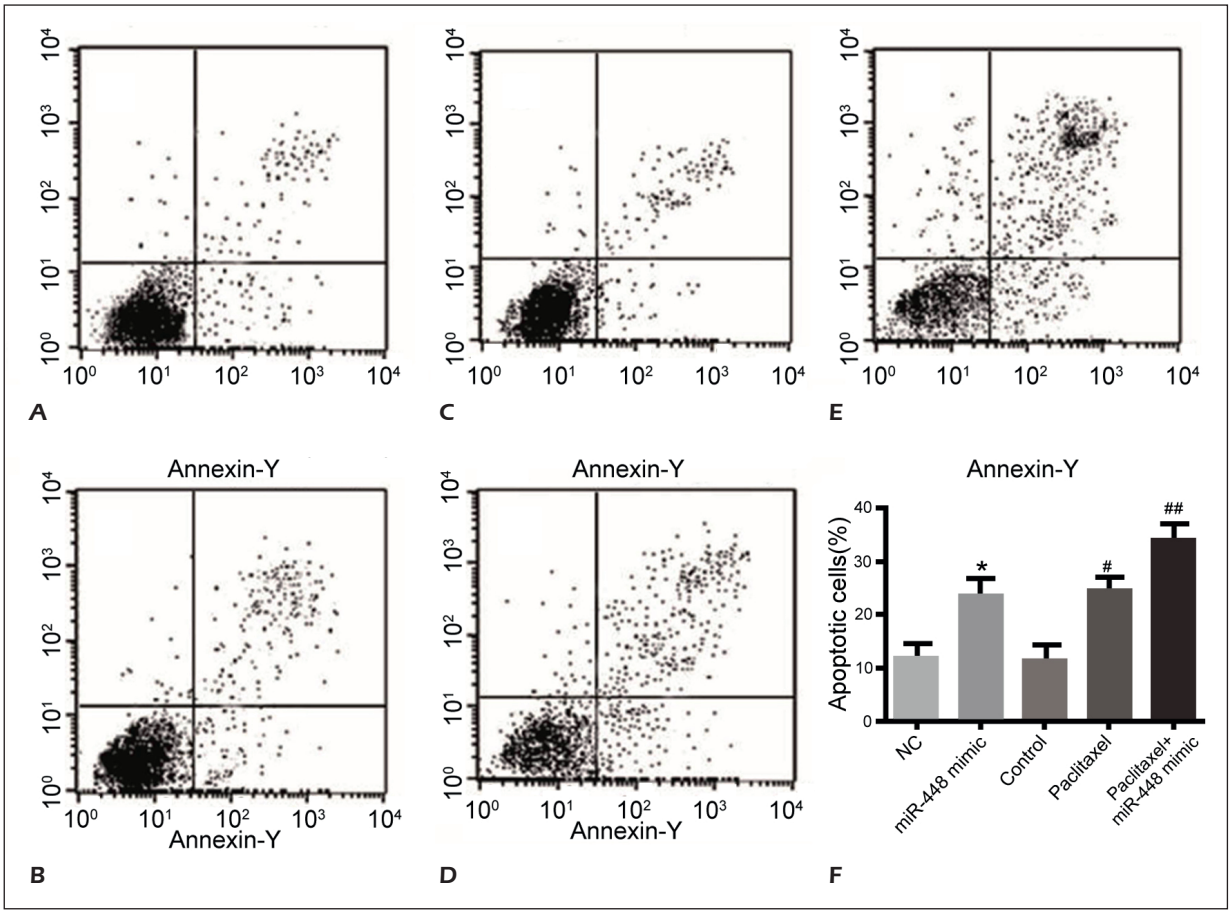

Figure 6. The impact of paclitaxel combined with miR-448 on EJ cell apoptosis. **A**, NC group; **B**, miR-448 mimic group; **C**, Control; **D**, Paclitaxel group; **E**, Paclitaxel + miR-448 mimic group; **F**, Cell apoptosis rate. *p<0.05, miR-448 mimic group compared with NC group. #p<0.05, paclitaxel group compared with control. ##p<0.05, paclitaxel combined with miR-448 mimic group compared with paclitaxel group.

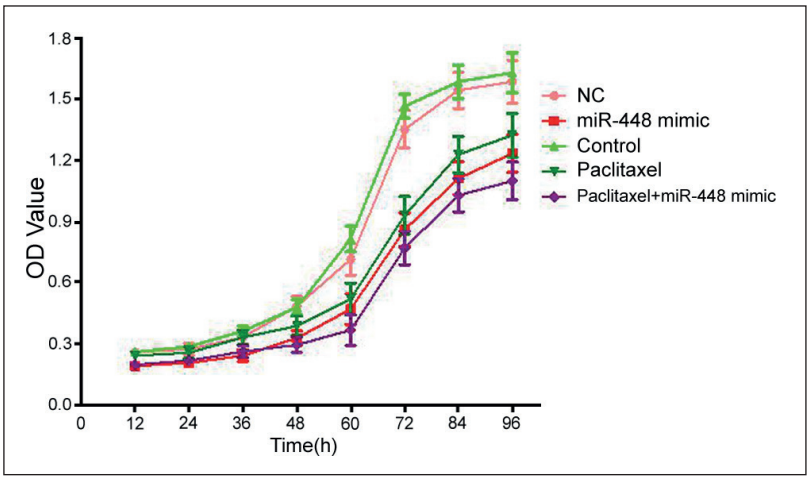

Figure 7. The influence of paclitaxel combined with miR-448 on EJ cell proliferation.

apoptosis by down-regulating the expression of Bcl-2 gene, thus is one of the mechanisms that inhibit cell proliferation[21].

This study observed that Bcl-2 gene expression decreased and apoptosis rate elevated in paclitaxel group compared with the control group ($p<0.05$). Further studies showed that paclitaxel did not induce miR-448 expression changes ($p>0.05$). However, paclitaxel combined with miR-448 significantly decreased the expression of Bcl-2 gene, enhanced apoptosis, and reduced cell proliferation compared with paclitaxel group ($p<0.05$). Paclitaxel combined with miR-448 significantly increased apoptosis and inhibited cell proliferation, which may be related to the regulation of Bcl-2 gene expression by miR-448.

Conclusions

We showed that Bcl-2 gene is the target gene of miR-448. Paclitaxel combined with miR-448 can enhance paclitaxel-induced bladder cancer EJ cell apoptosis and inhibit cell proliferation. This effect may be related to the regulation of Bcl-2 gene expression by miR-448. The therapeutic effect of paclitaxel on bladder cancer is multifaceted. In this study, paclitaxel combined with miR-448 inhibits the growth, and proliferation of cells is a part of its mechanism. The mechanism of paclitaxel for bladder cancer treatment needs further investigation.

Conflict of interest

The Authors declare that they have no conflict of interests.

References

1) Lerner SP, Bajorin DF, Dinney CP, Efstathiou JA, Grossen S, Hahn NM, Hansel D, Kwiatkowski D, O'Donnell M, Rosenberg J, Svatek R, Abrams JS, Al-Ahmadie H, Apolo AB, Bellmunt J, Callahan M, Cha EK, Drake D, Jarow J, Kamat A, Kim W, Knowles M, Mann B, Marchionni L, McConkey D, McShane L, Ramirez N, Sharabi A, Sharpe AH, Solit D, Tangen CM, Amiri AT, Van Allen E, West PJ, Witjes JA, Quale DZ. Summary and Recommendations from the National Cancer Institute's Clinical Trials Planning Meeting on Novel Therapeutics for Non-Muscle Invasive Bladder Cancer. Bladder Cancer 2016; 2: 165-202.
2) Chen CL, Chung T, Wu CC, Ng KF, Yu JS, Tsai CH, Chang YS, Liang Y, Tsui KH, Chen YT. Comparative tissue proteomics of microdissected specimens reveals novel candidate biomarkers of bladder cancer. Mol Cell Proteomic 2015; 14: 2466-2478.
3) Braicu C, Cojocneanu-Petric R, Chira S, Truta A, Floares A, Petrut B, Achimas-Cadariu P, Berindan-Neagoe I. Clinical and pathological implications of miRNA in bladder cancer. Int J Nanomed 2015; 10: 791-800.
4) Ye F, Wang L, Castillo-Martin M, McBride R, Galsky MD, Zhu J, Boffetta P, Zhang DY, Cordon-Cardo C. Biomarkers for bladder cancer management: present and future. Am J Clin Exp Urol 2014; 2: 1-14.
5) Al-Zalabani AH, Stewart KF, Wesselius A, Schols AM, Zeegers MP. Modifiable risk factors for the prevention of bladder cancer: a systematic review of meta-analyses. Eur J Epidemiol 2016; 31: 811-851.
6) Premo C, Apolo AB, Agarwal PK, Citrin DE. Trimodality therapy in bladder cancer: who, what, and when? Urol Clin North Am 2015; 42: 169-180.
7) Kampan NC, Madondo MT, McNally OM, Quinn OM, Plebanski M. Paclitaxel and its evolving role in the management of ovarian cancer. Biomed Res Int 2015; 2015: 413076.
8) Lisse TS, Middleton LJ, Pellegrini AD, Martin PB, Spaulding EL, Lopes O, Brochu EA, Carter EV, Waldron A, Rieger S. Paclitaxel-induced epithelial damage and ectopic MMP-13 expression pro-

motes neurotoxicity in zebrafish. Pro Natl Acad Sci USA 2016; 113: E2189-e2198.
9) Steinkraus BR, Toegel M, Fulga TA. Tiny giants of gene regulation: experimental strategies for microRNA functional studies. Wiley Interdiscip Rev Dev Biol 2016; 5: 311-362.
10) Akhtar MM, Micolucci L, Islam MS, Olivieri F, Procopio AD. Bioinformatic tools for microRNA dissection. Nucleic Acids Res 2016; 44: 24-44.
11) Bertoli G, Cava C, Castiglioni I. MicroRNAs: new biomarkers for diagnosis, prognosis, therapy prediction and therapeutic tools for breast cancer. Theranostics 2015; 5: 1122-1143.
12) Li B, Ge L, Li M, Wang L, Li Z. miR-448 suppresses proliferation and invasion by regulating IGF1R in colorectal cancer cells. Am J Transl Res 2016; 8: 3013-3022.
13) Li YH, Li XM, Lu MS, Lv MF, Jin X. The expression of the BRM and MMP2 genes in thoracic aortic aneurysm and aortic dissection. Eur Rev Med Pharmacol Sci 2017; 21: 2743-2748.
14) Jones C, Filloux A. Gene amplification and qRT-PCR. Methods Mol Biol 2014; 1149: 457-468.
15) Liu H, Chang HK, Hou JQ, Zhao ZH, Zhang LD. Inhibition of miR-221 influences bladder cancer cell proliferation and apoptosis. Eur Rev Med Pharmacol Sci 2017; 21: 3193-3199.
16) Wang SL, Huang JA, Liu XQ. Mechanism of action of KIAA1456 gene on the proliferation and apoptosis of alveolar epithelial cells. Eur Rev Med Pharmacol Sci 2017; 21: 600-605.
17) Tomlinson B, Lin TY, Dall'Era M, Pan CX. Nanotechnology in bladder cancer: current state of development and clinical practice. Nanomedicine 2015; 10: 1189-1201.
18) Mao XP, Zhang LS, Huang B, Zhou SY, Liao J, Chen LW, Qiu SP, Chen JX. Mir-135a enhances cellular proliferation through post-transcriptionally regulating PHLPP2 and FOXO1 in human bladder cancer. J Transl Med 2015; 13: 86.
19) Park SY, Kim MJ, Park SA, Kim SA, Min KN, Kim DK, Lim W, Nam JS, Sheen YY. Combinatorial TGF-beta attenuation with paclitaxel inhibits the epithelial-to-mesenchymal transition and breast cancer stem-like cells. Oncotarget 2015; 6: 37526-37543.
20) Foight GW, Keating AE. Locating Herpesvirus Bcl-2 Homologs in the Specificity Landscape of anti-apoptotic Bcl-2 proteins. J Mol Biol 2015; 427: 2468-2490.
21) Kwon CH, Park HJ, Choi Y, Won YJ, Lee SJ, Park DY. TWIST mediates resistance to paclitaxel by regulating Akt and Bcl-2 expression in gastric cancer cells. Tumour Biol 2017; 39: 1010428317722070.