Abstract. Multidrug resistance (MDR) is the major obstacle to bladder cancer chemotherapy. Several mechanisms have been implicated in the development of MDR, including extrusion of the drug by cell membrane pumps, associated with P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP); increased DNA damage repair, associated with topoisomerase II (Topo II); suppression of drug-induced apoptosis, associated with p53; and regulation of cancer cell growth, associated with vascular endothelial growth factor (VEGF). In the present study, the expression levels of these five markers were detected in an adriamycin (ADM)-resistant human bladder cancer cell line (pumc-91/ADM) and its parental cell line (pumc-91), in order to determine which marker is more important, or whether all of them participate in drug resistance. The expression levels of P-gp, MRP, Topo II, VEGF and p53 were measured in the two cell lines by reverse transcription-quantitative polymerase chain reaction, western blotting and immunohistochemistry. A significant increase in P-gp, MRP and VEGF, and a decrease in Topo II mRNA expression were detected in the pumc-91/ADM drug-resistant cell line compared with the pumc-91 cell line; however, no difference in p53 mRNA expression was detected between the cells. In pumc-91/ADM cells, the protein expression levels of P-gp and MRP were upregulated, whereas Topo II was significantly decreased. However, no marked differences in p53 or VEGF expression were detected between the two cell lines at the protein level. The cytoplasmic and cell membrane localization of P-gp and MRP, the cytoplasmic localization of VEGF, and the nuclear localization of p53 and Topo II were confirmed in the two cell lines. The present study detected increased P-gp and MRP, and reduced Topo II expression in pumc-91/ADM cells compared with pumc-91 cells; however, no difference was detected in p53 and VEGF expression between the cell lines. In conclusion, a significant upregulation of MRP and downregulation of Topo II were detected in the ADM-resistant human bladder cancer cell line (pumc-91/ADM) compared with in the parental cell line (pumc-91).

Introduction

Bladder cancer, which is the second most common malignant tumor of the urinary system, is ranked as the fourth most common cancer in males and the eighth leading cause of cancer-associated deaths worldwide (1). Intravesical administration of chemotherapy drugs and adjuvant chemotherapy after surgery have been the standard treatment of bladder cancer for >20 years (2). The five-year survival rate of patients with bladder cancer is 72.8% in men and 69.3% in women (3). Although chemotherapeutic drugs, including doxorubicin, vincristine, cisplatin and methotrexate, are widely used in the radical and palliative treatment of human bladder cancer, multidrug resistance (MDR) is one of the major drawbacks of bladder cancer chemotherapy. MDR is a significant obstacle to successful cancer treatment, which contributes to >90% of all treatment failures in patients with bladder cancer (4). Therefore, further studies regarding MDR in bladder cancer are urgently required.

MDR is a complex process associated with numerous factors in various types of cancer. In several instances, resistance is achieved by an increased efflux of chemotherapeutic agents out of the tumor. For example, P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP) are two classical efflux proteins, which result in drug resistance by pumping drugs out of the cell. Alterations to the drug target enzyme activity are also associated with drug resistance. Dysregulation of topoisomerase II (Topo II) results in the dissociation of cleavable complexes and reduction of DNA damage, which consequently induces drug resistance. Apoptotic cell death is the most common underlying
mechanism of antineoplastic drugs; however, the excessive expression of anti-apoptotic genes, such as p53, may lead to chemotherapy resistance. Microenvironmental resistance is another important factor; changes to the tumor microenvironment due to the action of vascular endothelial growth factor (VEGF) may also result in MDR (5-9).

Our laboratory has successfully established an adriamycin (ADM)-resistant human bladder cancer cell line (pumc-91/ADM) from the parental cell line pumc-91. According to drug resistance spectrum analysis, the pumc-91/ADM cell line exhibited the characteristics of MDR (10). In the present study, the expression levels of the five markers were analyzed in pumc-91/ADM and pumc-91 cells in order to determine the relationship between protein expression and drug resistance. In addition, the possible pathways that may lead to bladder cancer MDR were evaluated. The results may provide the basis for the knockdown or transfection of a target to reverse MDR in the future.

Materials and methods

**Cell culture.** The pumc-91 human bladder cancer cell line was generously provided by the Peking Union Medical College Hospital (Beijing, China). The increasing concentration gradient method was adopted in vitro to induce resistance. Increasing ADM concentrations (0.3, 0.6 and 1.0 µg/ml) were used to establish pumc-91/ADM multidrug-resistant bladder cell lines. The half maximal inhibitory concentration (IC₅₀) values for ADM treatment in pumc-91 and pumc-91/ADM cells were 0.61 and 6.02 µM, respectively. The IC₅₀ for pumc-91/ADM was 9.86-fold higher than that of pumc-91, thus indicating that the ADM-resistant cell line pumc-91/ADM (1.0 µg/ml) was successfully established. Pumc-91 and pumc-91/ADM cells were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Beijing Dingguo Biotechnology Co., Ltd., Beijing, China), respectively. Cells were maintained at 37°C in an atmosphere containing 5% CO₂.

**Drug cytotoxicity analysis.** To analyze drug cytotoxicity, 2.0×10⁴ cells/well were cultured with a concentration gradient (0.3, 0.6 and 1.0 µg/ml) of ADM (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) in 96-well plates at 37°C. Following 72 h of drug treatment, 20 µl 3-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to the cells. Following a 4 h incubation at 37°C, absorbance was measured in the presence of dimethyl sulfoxide at 450 nm. Cell survival in the absence of the drug was defined as 100% cell survival. The IC₅₀ values of ADM were determined from the corresponding absorbance.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated from the cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) as previously described (11). Total RNA (2 µg) was then reverse transcribed to cDNA using the Reverse Transcription system according to the manufacturer's protocol (Promega Corporation, Madison, WI, USA). TransStart Top Green qPCR SuperMix (Beijing TransGen Biotech Co., Ltd., Beijing, China) and Roche LightCycler 480 Real Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used to conduct RT-qPCR, according to the manufacturers' protocols. The PCR conditions were as follows: 45 cycles of annealing at 55°C for 1 min, denaturation at 95°C for 40 sec, and elongation at 72°C for 1 min. The PCR primer sequences are presented in Table I. The 2⁻ΔΔCq method was applied for relative quantitative analysis (12). All samples were independently analyzed at least three times in duplicate.

**Western blot analysis.** Pumc-91 and pumc-91/ADM cells were grown to 80-90% confluence. Cells were harvested, washed three times with phosphate-buffered saline (PBS), and lysed with radioimmunoprecipitation assay cell lysis buffer (Beijing Dingguo Biotechnology Co., Ltd.). The protein was quantified using Bicinchoninic Acid kit (Sigma-Aldrich; Merck Millipore) and protein samples (60 µg) were loaded onto an 8% sodium dodecyl sulfate-polyacrylamide gel and were electrotransferred to polyvinylidene fluoride membranes. After blocking with 5% nonfat dry milk for 2 h at room temperature, the membranes were incubated with the following primary antibodies: P-gp (1:2,000; cat. no. ab170903; Abcam, Cambridge, MA, USA), Topo II (1:10,000; cat. no. ab12318; Abcam), MRP (1:500; cat. no. sc-18835; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), p53 (1:500; cat. no. sc-126; Santa Cruz Biotechnology, Inc.), VEGF (1:500; cat. no. sc-65617; Santa Cruz Biotechnology, Inc.) and β-actin (1:400; cat. no. TA-09; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) at 4°C overnight. Subsequently, the membranes were incubated with goat anti-mouse immunoglobulin (Ig) G (1:1,000; Beijing Dingguo Biotechnology Co., Ltd.; cat. no. IH-0031) or goat anti-rabbit IgG (1:1,000; Beijing Dingguo Biotechnology Co., Ltd.; cat. no. IH-0011) for 30 min at 37°C. After extensive washing with PBS with Tween 20, proteins were visualized using an enhanced horseradish peroxidase-3,3′-diaminobenzidine (DAB) chromogenic kit (Tiangen Biotech Co., Ltd., Beijing, China), according to the manufacturer's protocol. Lane 1D gel analysis software (Beijing Sage Creation Science Co., Ltd., Beijing, China) was used to analyze the bands.

**Immunohistochemistry.** For staining, the pumc-91 and pumc-91/ADM cell slides were fixed with 4% paraformaldehyde for 15 min at room temperature. After washing with PBS, the slides were treated with 50 µl hydrogen peroxide (Beijing Dingguo Biotechnology Co., Ltd.) for 20 min at room temperature to block the action of endogenous peroxidases. Subsequently, the slides were blocked in nonimmune serum (Beijing Dingguo Biotechnology Co., Ltd.) at 37°C for 30 min. The specimens were then incubated with the following primary antibodies: P-gp (1:400), Topo II (1:10,000), MRP (1:400), p53 (1:500), VEGF (1:500) and β-actin (1:200) at 37°C for 1 h, followed by incubation with secondary antibodies (1:1,000; EnVision Detection systems, Peroxidase/DAB, Rabbit/Mouse; Dako, Carpinteria, CA, USA) for 30 min at 37°C. A DAB kit was used to visualize the immunohistochemical staining, and slides were counterstained with hematoxylin. Finally, specimens were dehydrated in gradient alcohol. The slides were observed using an Olympus CX31 microscope (Olympus Corporation, Tokyo, Japan) and the staining intensity and percentage of stained cells were quantified according to a
previously described approach (13). Professional image analysis software, Image Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD USA) was used to evaluate the results.

**Statistical analysis.** All data are presented as the mean ± standard deviation of at least three independent experiments. The differences between groups were assessed by Student’s t-test. Statistical analysis was carried out using SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). *P*<0.05 was considered to indicate a statistically significant difference.

**Results**

**Differential expression of mRNA in the two cell lines.** The mRNA expression levels of P-gp, MRP, Topo II, p53 and VEGF were detected by RT-qPCR. Glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous control. The mRNA expression levels of P-gp, MRP and VEGF were markedly upregulated, whereas Topo II was downregulated in the pumc-91/ADM cells compared with the pumc-91 cell line (P=0.017, 0.045, 0.029 and 0.004, respectively; Fig. 1). These differences were statistically significant. However, no difference was detected in p53 expression at the genetic level between the two cell lines (P=0.722; Fig. 1).

**Differential expression of proteins in the two cell lines.** The corresponding protein expression levels were confirmed by western blot analysis in the two cell lines. Compared with pumc-91 cells, a significant increase in P-gp and MRP, and decrease in Topo II was detected in the pumc-91/ADM drug-resistant cell line (P=0.008, 0.031 and 0.014, respectively; Fig. 2). These differences were statistically significant. However, no evident differences in p53 and VEGF expression were detected between the two cell lines at the protein level (P=0.103 and 0.700, respectively; Fig. 2).

**Immunohistochemical protein expression in the two cell lines.** The results of the expression analyses were further confirmed by localization of P-gp, MRP, Topo II, p53 and VEGF by immunohistochemistry in the two cell lines. Image Pro Plus 6.0 was used to evaluate the results. β-actin was used as a positive control, whereas PBS was used instead of the primary antibodies as a negative control. The cytoplasmic and cell membrane localization of P-gp and MRP,
Figure 2. (A) P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), topoisomerase II (Topo II), p53 and vascular endothelial growth factor (VEGF) protein expression levels were verified by western blotting. β-actin was used as an internal control. (B) Integrated optical density (IOD) value of each band was analyzed using Lane 1D gel analysis software. All samples were independently analyzed three times in duplicate. Data are presented as the mean ± standard deviation. *P<0.05 vs. pumc-91 cells. ADM, adriamycin.

Figure 3. Immunohistochemical staining of P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), topoisomerase II (Topo II), p53 and vascular endothelial growth factor (VEGF) expression in pumc-91 and adriamycin (ADM)-resistant pumc-91 ADM cell lines. P-gp in (A) pumc-91 and (B) pumc-91/ADM cells. MRP in (C) pumc-91 and (D) pumc-91/ADM cells. Topo II in (E) pumc-91 and (F) pumc-91/ADM cells, p53 in (G) pumc-91 and (H) pumc-91/ADM cells. VEGF in (I) pumc-91 and (J) pumc-91/ADM cells. (K) β-actin was used as a positive control. (L) Phosphate-buffered saline instead of primary antibodies was used as a negative control. (M) Protein expression was presented as integrated optical density (IOD)/area of at least three independent experiments. Data are presented as the mean ± standard deviation. *P<0.05 vs. pumc-91 cells. Magnification, x100.
the cytoplasmic localization of VEGF, and the nuclear localization of p53 and Topo II were observed in the two cell lines. Consistent with the results of western blotting, P-gp and MRP were upregulated, and Topo II was downregulated in pumc-91/ADM cells compared with in pumc-91 cells (P=0.014, 0.048 and 0.039, respectively; Fig. 3). No significant difference in p53 and VEGF expression was detected between the two cell lines (P=0.316 and 0.113, respectively; Fig. 3).

Discussion

Adjuvant chemotherapy following surgery is considered necessary for the treatment of bladder cancer; however, long-term chemotherapy decreases the response of cancer cells to anticancer agents, thus leading to the occurrence of tumor escape. In the majority of cancer types, several key molecules participate in the development of MDR, including P-gp, MRP, Topo II, p53 and VEGF (14-19). The present study investigated the relationship between the expression levels of these five markers and drug resistance in ADM-resistant (pumc-91/ADM) and parental (pumc-91) human bladder cancer cell lines. The results provided evidence regarding the possible pathways that lead to bladder cancer MDR. In addition, the results may provide useful information for the reversal of MDR in bladder cancer.

Among the five molecules evaluated in the present study, aberrant expression of P-gp and MRP has been the most extensively reported. P-gp and MRP are two important members of the ATP-binding cassette transporter superfamily, which have been reported to confer resistance against various chemotherapeutic agents (20). By affecting the intracellular drug concentration through drug eflux alterations, P-gp and MRP are associated with drug resistance in various types of cancer. The results of the present study suggested that P-gp and MRP were upregulated in pumc-91/ADM cells compared with in pumc-91 cells. Notably, MRP demonstrated a more significant increasing trend. The mechanisms underlying the effects of MRP on MDR include effects on drug eflux and microenvironment alterations. MRP is able to redistribute intracellular drugs, via altering the pH microenvironment of the cytoplasm and organelles, which may trigger resistance (21). Therefore, in further studies regarding the reversal of MDR, researchers have selected MRP as the first factor to target by transfection or expression knockdown. Copsel et al reported that knockdown of MRP by short hairpin RNA could regulate drug resistance in the U937 acute myeloid leukemia cell line (22). In addition, Su and Pasternak revealed that downregulation of MRP by short hairpin RNA could regulate drug resistance in the U937 acute myeloid leukemia cell line (22). In conclusion, the present study detected a significant upregulation of MRP in ADM-resistant human bladder cancer cells (pumc-91/ADM) compared with in the parental cells (pumc-91). Further studies are required to explore the specific mechanisms underlying MDR in bladder cancer. In addition, how to reverse MDR based on the targets examined in the present study will be our future aim.

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References