The Establishment of Two Paclitaxel-Resistant Prostate Cancer Cell Lines and the Mechanisms of Paclitaxel Resistance withTwo Cell Lines

Masashi Takeda,[1] Atsushi Mizokami,[1]* Kiminori Mamiya,[1] You Qiang Li,[1] Jian Zhang,[2] Evan T. Keller,[3] and Mikio Namiki[1]

[1]*Department of Integrative Cancer Therapy and Urology, Kanazawa University Graduate School of Medical Sciences, Kanazawa, Ishikawa, Japan*
[2]*Department of Medicine, Division of Hematology/Oncology, University Drive, Pittsburgh*
[3]*Unit for Laboratory Animal Medicine and Department of Pathology, University of Michigan, Ann Arbor, Michigan*

BACKGROUND. Although paclitaxel is used for hormone-resistant prostate cancer, relapse definitely occurs later. Details of the molecular mechanism responsible for paclitaxel- resistance remain unclear.
METHODS. We established paclitaxel-resistant cells, DU145-TxR and PC-3-TxR from parent DU145 and PC-3. To characterize these cells, we examined cross-resistance to other anticancer drugs. Expression of several potential genes that had been related to drug-resistance was compared with parent cells by RT-PCR and Western blotting. Methylation analysis of multiple drug resistance (MDR1) promoter was carried out using bisulfite-modified DNA from cell lines. Knockdown experiments using small interfering RNA (siRNA) were also performed to confirm responsibility of drug-resistance. Finally, cDNA microarray was performed to quantify gene expression in PC-3 and PC-3-TxR cells.
RESULTS. The IC_{50} for paclitaxel in DU145-TxR and PC-3-TxR was 34.0- and 43.4-fold higher than that in both parent cells, respectively. Both cells showed cross-resistance to some drugs, but not to VP-16 and cisplatin. Methylation analysis revealed that methylated CpG sites of MDR1 promoter in DU145 and PC-3 cells were demethylated in DU145-TxR cells, but not in PC-3-TxR cells. Knockdown of P-glycoprotein (P-gp), which was up-regulated in resistant cells, by MDR-1 siRNA restored paclitaxel sensitivity in DU145-TxR but not in PC-3-TxR, indicating that up-regulation of P-gp was not always main cause of paclitaxel-resistance. Microarray analysis identified 201 (1.34%) up-regulated genes and 218 (1.45%) out of screened genes in PC-3-TxR.
CONCLUSIONS. Our data will provide molecular mechanisms of paclitaxel-resistance and be useful for screening target genes to diagnose paclitaxel sensitivity. *Prostate 67: 955–967, 2007.*

KEY WORDS: **prostate cancer; paclitaxel resistance; MDR-1; cDNA microarray**

INTRODUCTION

Prostate cancer (PCa) is the most common malignancy and the second most frequent cause of cancer-related death of men in the United States [1]. Androgen deprivation treatment is very effective for more than 80% of advanced PCa. More than half of those cases of advanced PCa become resistant to deprivation treatment after several years and then several other

*Correspondence to: Atsushi Mizokami, MD, PhD, Department of Integrative Cancer Therapy and Urology, Kanazawa University Graduate School of Medical Sciences, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan. E-mail: mizokami@med.kanazawa-u.ac.jp
Received 24 November 2006; Accepted 14 February 2007
DOI 10.1002/pros.20581
Published online 17 April 2007 in Wiley InterScience (www.interscience.wiley.com).

palliative treatments, such as estramustine phosphate (EMP), steroids, are employed for these patients. However, the results are very disappointing because a half of those cases lead to death within a year or 2 years.

Recently, the taxanes [paclitaxel or docetaxel (DTX)] with other agents, such as EMP or predonisone have been used for hormone-resistant prostate cancer (HRPC) and have shown good response [2–5]. Paclitaxel, which is purified from *Taxus brevifolia*, stabilize microtubule and causes apoptosis [6]. The response rates of taxane-based combination therapies are better than combination therapies with other anticancer agents. However, even HRPC treated with paclitaxel-based chemotherapy also relapses as occurred using other anticancer agents. Then the prognosis of the patients after the relapse is extremely poor.

In order to investigate the mechanisms of paclitaxel-resistance, several paclitaxel-resistance cell lines have been generated in ovarian cancer, breast cancer, and lung cancer [7,8]. Some of major mechanisms of taxane-resistance are overexpression of multiple drug resistance (MDR1), and multidrug resistance protein (MRP) family [9]. Especially accumulation of P-glycoprotein (P-gp) encoded from MDR1 might cause resistance of several drugs in some cancers. The microtubule dynamics may also be important for paclitaxel-resistance because the target of paclitaxel is the microtubule [10]. As for the role of bcl-2 as a modulator of paclitaxel sensitivity remains controversial. In human paclitaxel-resistant hepatocellular carcinoma cells bcl-2 was overexpressed [11]. Whereas bcl-2 expression was consistently down-regulated in T47-D breast cancer cells [12]. In PCa, although Bcl-2/Bcl-xL bispecific antisense oligonucleotide also enhanced paclitaxel chemosensitivity in PC-3 and LNCaP cells [13,14], involvement to paclitaxel-resistance of Bcl-2/Bcl-xL in PCa is not clear. Recently, cDNA microarray analyses were performed in order to reveal the key genes that are related with paclitaxel resistance. Not only MDR-1 gene but also Rho guanine dinucleotide phosphate dissociation inhibitor beta (RhoGDI) and insulin-like growth factor-binding protein 3 (IGFBP-3) were up-regulated in paclitaxel-resistant ovarian cancer cell lines [15]. Villeneuve et al.[16]described that 1.9% of 1,728 genes were regulated in paclitaxel-resistant MCF-7 breast cancer cells. Thus it is very important to know the mechanisms of paclitaxel-resistance in PCa.

In the present study, we established two paclitaxel-resistant cell lines from androgen-independent DU145 and PC-3 PCa cell lines by increasing concentration of paclitaxel gradually. Although both cell lines showed resistance to paclitaxel over 30 times more than parents cells and cross-resistance to other anticancer drugs, the mechanism of resistance was different.

MATERIALS AND METHODS

Cell Culture and Cell Proliferation Assay

DU145 and PC-3 cells purchased from American type culture collection were cultured in Dulbecco's modified Eagle medium (DMEM) and RPMI1640 containing 5% fetal calf serum (FCS) and penicillin/streptomycin (Invitrogen, CA, USA). Cell growth inhibition assay was preformed by plating 1×10^5 cells on 6-well plates. Twenty-four hours later, cells were treated with the indicated concentration of anticancer agents, and cultured for an additional 48 hr. At the end of the culture period, the cells were trypsinized and counted with a hemocytometer.

Establishment of Paclitaxel-resistant DU145 and PC-3 Cell Lines

Paclitaxel-resistant cancer cells were obtained by stepwise increased concentrations of paclitaxel. DU145 and PC-3 cells maintained as described above were incubated with 10 nM paclitaxel for 2 days. Then the medium was changed to fresh one without paclitaxel and cells were cultured cells grow well. Whenever we subcultured, the cells were incubated with gradual increasing concentration of paclitaxel for 2 days and cultured without paclitaxel until cells grow well. Some aliquots of the cells were stored whenever we subcultured it. When cells were killed by increased paclitaxel, the aliquots were subcultured again and lower concentration of paclitaxel was used for treatment. Cells that grew at the maximum concentration of paclitaxel were stored for further analyses. For maintenance of paclitaxel-resistant cells, 10 nM paclitaxel was added into the normal medium every time.

RNA extraction and RT-PCR. Twenty-four hours after plating of 1×10^6 DU145 or PC-3 cells, total RNA was purified with RNeasy mini kit (Qiagen, Maryland, USA). Complementary DNA (cDNA) was made by reverse-transcription (RT) of 1 μg each total RNA using ThermoScript RT-PCR system (Invitrogen). Each cDNA sample was amplified with ExTaq (Takara, Japan). PCR reactions for indicated genes were carried out using the following forward (F) and reverse (R) in Table I. Each of the amplified PCR products was determined by electrophoresis on an 1.5% agarose gel.

Western blot analysis. Twenty-four hours after plating 1×10^6 DU145, DU145-TxR or PC-3, and PC-3-TxR cells on 6 cm dishes in DMEM-5% FBS, the cells were lysed with 200 μl hypotonic buffer (20 mM Tris-HCl (pH 7.6), 10 mM NaCl, 1 mM $MgCl_2$, and 0.5% NP-40) and the membrane and cytosol fractions were collected by centrifugation as described previously [17]. To

TABLE I. The Primers Used for RT-PCR Analysis

Gene	Forward	Reverse
GAPDH	5′-GACCACAGTCCATGCCATCA-3′	5′-TCCACCACCCTGTTGCTGTA-3′
MDR-1	5′-ATGCTCTGGCCTTCTGG ATG GGA-3′	5′-ATGGCGATCCTCTGCTTCTGCCCA C-3′
MRP-1	5′-GCATGA TCCCTGAAGACGA-3′	5′-TAGAGCTGG CCCTTGTACTC-3′
MRP2	5′-TAGAGCTGGCCCTTGTACTC-3′	5′-TCAACTTCCCAGACATCCTC-3′
MRP-3	5′-CGCCTGTTTTTCTGGTGGTT-3′	5′-TCCCCCAGTCACAAAGATG -3′
MRP-4	5′-GCTGAGAATGACGCACAGAA-3′	5′-TCCCAGCAAGGCACGATATT-3′
MRP-5	5′-GTCCTGGGTATAGAAGTGTG-3′	5′-CAGAAGATCCACACAACCCT-3′
MRP-6	5′-TTGGATTCGCCCTCATAGTC-3′	5′-TCTTTTGGTCTCAGTGGCCT-3′
MRP-7	5′-CTCCCACTGGATCTCTCAGC-3′	5′-TCGCATACACGGTGAGGTAG-3′
Fas	5′-CAGGCTAACCCCACTCTATG-3′	5′-TGGGGGTGCATTAGGCCATT-3′
Caspase-8	5′-ACTTCAGACACCAGGCAGGGC T-3′	5′-GCCCCTGCATCCAAGTGTGTTC-3′
Bcl-2	5′-ATGTCCAGCCAGCTGCACCTGAC-3′	5′-GCAGAGTCTTCAGAGACAGCCAGG-3′
Bax	5′- GCTTCAGGGTTTCATCCAGG-3′	5′-AAAGTAGGAGAGGAGGCCGT-3′
c-jun	5′- GGAAA GACCTTCTATGACGATGC -3′	5′-GAACCCCTCCTGCTCATCTGT CAC-3′
YB-1	5′-GACTGCCATAGAGAATAACCCCAG-3′	5′-CTCTCTAGGCTGTTTTGGGCGAGGA-3′
Sp-1	5′-GCTGCCGCTCCCAACTTACA-3′	5′-ATCGTGACTGCCTGAGAGCT-3′

extract nuclear protein, the centrifuged pellet after separating cytosol fraction was lysed with 50 μl hypertonic buffer (20 mM Tris-HCl (pH 7.6), 0.42 M NaCl, 1 mM EDTA, and 0.5% NP-40) and nuclear fraction were collected by centrifugation. To extract whole cell protein, cells were lysed with hypertonic buffer directly. Fifty micrograms of cytosol protein, 50 μg of whole cell protein, or 10 μg of nuclear protein was loaded in each lane of 7.5 or 12.5% Ready Gel J (Bio-Rad, NY), subjected to electrophoresis, then electrotransferred to a PVDF membrane (Bio-Rad). The immobilized proteins were incubated with primary antibody, P-gp (rabbit polyclonal IgG, 200-fold dilution; Santa Cruz, CA), YB-1 (goat polyclonal IgG, 200-fold dilution; Santa Cruz), or GAPDH (rabbit polyclonal IgG, 1,000-fold dilution; Trevigen, MD). The presence of primary antibody was visualized by Super signal west pico luminol/enhancer solution (Pearce, IL).

Methylation analysis of MDR1 promoter. Genomic DNA from PC-3, PC-3-TxR, DU145, and DU145-TxR was purified using Blood and cell culture DNA mini kit (Quiagen) 24 hr after 5×10^5 cells were plated on 6 cm dish. One microgram of DNA was subjected to sodium bisulfite modification kit (BisulFast DNA Modification Kit, Toyobo, Osaka, Japan). MDR-1 (223 bp) promoter region (−183 to +40 of transcription initiation site) was amplified from bisulfite-modified DNA as described by Enokida et al. [18,19]. The amplified DNA was further amplified using methylation-specific primer (MSP) or unmethylation-specific primer (USP) after 100-fold dilution of the amplified DNA [19]. PCR reaction was modified to 94°C 15 s, 70°C 30 s, 72°C and 20 cycles for MSP primers and 94°C 15 s, 68°C 30 s, 72°C and 20 cycles for USP primers. Then DNA sequence analysis was also carried out using the amplified 223 bp PCR products.

Small interfering RNA transfection. MDR-1 small interfering RNA (siRNA), lamina/C siRNA, non-targeting siRNA were purchased from Dharmacon (Lafayette, CO). After 3×10^4 DU145-TxR and PC-3-TxR cells or 3×10^5 those cells were cultured on 24-well plates or in 6-well plates for total RNA purification or for protein extraction, respectively, cells were transfected with 0, 10, 20, or 30 nM MDR-1 siRNA, 30 nM lamina/C siRNA, and 30 nM non-targeting siRNA by X-treme GENE siRNA Transfection Reagent (Roche). Forty-eight hours after transfection, total RNA and protein was extracted. In order to see the effect of siRNA on drug resistance, cells were transfected with 30 nM MDR-1 siRNA or non-targeting siRNA 24 hr after plating on 24-well plates. Twenty-four hours later cells were treated with 0, 1, 3, 10, 30, 100, 300, and 1,000 nM paclitaxel and cultured for 48 hr. Then the cells were trypsinized and counted with a hemocytometer.

cDNA Microarray Analysis

Twenty-four hours after plating of 5×10^5 PC-3 cells, total RNA was purified with RNeasy mini kit (Qiagen,). RNA samples were sent to Hokkaido system science (Sapporo, Japan) and analyzed by Agilent technologies (human 1A microarray kit).

RESULTS

Establishment of Paclitaxel-resistant Cell Lines

When we examined the sensitivity for paclitaxel of parent DU145 and PC-3 cells, IC_{50} values of these cells were 11.3 and 5.0 nM, respectively

(Tables II and III). We established paclitaxel-resistant DU145 (DU145-TxR) and PC-3 (PC-3-TxR) cells by stepwise exposure method (from 10 nM paclitaxel) for 9 and 15 months, respectively. Cell growth inhibition assay demonstrated that these DU145-TxR and PC-3-TxR cells become 34.0-fold (IC_{50}: 384.2 nM) and 43.4-fold (IC_{50}: 217.1 nM) more paclitaxel resistant than parent cells (Tables II and III and Fig. 1). We also compared the cross-resistance to other anticancer drugs [EMP, vinblastin (VBL), doxorubicin (DOX), DTX, VP-16, and cisplatin] between parent and paclitaxel-resistant cells (Figs. 2 and 3, Tables II and III). Both of DU145-TxR and PC-3-TxR cells showed almost same cross-resistance to EMP, VBL, DOX, and DTX. However, cross-resistance to cisplatin and VP-16 was hardly observed.

Expression of Several Potential Chemoresistant Genes

Cellular mechanisms of drug resistance include in decreasing intracellular drug concentrations by increased efflux or decreased influx. The drug distribution in an organism is highly dependent on transporters which play a role in absorption and elimination. P-gp and MRP which belong to the ATP-binding cassettes (ABC) family are well-known typical transporters. We evaluated the expression of MDR-1 and MRP1 to MRP7 of DU145-TxR and PC-3-TxR cells by RT-PCR. Only MDR-1 mRNA was overexpressed in both cells (Fig. 4A). Since MDR-1 mRNA was overexpressed in both cells, we confirmed the expression of P-gp which was encoded from MDR-1 mRNA. P-gp as well as MDR-1 mRNA was overexpressed in DU145-TxR and PC-3-TxR cells but not in parent cells (Fig. 4B). Moreover, the level of P-gp in DU145-TxR cells was more expressed than PC-3 cells. Since the cell death by paclitaxel is associated with apoptosis, we also compared the expression of major apoptosis-related genes, Bcl-2, Bax, Fas, and Capase-8 in these cells. However, expression level of all of these genes was not changed between parent and resistant cells (Fig. 4C).

Mechanisms of MDR1 Overexpresssion in DU145-TxR and PC-3-TxR Cells

One of mechanisms by which of MDR-1 is overexpressed in paclitaxel-resistant cells is the induction by Y-box-binding protein 1 (YB-1). YB-1 is mainly located in the cytoplasm [20]. Once cells are exposed to UV irradiation and anticancer drugs, such as paclitaxel, YB-1 tanslocates into nucleus, bind to a *cis*-acting element of the MDR-1 promoter, and induce MDR-1 mRNA expression [21]. In order to see the nuclear localization of YB-1 protein, we performed Western blot analysis. The YB-1 protein level in nucleus was about three times higher in DU145-TxR cells than in DU145 cells and it was almost at the same level between PC-3 and PC-3-TxR cells (Fig. 5A). Nuclear localization of YB-1 was less dramatic compared to the MDR-1 expression in paclitaxel-resistant cells.

Next, we investigated methylation status of CpG sites at the MDR1 promoter region because some

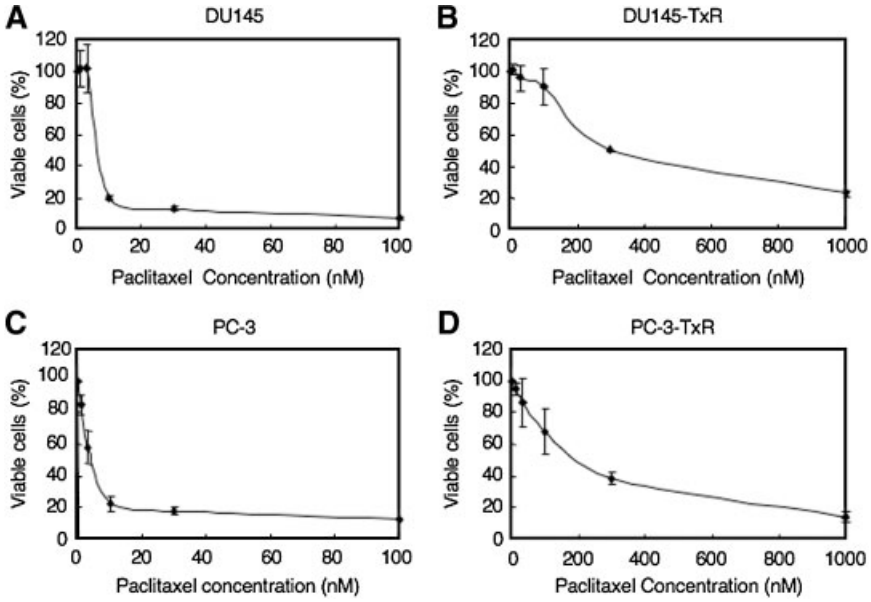

Fig. 1. Establishment of paclitaxel-treated cell lines. DU145 (**A**), paclitaxel-resistant DU145-TxR (**B**), PC-3 (**C**), and paclitaxel-resistant PC-3-TxR (**D**) cells were exposed with indicated concentrations of paclitaxel for 24 hr and counted 2 days after exposure.

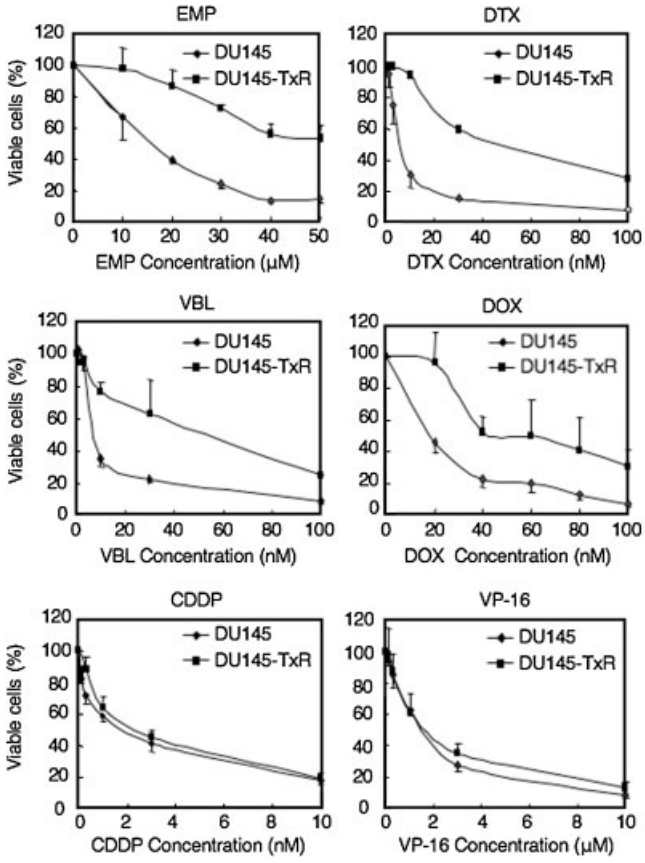

Fig. 2. Cross-resistance of DU145 and DU145-TxR cells. DU145 and DU145-TxR cells were exposed with indicated concentrations of EMP, docetaxel (DTX), vinblastin (VBL), doxorubicin (DOX), cisplatin (CDDP), and etoposide (VP-16) for 24 hr and counted 2 days after exposure.

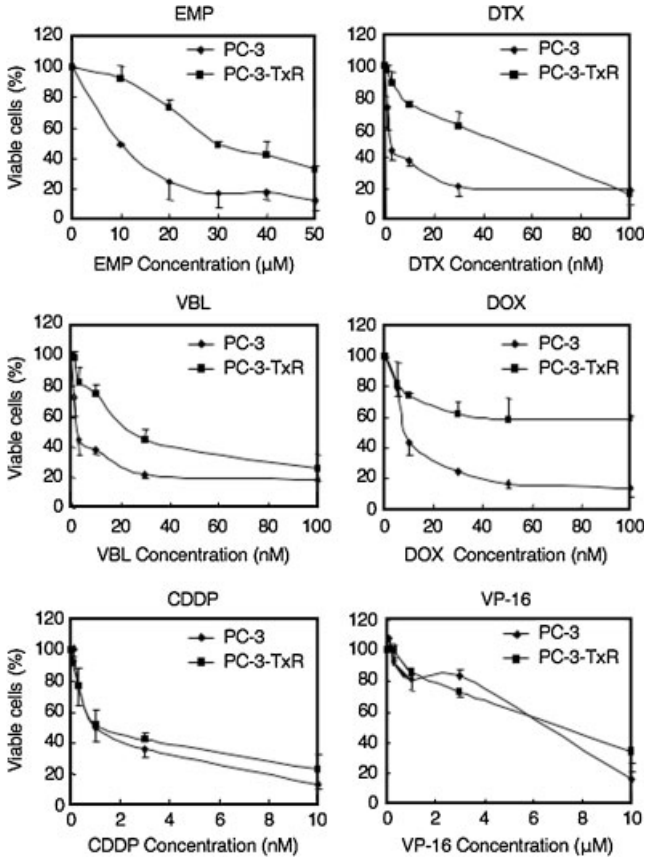

Fig. 3. Cross-resistance of PC-3and PC-3-TxR cells. PC-3 and PC-3-TxR cells were exposed with indicated concentrations of EMP, docetaxel (DTX), vinblastin (VBL), doxorubicin (DOX), cisplatin (CDDP), and etoposide (VP-16) for 24 hr and counted 2 days after exposure.

groups reported inverse correlation between methylation and MDR1 expression in [19,22,23]. Since DU145-TxR and PC-3-TxR cells overexpressed MDR1 mRNA compared to parent cells, we expected that paclitaxel-resistance might cause demethylation of CpG sites at MDR1 promoter. Although MSP published by Enokida et al. detected PCR products from bisulfite-modified DNA in both parent cells and paclitaxel-resistant cells, USP detected stronger PCR band in DU145-TxR cells than in DU145 cells, suggesting that MDR1 promoter in DU145-TxR cells is less methylated than in DU145 cells. However, USP did not detect PCR band in PC-3-TxR cells compared to PC-3 (Fig. 5B). To further confirm the methylated CpG site at the MDR1 promoter, we performed DNA sequence analysis using bisulfite-modified DNA. The MDR1 promoter region of DU145 cells was methylated at the CpG sites of −134, −105, −59, −56, −51, −34, and −29 of the transcription initiation site. The MDR1 promoter region of DU145-TxR cells was methylated only at the CpG site of -105

TABLE II. IC_{50} Value of DU145 and DU145-TxR Cells

Drug	DU145	DU145-TxR	Fold difference
PTX (nM)	11.3	384.2	34.0
EMP (μM)	15.1	49.6	3.28
DTX (nM)	8.30	55.6	6.70
VBL (nM)	14.1	40.8	2.89
DOX (nM)	17.5	61.1	3.49
VP-16 (μM)	0.83	1.10	1.33
CDDP (μM)	1.32	1.97	1.49

TABLE III. IC_{50} Value of PC-3 and PC-3-TxR Cells

Drug	PC-3	PC-3-TxR	Fold difference
PTX (nM)	5.00	217.1	43.4
EMP (μM)	8.57	33.0	3.85
DTX (nM)	3.67	28.2	7.68
VBL (nM)	8.00	27.4	2.43
DOX (nM)	121.3	1,218.2	10.0
VP-16 (μM)	4.40	5.95	1.35
CDDP (μM)	1.47	1.66	1.13

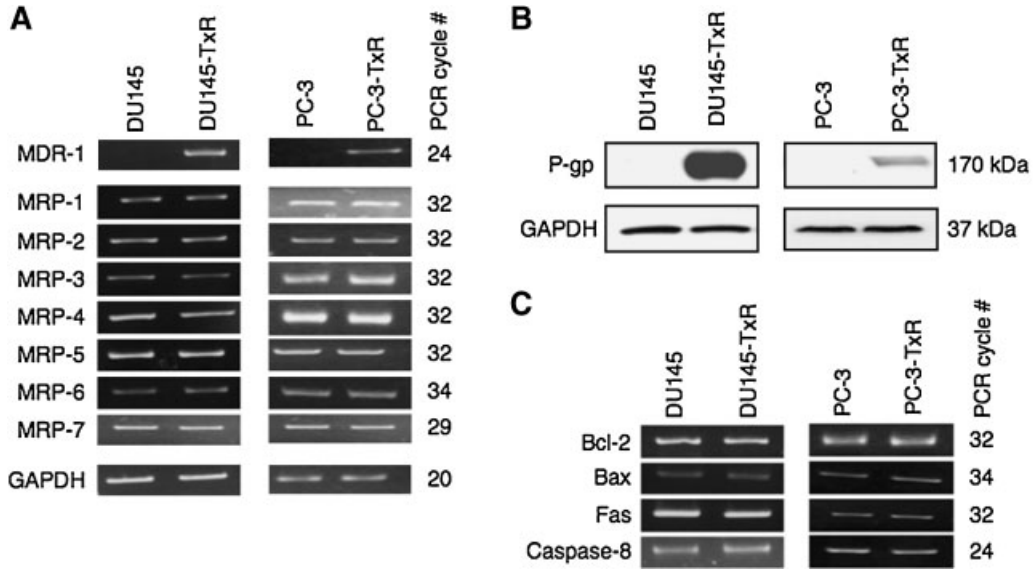

Fig. 4. Expression of various drug-resistance-related genes in parent and paclitaxel-resistant cells. (**A**) RT-PCR of MDR and MRPI-7 mRNA in DUI45, DUI45-TxR, PC-3, and PC-3-TxR cells. After mRNA was purified from these cells, RT-PCR was performed using primers as described in Table I. (**B**) Expression of P-gp. Cells were cultured for 12 h in the presence of indicated concentration of DHT or Adiol and harvested. Membrane and cytosol protein were extracted as described in Materials and Methods and loaded on an 7.5% SDS-polyacrylamide gel for Western blot analysis. After protein was transferred to PVDF-membrane, anti-P-gp antibody and anti-GAPDH antibody were employed for detection of 170 kDa P-gp and 37 kDa GAPDH protein, respectively. (**C**) RT-PCR of bcl-2, Bax, Fas, and capase-8 mRNA in DUI45, DUI45-TxR, PC-3, and PC-3-TxR cells.

(data not sown). Especially, the important region for MDR1 transcriptional regulation that included a G-box (−59, −56, and −51) [24] was demethylated in DU145-TxR cells (Fig. 5C). This demethylation of MDR1 promoter in DU145-TxR cells was coincident with the enhanced MDR1 expression. Whereas DNA sequence analysis of the amplified PCR product showed that the MDR1 promoter regions of PC-3 and PC-3-TxR cells were methylated at the CpG sites of −134, −110, −59, −51, −34, and −29 and at the CpG sites of, −110, −105, −59, −56, −51, and −29, respectively. Much difference was not observed in the methylated sites and the number between PC-3 and PC-3-TxR promoter region.

Recovery of Paclitaxel Sensitivity by MDR-1 Knockdown

In order to investigate if MDR-1 mRNA overexpression in TxR cells is the main cause of paclitaxel resistance, we knocked-down the MDR-1 mRNA by MDR-1 siRNA. Ten to thirty nanometer MDR-1 siRNA down-regulated MDR-1 mRNA in DU145-TxR and PC-3-TxR cells 48 h after transfection (Fig. 5A and C). Non-targeting siRNA and laminin siRNA failed to inhibit MDR-1 mRNA expression. MDR-1 mRNA down-regulation by MDR-1 siRNA treatment also inhibited the expression of P-gp protein.

Since MDR-1 siRNA down-regulated P-gp, we confirmed if MDR-1 down-regulation could restore paclitaxel sensitivity. As shown in Table IV and Figure 5B and D, IC_{50} of in parent DU145 and PC-3 cells was not changed when non-target (NT) siRNA or MDR-1 siRNA was transfected. Transfection with MDR-1 siRNA into DU145-TxR cells after 48 hr restored paclitaxel sensitivity compared to transfection with NT siRNA (Fig. 6B). IC_{50} of paclitaxel of DU145-TxR was reduced from 537.9 nM to 60.8 nM and recovery ratio became 88.7% 48 hr after transfection (Table IV). Whereas transfection with MDR-1 siRNA into PC-3-TxR cells hardly changed paclitaxel sensitivity. IC_{50} of paclitaxel of PC-3-TxR was reduced only from 198.4 nM to 140.6 nM and recovery ratio became 229.1% (Table IV and Fig. 6D) sensitivity.

Mechanisms of Paclitaxel Resistance in PC-3-TxR Cells

Although P-gp overexpression played important role on paclitaxel resistance in DU145-TxR cells, this was not an important factor in PC-3-TxR cells. There should be P-gp-independent pathway to become paclitaxel-resistance. In order to identify the genes that are associated with on paclitaxel resistance in PC-3-TxR cells, we performed cDNA microarray using mRNA from parent PC-3 and PC-3-TxR cells and compared differentially expressed genes as described in Materials and Methods. Approximately 15,000 genes were screened by microarray analysis. two hundred and one (1.34%) of screened genes were induced more than

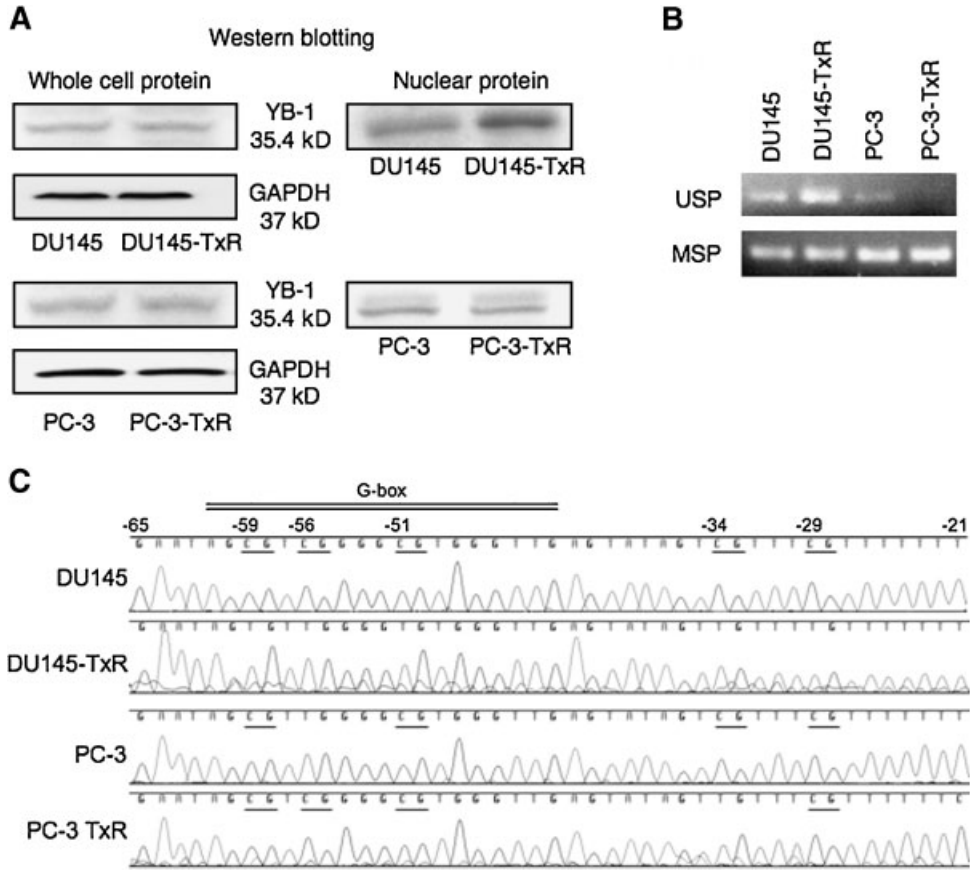

Fig. 5. Expression of YB-1 protein and methylation status of *MDR1* promoter. (**A**) Western blotting of YB-1 protein. Whole cell protein and nuclear protein were extracted as described in Materials and Methods and loaded on a 12.5% SDS-polyacrylamide gel for Western blotting. After protein was transferred to PVDF membrane, anti-YB-1 or GAPDH antibody was employed for detection of 35.4 or 37 kDa YB-1 or GAPDH protein, respectively. **B**: Detection of methylated and unmethylated promoter of MDR1 genes. USP and MSP were employed for detection of unmethylated and methylated MDR1 promoter after the 223 bp MDR1 promoter region was amplified from bisulfite-modified DNA. **C**: Bisulfite-modified DNA sequence of MDR1 promoter. The sequences of bisulfite-modified MDR1 promoter regions from DU145, DU145-TxR, PC-3, and PC-3-TxR cells were shown from −65 to −21 of transcription initiation site. Underlines and double underline show methylated CpG sites and G-box, respectively.

two-fold and 218 (1.45%) of genes were reduced more than two-fold in PC-3-TxR cell line compared with parent PC-3 cell line. Tables V and VI describe the major 30 genes that showed up-regulated and down-regulated expression in PC-3-TxR cells compared with PC-3 cells. As we confirmed in Figure 4, MDR-1 genes was up-regulated to 6.0-fold in PC-3-TxR cells. Some microtubule-related genes, tubuline β6, β2, and β4, were up-regulated to 3.5-, 2.2-, and 2.1-fold in PC-3-TxR cells, respectively. Calcium is an important factor that is associated with microtubule polymerization. Calcium-binding protein, S100A9 and S100A8 were

TABLE IV. IC_{50} Value of Paclitaxel in iMDR-1-Transfected TxR Cells

Transfected cells	IC_{50} (nM)	Relative resistant ratio	Recovery ratio
DU145 (NT siRNA)	9.74	1.0	
DU145 (iMDR-1)	9.11	0.94	6%
DU145-TxR (NT siRNA)	537.9	55.2	
DU145-TxR (iMDR-1)	60.8	6.24	88.7%
PC-3 (NT siRNA)	10.5	1.0	
PC-3 (iMDR-1)	10.0	0.95	5%
PC-3-TxR (NT siRNA)	198.4	18.9	
PC-3-TxR (iMDR-1)	140.6	13.4	29.1%

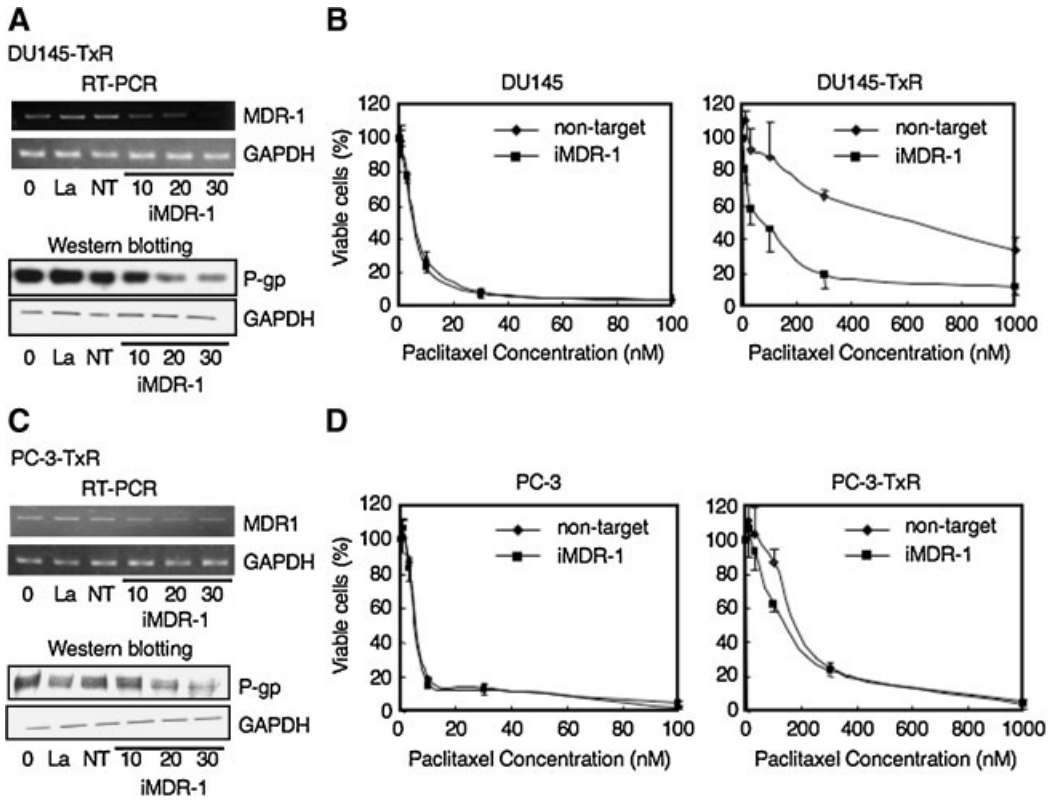

Fig. 6. Paclitaxel in iMDR-1 transfected TxR cells. **A and C**: Forty-eight hours after transfection with 0, 10, 20, or 30 nM MDR-1 siRNA, 30 nM LaminA/C siRNA (La), and 30 nM non-targeting siRNA (La), total RNA and protein was extracted according to the Materials and Methods. **B and D**: In order to see the effect of siRNA on drug resistance, cells were transfected with 30 nM MDR-1 siRNA or non-targeting siRNA 24 hr after plating on 24-well plates. Twenty-four hours after transfection with 30 nM non-targeting iRNA or iMDR-1, cells were treated with 0, 1, 3, 10, 30, 100, 300, and 1,000 nM paclitaxel and cultured for 48 hr. Then the cells were counted with a hemocytometer. The data represent mean of triplicate experiments and the bars show SD. The data were described in Table IV.

down-regulated to 4.34- and 2.56-fold in PC-3-TxR cells, respectively. Other calcium-related genes, tumor-associated calcium signal transducer 1 (TACSTD1), S100P, and S100A2 mRNA were also down-regulated in PC-3-TxR cells. MMP-1 that is related with cancer invasion is overexpressed in multiple drug-resistant cell lines [25]. We also observed overexpression of MMP-1 in PC-3-TxR cells (4.77-fold).

DISCUSSION

In order to elucidate the mechanisms of paclitaxel-resistant in hormone refractory PCa, we established two paclitaxel-resistant cell lines from androgen-independent cell lines. Several potential mechanisms have been proposed for resistance to taxans. The result that cross-resistance to cisplatin and VP-16 was not observed in both paclitaxel-resistant cell lines indicates that resistance to paclitaxel is resulted from different pathways from resistance to cisplatin and VP-16. Although paclitaxel induces apoptosis, we could not detect differences of expression in apoptosis-related genes, such as bcl-2, bax, caspase 8 between parent cells and TxR cells. One of major mechanisms of paclitaxel-resistance is overexpression of P-gp [9]. The MDR-1 overexpression was the important factor as a responsible gene when DU145 cells became paclitaxel resistance. Since MDR-1 siRNA almost restored paclitaxel sensitivity in DU145-TxR cells, P-gp overexpression is the main reason of paclitaxel resistance in this cell line.

Our results showed that one of main mechanisms by which of MDR-1 was overexpressed in paclitaxel-resistant DU145 cells was the demethylation of CpG sites at the MDR1 promoter region. Originally CpG sites at the MDR1 promoter region in parent DU145 cells were hypermethylated [19]. Because it is rare, as for the necessity of MDR1, expression of MDR1 is inhibited for cancer cell by methylation of MDR1 promoter. However, when cells can leave damage by paclitaxel, demethylation of MDR1 promoter, especially G-box that includes Sp1-binding site and EGR-1-binding site and is very important for transcription [24], is promoted and induces expression of MDR1 so that cell themselves survives it, then cells may be going to remove paclitaxel from intracellular. However, it remains unclear why PC-3-TxR cells overexpressed

TABLE V. List of Genes which were Overexpressed in PC-3-TxR Cells

Gene name	Systematic name	PC-3-TxR signal	PC-3 signal	Fold change	Description
TNS	NM_022648	704	97	7.23	Tensin
ABCB1	NM_000927	5,902	980	6.02	ATP-binding cassette, subfamily B (MDR/TAP)
LAMA4	NM_002290	10,609	1,991	5.33	Laminin, alpha 4 (LAMA4)
IGSF4	NM_014333	2,586	486	5.32	Immunoglobulin superfamily, member 4
CD33L3	AK092746	22,410	4,403	5.09	cDNA FLJ35427 fis, clone SMINT2001731
MMP1	NM_002421	10,946	2,293	4.77	Matrix metalloproteinase 1 (interstitial collagenase)
TIMP4	NM_003256	2,786	644	4.33	Tissue inhibitor of metalloproteinase 4
AUTS2	NM_015570	821	199	4.12	Autism susceptibility candidate 2
PLA2G7	NM_005084	4,353	1,068	4.08	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)
ROBO4	NM_019055	2,058	508	4.05	Roundabout homolog 4, magic roundabout (Drosophila)
IL1RL1	NM_016232	1,121	286	3.92	Interleukin 1 receptor-like 1 (IL1RL1), transcript variant 1
POU4F3	NM_002700	557	148	3.76	POU domain, class 4, transcription factor 3
SLC35F2	NM_017515	31,443	8,611	3.65	Solute carrier family 35, member F2
FZD4	NM_012193	9,899	2,791	3.55	Frizzled homolog 4 (Drosophila) (FZD4)
MGC4083	NM_032525	20,402	5,787	3.53	Tubulin beta MGC4083
TFPI2	AK092499	21,336	6,056	3.52	cDNA FLJ35180 fis, clone PLACE6014882, similar to Tissue Factor pathway inhibitor 2
CHKA	NM_001277	893	262	3.41	Choline kinase
PFTK1	NM_012395	2,295	705	3.26	PFTAIRE protein kinase 1
BNIP3	NM_004052	26,713	8,310	3.21	BCL2/adenovirus E1B 19 kDa interacting protein 3
C14orf149	NM_144581	25,746	8,094	3.18	Hypothetical protein FLJ25436
H19	AK056774	2,557	815	3.14	cDNA FLJ32212 fis, clone PLACE6003399, weakly similar to SPIDROIN 1
MGC2574	NM_024098	24,317	7,812	3.11	Hypothetical protein MGC2574
LOC114990	NM_138440	1,953	647	3.02	Hypothetical protein BC013767
MGC10981	BC004397	776	259	2.99	Hypothetical protein MGC10981
PRSS11	NM_002775	14,523	5,115	2.84	Protease, serine, 11 (IGF binding)
DPYSL4	NM_006426	776	274	2.83	Dihydropyrimidinase-like 4
GPR56	NM_005682	32,764	11,683	2.80	G protein-coupled receptor 56
BAG3	NM_004281	3,952	1,425	2.77	BCL2-associated athanogene 3
SC5DL	BC012333	4,652	1,685	2.76	Sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, fungal)-like
C10orf125	NM_198472	3,036	1,101	2.76	FLJ26016 protein (FLJ26016)
MTVR1	NM_152832	8,897	3,302	2.69	Mouse mammary turmor virus receptor homolog 1
GPR4	NM_005282	1,507	565	2.66	G protein-coupled receptor 4
FAHD1	NM_031208	6,677	2,517	2.65	Hypothetical protein DKFZp566J2046
FLJ37078	NM_153043	395	149	2.64	Hypothetical protein FLJ37078
POLA2	NM_002689	10,368	3,935	2.64	Polymerase (DNA-directed), alpha (70kD)
LOC201194	AK022617	540	207	2.61	cDNA FLJ12555 fis
MGC16291	NM_032770	3,167	1,228	2.58	Hypothetical protein MGC16291
ESM1	NM_007036	7,643	2,966	2.58	Endothelial cell-specific molecule 1
CDCA5	NM_080668	15,949	6,219	2.56	Cell division cycle associated 5
DGAT2	NM_032564	530	207	2.56	Diacylglycerol O-acyltransferase homolog 2
SLC35F2	AK128062	2,548	998	2.55	cDNA FLJ46182 fis
LOC201194	AK022617	488	192	2.54	cDNA FLJ12555 fis, clone NT2RM4000764
ESM1	NM_007036	6,500	2,560	2.54	Endothelial cell-specific molecule 1
LOC201194	AK022617	553	218	2.53	cDNA FLJ12555 fis, clone NT2RM4000764
EHBP1L1	AL834433	2,554	1,008	2.53	cDNA DKFZp762C186
FANCA	NM_000135	874	348	2.51	Fanconi anemia, complementation group A
ESM1	NM_007036	10,124	4,042	2.50	Endothelial cell-specific molecule 1 (ESM1), mRNA

TABLE VI. List of Genes which were Repressed in PC-3-TxR

Gene name	Systematic name	PC-3-TxR signal	PC-3 signal	Fold change	Description
IL23A	NM_016584	988	16,873	17.07	Interleukin 23, alpha subunit p19
CALB1	NM_004929	482	7,509	15.57	Calbindin 1, 28 kDa
CTEN	NM_032865	591	6,318	10.69	C-terminal tensin-like
PLAC8	NM_016619	290	2,886	9.95	Placenta-specific 8
LXN	NM_020169	214	1,957	9.13	Latexin protein
CDH1	NM_004360	626	4,957	7.92	Cadherin 1, type 1, E-cadherin (epithelial)
S100A2	NM_005978	2,579	17,878	6.93	S100 calcium-binding protein A2
KLK6	NM_002774	140	930	6.66	Kallikrein 6 (neurosin, zyme)
IL6	NM_000600	1,592	10,288	6.46	Interleukin 6 (interferon, beta 2)
LCN2	NM_005564	1,277	7,457	5.84	Lipocalin 2 (oncogene 24p3)
IL13RA2	NM_000640	143	752	5.26	Interleukin 13 receptor, alpha 2
CSF2	NM_000758	1,595	8,099	5.08	Colony stimulating factor 2 (granulocyte-macrophage)
CD33	NM_001772	205	1,037	5.07	CD33 antigen (gp67)
SERPINB4	NM_002974	708	3,545	5.01	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 4
CYP1B1	NM_000104	3,090	15,173	4.91	Cytochrome P450, family 1, subfamily B, polypeptide 1
NOX5	NM_024505	1,707	7,840	4.59	NADPH oxidase, EF hand calcium-binding domain 5
CRB3	NM_174882	390	1,774	4.55	Crumbs homolog 3 (Drosophila) (CRB3)
NMES1	NM_032413	3,567	16,181	4.54	Normal mucosa of esophagus specific 1
PTAFR	NM_000952	297	1,344	4.53	Platelet-activating factor receptor
THBD	NM_000361	165	749	4.53	Thrombomodulin
SAT	NM_002970	2,227	10,029	4.50	Spermidine/spermine N1-acetyltransferase
FXYD6	NM_022003	594	2,674	4.50	FXYD domain containing ion transport regulator 6
SAA1	NM_000331	410	1,834	4.47	Serum amyloid A1 (SAA1)
TACSTD1	NM_002354	4,722	21,027	4.45	Tumor-associated calcium signal transducer 1
IL1F7	NM_014439	1,532	6,811	4.45	Interleukin 1 family, member 7 (zeta)
ADMP	NM_145035	504	2,213	4.39	ADMP
IFI27	NM_005532	211	915	4.35	Interferon, alpha-inducible protein 27
S100A9	NM_002965	2,678	11,624	4.34	S100 calcium-binding protein A9 (calgranulin B)
AY358920	AY358920	216	931	4.31	Clone DNA129549 ALGV3072 (UNQ3072)
IFI27	NM_005532	139	594	4.27	Interferon, alpha-inducible protein 27
ALOX5AP	NM_001629	2,028	8,503	4.19	Arachidonate 5-lipoxygenase-activating protein
AREG	NM_001657	1,461	6,122	4.19	Amphiregulin (schwannoma-derived growth factor)
ANKRD1	NM_014391	131	545	4.16	Ankyrin repeat domain 1 (cardiac muscle)
SGNE1	NM_003020	748	3,076	4.11	Secretory granule, neuroendocrine protein 1 (7B2 protein)
IFITM1	NM_003641	698	2,783	3.99	Interferon induced transmembrane protein 1 (9–27)
FCGR2C	NM_201563	198	785	3.96	Fc fragment of IgG, low affinity IIc, receptor for (CD32)
FLJ31204	NM_174912	150	584	3.89	Hypothetical protein FLJ31204
S100P	NM_005980	2,465	9,570	3.88	S100 calcium-binding protein P
HIST1H1C	NM_005319	3,943	14,847	3.77	Histone 1, H1c
CD33	AY162464	500	1,875	3.75	Sialic acid-binding immunoglobulin-like lectin 3
CXCL2	NM_002089	4,477	16,726	3.74	Chemokine (CXC motif) ligand 2
CXCL3	NM_002090	4,728	17,552	3.71	Chemokine (CXC motif) ligand 3
SERPINB3	NM_006919	1,206	4,461	3.70	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 3
CCL20	NM_004591	844	3,109	3.68	Chemokine (CC motif) ligand 20
KLF5	NM_001730	2,148	7,908	3.68	Kruppel-like factor 5 (intestinal)
AMIGO2	NM_181847	852	3,108	3.65	Homo sapiens amphoterin induced gene 2
AIM2	NM_004833	232	845	3.64	Absent in melanoma 2
THC1991570	THC1991570	1,416	5,074	3.58	AY140952 G-protein coupled receptor GPR110
SAA2	NM_030754	450	1,591	3.54	Serum amyloid A2
TGFA	NM_003236	1,294	4,544	3.51	Transforming growth factor, alpha
FGFBP1	NM_005130	1,619	5,680	3.51	Heparin-binding growth factor-binding protein

MDR1 mRNA compared to PC-3 cells although the expression level in PC-3-TxR cells was lower than in DU145-TxR cells. It will be very interesting to study why paclitaxel exposure causes demethylation of the MDR1 promoter region of DU145 cells.

Inhibition of MDR-1 hardly restored resistance in PC-3-TxR although PC-3-TxR cells overexpressed P-gp compared to parent PC-3 cells. Only by overexpression of p-gp, there is not explanation of the mechanism that PC -3 cells become paclitaxel resistance. Other mechanisms should be involved in paclitaxel-resistance in PC-3-TxR cells. Lin et al.[26] demonstrated that DOX resistance rat PCa cell line expressed more Id-1, MIF, and GSTpi mRNA than parent cell line . They also showed that overexpression of Id-1 caused paclitaxel-resistance in the cell line. However, we could not detect the difference of Id-1 expression between PC-3 and PC-3-TxR cells although Id-1 mRNA was temporally down-regulated by paclitaxel treatment in PC-3 cells.

In order to investigate what genes are involved in paclitaxel resistance, we compared gene expression profile between PC-3 and PC-3-TxR cells. To the best of our knowledge, this is the first report that compared gene expression profile about paclitaxel-resistance in hormone refractory PCa cell line. Expressions of many genes were also altered in paclitaxel-resistant breast cancer cells [16]. Expression patterns were similar in some of these genes, such as MDR1 and S100P. However, those in PC-3-TxR cells were different from breast cancer cells, suggesting that different mechanisms are involved in becoming paclitaxel-resistance in different cancers.

Paclitaxel shows the effect as an anticancer drug by stabilizing polymer of microtubule [27]. Alterations of microtubule formation in resistant cells is also important factors [10,28]. Li et al.[29] demonstrated by microarray analysis that taxotere regulated many genes including microtubule, apoptosis, and cell cycle-related genes in PCa cell lines, PC-3 and LNCaP cells. Especially, microtubule-related genes are down-regulated in those cells. They treated cells with taxotere transiently and compare the regulated genes before and after treatment. Down-regulated genes after treatment may be the genes which, as a result of having been impaired, were inhibited by taxotere. Or up-regulated genes may be the genes which, as a result, are elevated when apoptosis by taxotere is induced. Ranganathan et al.[30] demonstrated that increase in tubulin βIII (nine-fold) and βIVa (five-fold) were observed in DU145 cells that became paclitaxel-resistance. Orr et al.[10] also reviewed that alterations in tubulin composition expression were associated with paclitaxel resistance. We also confirmed the up-regulation of some tubulin β-6 (3.53-fold), −2 (2.22-fold), and −4 (2.13-fold) in PC-3-TxR cells by cDNA microarray analysis. However, overexpression of βIII isotype in human prostate carcinoma cells by stable transfection failed to confer antimicrotubule drug resistance to these cells [31]. Interestingly, overexpression of tubulin β are related with poor prognosis and resistance [32]. At least overexpression of tubulin βs may be thought with a good marker predicting with reactivity for paclitaxel and prognosis. We will investigate if overexpression of tubulin βs causes paclitaxel resistance and progression in PC-3 cells.

Paclitaxel is known to repress influx of calcium into cytoplasm [33,34]. Reduction of calcium-associated proteins expression may be a cause of repression of calcium influx by paclitaxel and may not be a mechanism of paclitaxel resistance. However, calcium dynamics which is associated with microtubule polymerization is important factor for paclitaxel-resistance. Moreover, altered intracellular calcium homeostasis may contribute to the paclitaxel-resistant phenotype [35]. Microarray analysis in this study revealed a decline of S100A8/S100A9 expression in PC-3-TxR cells compared with parent PC-3 cells. Calcium-induced complexes of S100A8 and S100A9 have been shown to colocalize with microtubules during activation of monocytes. They directly bind to tubulin and promote tubulin polymerization in a calcium-dependent manner [36]. Then failure of tetramer formation was associated with a lack of functional activity of S100A8/S100A9 complexes in promoting the formation of microtubules [37]. A decline of S100A8/S100A9 expression would also inhibit the formation of microtubules. Therefore, since paclitaxel cannot stabilize the formation of microtubules due to a decline of S100A8/S100A9 expression in PC-3-TxR cells, paclitaxel might not be able to show effect as an anticancer drug in resistant cells.

In conclusions, after we established paclitaxel-resistant hormone refractory PCa cell lines, we compared resistant cells with parent cells. This comparison will make it more possible to identify the genes which cause paclitaxel resistance except MDR-1. Not only MDR-1 gene but also many genes were up-regulated and down-regulated. We have to still distinguish the genes that are responsible for resistance from the genes that are regulated as a result one by one. Nevertheless, identification of these genes will be useful for thinking strategies using taxanes to individual hormone refractory PCa.

ACKNOWLEDGMENTS

We thank Saeko Fujii, Yukari Kawabuchi, and Chiharu Shimoda for assistance. This work was supported in part by Japan Society for the Promotion of Science Grant 17591669 (A. Mizokami).

REFERENCES

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ. Cancer statistics, 2006. CA Cancer J Clin 2006;56(2) 106–130.
2. Obasaju C, Hudes GR. Paclitaxel and docetaxel in prostate cancer. Hematol Oncol Clin North Am 2001;15(3):525–545.
3. Petrylak DP, Tangen CM, Hussain MH, Lara PN Jr, Jones JA, Taplin ME, Burch PA, Berry D, Moinpour C, Kohli M, Benson MC, Small EJ, Raghavan D, Crawford ED. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. N Engl J Med 2004;351(15): 1513–1520.
4. Tannock IF, de Wit R, Berry WR, Horti J, Pluzanska A, Chi KN, Oudard S, Theodore C, James ND, Turesson I, Rosenthal MA, Eisenberger MA. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med 2004;351(15):1502–1512.
5. Oudard S, Banu E, Beuzeboc P, Voog E, Dourthe LM, Hardy-Bessard AC, Linassier C, Scotte F, Banu A, Coscas Y, Guinet F, Poupon MF, Andrieu JM. Multicenter randomized phase II study of two schedules of docetaxel, estramustine, and prednisone versus mitoxantrone plus prednisone in patients with metastatic hormone-refractory prostate cancer. J Clin Oncol 2005;23(15):3343–3351.
6. Fulton B, Spencer CM. Docetaxel. A review of its pharmacodynamic and pharmacokinetic properties and therapeutic efficacy in the management of metastatic breast cancer. Drugs 1996;51(6): 1075–1092.
7. Duan Z, Lamendola DE, Duan Y, Yusuf RZ, Seiden MV. Description of paclitaxel resistance-associated genes in ovarian and breast cancer cell lines. Cancer Chemother Pharmacol 2005;55(3):277–285.
8. Teraishi F, Wu S, Sasaki J, Zhang L, Zhu HB, Davis JJ, Fang B. P-glycoprotein-independent apoptosis induction by a novel synthetic compound, MMPT [5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone]. J Pharmacol Exp Ther 2005;314(1):355–362.
9. Hopper-Borge E, Chen ZS, Shchaveleva I, Belinsky MG, Kruh GD. Analysis of the drug resistance profile of multidrug resistance protein 7 (ABCC10): resistance to docetaxel. Cancer Res 2004;64(14):4927–4930.
10. Orr GA, Verdier-Pinard P, McDaid H, Horwitz SB. Mechanisms of Taxol resistance related to microtubules. Oncogene 2003;22(47):7280–7295.
11. Chun E, Lee KY. Bcl-2 and Bcl-xL are important for the induction of paclitaxel resistance in human hepatocellular carcinoma cells. Biochemical and Biophysical Research Communications 2004; 315(3):771–779.
12. Ferlini C, Raspaglio G, Mozzetti S, Distefano M, Filippetti F, Martinelli E, Ferrandina G, Gallo D, Ranelletti FO, Scambia G. Bcl-2 down-regulation is a novel mechanism of paclitaxel resistance. Mol Pharmacol 2003;64(1):51–58.
13. Yamanaka K, Rocchi P, Miyake H, Fazli L, So A, Zangemeister-Wittke U, Gleave ME. Induction of apoptosis and enhancement of chemosensitivity in human prostate cancer LNCaP cells using bispecific antisense oligonucleotide targeting Bcl-2 and Bcl-xL genes. BJU Int 2006;97(6):1300–1308.
14. Yamanaka K, Rocchi P, Miyake H, Fazli L, Vessella B, Zangemeister-Wittke U, Gleave ME. A novel antisense oligonucleotide inhibiting several antiapoptotic Bcl-2 family members induces apoptosis and enhances chemosensitivity in androgen-independent human prostate cancer PC3 cells. Mol Cancer Ther 2005;4(11):1689–1698.
15. Goto T, Takano M, Sakamoto M, Kondo A, Hirata J, Kita T, Tsuda H, Tenjin Y, Kikuchi Y. Gene expression profiles with cDNA microarray reveal RhoGDI as a predictive marker for paclitaxel resistance in ovarian cancers. Oncol Rep 2006;15(5):1265–1271.
16. Villeneuve DJ, Hembruff SL, Veitch Z, Cecchetto M, Dew WA, Parissenti AM. cDNA microarray analysis of isogenic paclitaxel- and doxorubicin-resistant breast tumor cell lines reveals distinct drug-specific genetic signatures of resistance. Breast Cancer Res Treat 2006;96(1):17–39.
17. Mizokami A, Koh E, Fujita H, Maeda Y, Egawa M, Koshida K, Honma S, Keller ET, Namiki M. The adrenal androgen androstenediol is present in prostate cancer tissue after androgen deprivation therapy and activates mutated androgen receptor. Cancer Res 2004;64(2):765–771.
18. Ueda K, Pastan I, Gottesman MM. Isolation and sequence of the promoter region of the human multidrug-resistance (P-glycoprotein) gene. J Biol Chem 1987;262(36):17432–17436.
19. Enokida H, Shiina H, Igawa M, Ogishima T, Kawakami T, Bassett WW, Anast JW, Li LC, Urakami S, Terashima M, Verma M, Kawahara M, Nakagawa M, Kane CJ, Carroll PR, Dahiya R. CpG hypermethylation of MDR1 gene contributes to the pathogenesis and progression of human prostate cancer. Cancer Res 2004;64(17):5956–5962.
20. Bargou RC, Jurchott K, Wagener C, Bergmann S, Metzner S, Bommert K, Mapara MY, Winzer KJ, Dietel M, Dorken B, Royer HD. Nuclear localization and increased levels of transcription factor YB-1 in primary human breast cancers are associated with intrinsic MDR1 gene expression. Nat Med 1997;3(4):447–450.
21. Ohga T, Uchiumi T, Makino Y, Koike K, Wada M, Kuwano M, Kohno K. Direct involvement of the Y-box binding protein YB-1 in genotoxic stress-induced activation of the human multidrug resistance 1 gene. J Biol Chem 1998;273(11):5997–6000.
22. Nakayama M, Wada M, Harada T, Nagayama J, Kusaba H, Ohshima K, Kozuru M, Komatsu H, Ueda R, Kuwano M. Hypomethylation status of CpG sites at the promoter region and overexpression of the human MDR1 gene in acute myeloid leukemias. Blood 1998;92(11):4296–4307.
23. Tada Y, Wada M, Kuroiwa K, Kinugawa N, Harada T, Nagayama J, Nakagawa M, Naito S, Kuwano M. MDR1 gene overexpression and altered degree of methylation at the promoter region in bladder cancer during chemotherapeutic treatment. Clin Cancer Res 2000;6(12):4618–4627.
24. Cornwell MM, Smith DE. SP1 activates the MDR1 promoter through one of two distinct G-rich regions that modulate promoter activity. J Biol Chem 1993;268(26):19505–19511.
25. Yang JM, Xu Z, Wu H, Zhu H, Wu X, Hait WN. Overexpression of extracellular matrix metalloproteinase inducer in multidrug resistant cancer cells. Mol Cancer Res 2003;1(6):420–427.
26. Lin JC, Chang SY, Hsieh DS, Lee CF, Yu DS. The association of Id-1, MIF and GSTpi with acquired drug resistance in hormone independent prostate cancer cells. Oncol Rep 2005;13(5):983–988.
27. Dumontet C, Sikic BI. Mechanisms of action of and resistance to antitubulin agents: microtubule dynamics, drug transport, and cell death. J Clin Oncol 1999;17(3):1061–1070.
28. Drukman S, Kavallaris M. Microtubule alterations and resistance to tubulin-binding agents (review). Int J Oncol 2002;21(3): 621–628.
29. Li Y, Li X, Hussain M, Sarkar FH. Regulation of microtubule, apoptosis, and cell cycle-related genes by taxotere in prostate cancer cells analyzed by microarray. Neoplasia 2004;6(2):158–167.

30. Ranganathan S, Benetatos CA, Colarusso PJ, Dexter DW, Hudes GR. Altered beta-tubulin isotype expression in paclitaxel-resistant human prostate carcinoma cells. Br J Cancer 1998;77(4):562–566.
31. Ranganathan S, McCauley RA, Dexter DW, Hudes GR. Modulation of endogenous beta-tubulin isotype expression as a result of human beta(III)cDNA transfection into prostate carcinoma cells. Br J Cancer 2001;85(5):735–740.
32. Song JH, Choi CH, Yeom HJ, Hwang SY, Kim TS. Monitoring the gene expression profiles of doxorubicin-resistant acute myelocytic leukemia cells by DNA microarray analysis. Life Sci 2006;79(2):193–202.
33. Burke WJ, Raghu G, Strong R. Taxol protects against calcium-mediated death of differentiated rat pheochromocytoma cells. Life Sci 1994;55(16):313–319.
34. Furukawa K, Mattson MP. Taxol stabilizes [Ca2+]i and protects hippocampal neurons against excitotoxicity. Brain Res 1995; 689(1):141–146.
35. Padar S, van Breemen C, Thomas DW, Uchizono JA, Livesey JC, Rahimian R. Differential regulation of calcium homeostasis in adenocarcinoma cell line A549 and its Taxol-resistant subclone. Br J Pharmacol 2004;142(2):305–316.
36. Vogl T, Ludwig S, Goebeler M, Strey A, Thorey IS, Reichelt R, Foell D, Gerke V, Manitz MP, Nacken W, Werner S, Sorg C, Roth J. MRP8 and MRP14 control microtubule reorganization during transendothelial migration of phagocytes. Blood 2004;104(13): 4260–4268.
37. Leukert N, Vogl T, Strupat K, Reichelt R, Sorg C, Roth J. Calcium-dependent tetramer formation of S100A8 and S100A9 is essential for biological activity. J Mol Biol 2006;359(4):961–972.