The establishment of two paclitaxel-resistant prostate cancer cell lines and the mechanisms of paclitaxel resistance with two cell lines

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The establishment of two paclitaxel resistant prostate cancer cell lines and the mechanisms of paclitaxel resistance with two cell lines

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Key words: prostate cancer, paclitaxel resistance, MDR-1, cDNA microarray

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Abstract

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 MDR1 promoter was carried out using bisulfite-modified DNA from cell lines. Knock-down experiments using 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 IC₅₀ 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

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7 methylated CpG sites of MDR1 promoter in DU145 and PC-3 cells were demethylated in
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10 DU145-TxR cells, but not in PC-3-TxR cells. Knock down of P-gp, which was
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13 up-regulated in resistant cells, by MDR-1 siRNA restored paclitaxel sensitivity in
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17 DU145-TxR but not in PC-3-TxR, indicating that up-regulation of P-gp was not always
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20 main cause of paclitaxel-resistance. Microarray analysis identified 201 (1.34%)
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23 up-regulated genes and 218 (1.45%) out of screened genes in PC-3-TxR.
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28 **Conclusions.** Our data will provide molecular mechanisms of paclitaxel-resistance and
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32 be useful for screening target genes to diagnose paclitaxel sensitivity.
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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 prostate cancer. More than half of those cases of advanced prostate cancer become resistant to deprivation treatment after several years and then several other 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 two years.

Recently, the taxanes (paclitaxel or docetaxel) 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 anti-cancer agents.

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7 However, even HRPC treated with paclitaxel-based chemotherapy also relapses as
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10 occurred using other anti-cancer agents. Then the prognosis of the patients after the
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13 relapse is extremely poor.
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18 In order to investigate the mechanisms of paclitaxel-resistance, several
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21 paclitaxel-resistance cell lines have been generated in ovarian cancer, breast cancer, and
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24 lung cancer (7,8). Some of major mechanisms of taxane-resistance are overexpression of
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27 multiple drug resistance (MDR1), and multidrug resistance protein (MRP) family (9).
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30 Especially accumulation of P-glycoprotein encoded from MDR1 might cause resistance
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33 of several drugs in some cancers. The microtubule dynamics may also be important for
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36 paclitaxel-resistance because the target of paclitaxel is the microtubule (10). As for the
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39 role of bcl-2 as a modulator of paclitaxel sensitivity remains controversial. In
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42 human paclitaxel-resistant hepatocellular carcinoma cells bcl-2 was overexpressed (11).
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46 Whereas bcl-2 expression was consistently down-regulated in T47-D breast cancer cells
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50 (12). In prostate cancer, although Bcl-2/Bcl-xL bispecific antisense oligonucleotide also
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53 enhanced paclitaxel chemosensitivity in PC-3 and LNCaP cells (13, 14), involvement to
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56 paclitaxel-resistance of Bcl-2/Bcl-xL in prostate cancer is not clear. Recently cDNA
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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 described that 1.9% of 1728 genes were regulated in paclitaxel-resistant MCF-7 breast cancer cells (16). Thus it is very important to know the mechanisms of paclitaxel-resistance in prostate cancer.

In the present study, we established two paclitaxel-resistant cell lines from androgen-independent DU145 and PC-3 prostate cancer 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 h later, cells were treated with the indicated concentration of anticancer agents, and cultured for an additional 48 h. 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 aliquot 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 h 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 μ g 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 1. Each of the amplified PCR products was determined by electrophoresis on an 1.5% agarose gel.

Western blot analysis. Twenty-four h 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 MgCl₂, and 0.5% NP-40) and the membrane and cytosol fraction were collected by centrifugation as described previously (17). To 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 µg 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 & cell culture DNA mini kit (QUIAGEN) 24 h after 5×10^5 cells were plated on 6 cm dish. One μg of DNA was subjected to sodium bisulfite modification kit (BisulFast DNA Modification Kit, TOYOBO, Osaka Japan). 223 bp MDR-1 promoter region (-183 to +40 of transcription initiation site) was amplified from bisulfite-modified DNA as described by Enokida H 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 h 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 h after plating on 24-well plates. Twenty-four later cells were treated with 0, 1, 3, 10, 30, 100, 300, and 1000 nM paclitaxel and cultured for 48 h. Then the cells were trypsinized and counted with a hemocytometer.

cDNA microarray analysis

Twenty-four h after plating of 5×10^5 PC-3 cells, Total RNA was purified with RNeasy mini kit (QIAGEN, Maryland, USA). 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, IC₅₀ values of these cells were 11.3 nM and 5.0 nM, respectively (Table 2). We established paclitaxel-resistant DU145 (DU145-TxR) and PC-3 (PC-3-TxR) cells by stepwise exposure method (from 10 nM paclitaxel) for 9 months and 15 months, respectively. Cell growth inhibition assay demonstrated that these DU145-TxR and PC-3-TxR cells become 34.0-fold (IC₅₀: 384.2 nM) and 43.4-fold (IC₅₀: 217.1 nM) more paclitaxel-resistant than parent cells (Table 2 and Fig. 1). We also compared the cross-resistance to other anticancer drugs (estramustine phosphate, vinblastin, doxorubicin, docetaxel, VP-16, and cisplatin) between parent and paclitaxel-resistant cells (Fig. 2 and 3, Table 2 and 3). Both of DU145-TxR and PC-3-TxR cells showed almost same cross-resistance to estramustine phosphate, vinblastin, doxorubicin, and docetaxel. 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-glycoprotein (P-gp) and multidrug resistance associated protein (MRP) which belong to the ABC (ATP-binding cassettes) 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.

Mechanisms of MDR1 overexpression 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 translocates 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 3 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 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, methylation-specific primers (MSP) published by Enokida et al. detected PCR products from bisulfite-modified DNA in both parent cells and paclitaxel-resistant cells, unmethylation-specific primers (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 (data not shown). 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 30 nM 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 4 and Fig. 5B and 5D, IC₅₀ 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 h restored paclitaxel sensitivity compared to transfection with NT siRNA (Fig. 5B). IC₅₀ of paclitaxel of DU145-TxR was reduced from 537.9 nM to 60.8 nM and recovery ratio became 88.7% 48 h after transfection (Table 4). Whereas transfection with MDR-1 siRNA into PC-3-TxR cells hardly changed paclitaxel sensitivity. IC₅₀ of paclitaxel of PC-3-TxR was reduced only from 198.4 nM to 140.6 nM and recovery ratio became 29.1%.

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. 201 (1.34%) of screened genes were induced more than 2-fold and 218 (1.45%) of genes were reduced more than 2-fold in PC-3-TxR cell line compared with parent PC-3 cell line. Table 5 and 6 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 Fig. 4, MDR-1 genes was up-regulated to 6.0-fold in PC-3-TxR cells. Some microtubule-related genes, tubuline $\beta 6$, $\beta 2$, and $\beta 4$, were up-regulated to 3.5-fold, 2.2-fold, 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 down-regulated to 4.34-fold 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).

Discussions

In order to elucidate the mechanisms of paclitaxel-resistant in hormone refractory prostate cancer, 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 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.

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13 overexpression of p-gp, there is not explanation of the mechanism that PC -3 cells
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17 become paclitaxel resistance. Other mechanisms should be involved in
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20 paclitaxel-resistance in PC-3-TxR cells. Lin et al. demonstrated that doxorubicin
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23 resistance rat prostate cancer cell line expressed more Id-1, MIF, and GSTpi mRNA than
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26 parent cell line (26). They also showed that overexpression of Id-1 caused
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29 paclitaxel-resistance in the cell line. However, we could not detect the difference of Id-1
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32 expression between PC-3 and PC-3-TxR cells although Id-1 mRNA was temporally
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35 down-regulated by paclitaxel treatment in PC-3 cells.
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42 In order to investigate what genes are involved in paclitaxel resistance, we
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45 compared gene expression profile between PC-3 and PC-3-TxR cells. To our knowledge,
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48 this is the first report that compared gene expression profile about paclitaxel-resistance in
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51 hormone refractory prostate cancer cell line. Expressions of many genes were also
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54 altered in paclitaxel-resistant breast cancer cells (16). Expression patterns were similar in
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57 some of these genes, such as MDR1 and S100P. However, those in PC-3-TxR cells were
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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. demonstrated by microarray analysis that taxotere regulated many genes including microtubule, apoptosis, and cell cycle-related genes in prostate cancer cell lines, PC-3 and LNCaP cells (29). 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. demonstrated that increase in tubulin β III (9-fold) and β IVa (5-fold) were observed in DU145 cells that became paclitaxel-resistance (30). Orr et al. also reviewed that alterations in tubulin composition expression were associated with paclitaxel resistance (10). We also confirmed the up-regulation of some tubulin β -6 (3.53-fold), -2 (2.22-fold), and -4 (2.13-fold) in

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7 PC-3-TxR cells by cDNA microarray analysis. However, overexpression of β III isotype
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10 in human prostate carcinoma cells by stable transfection failed to confer antimicrotubule
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13 drug resistance to these cells (31). Interestingly, overexpression of tubulin β are related
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16 with poor prognosis and resistance (32). At least overexpression of tubulin β s may be
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19 thought with a good marker predicting with reactivity for paclitaxel and prognosis. We
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22 will investigate if overexpression of tubulin β s causes paclitaxel resistance and
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25 progression in PC-3 cells.
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32 Paclitaxel is known to repress influx of calcium into cytoplasm (33,34).
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35 Reduction of calcium-associated proteins expression may be a cause of repression of
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38 calcium influx by paclitaxel and may not be a mechanism of paclitaxel resistance.
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41 However, calcium dynamics which is associated with microtubule polymerization is
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44 important factor for paclitaxel-resistance. Moreover, altered intracellular calcium
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47 homeostasis may contribute to the paclitaxel-resistant phenotype (35). Microarray
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50 analysis in this study revealed a decline of S100A8/S100A9 expression in PC-3-TxR
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53 cells compared with parent PC-3 cells. Calcium-induced complexes of S100A8 and
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56 S100A9 have been shown to colocalize with microtubules during activation of monocytes.
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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 prostate cancer 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 prostate cancer.

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Legends

Table 1 The Primers used for RT- PCR analysis.

Table 2 IC₅₀ value of DU145 and DU145-TxR cells

Table 3 IC₅₀ value of PC-3 and PC-3-TxR cells

Table 4 IC₅₀ value of paclitaxel in iMDR-1-transfected TxR cells

Table 5 List of genes which were overexpressed in PC-3-TxR cells

Table 6 List of genes which were repressed in PC-3-TxR

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 h 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 estramustine phosphate (EMP), docetaxel (DTX), vinblastin (VBL), doxorubicin (DOX), cisplatin (CDDP), and etoposide (VP-16) for 24 h and counted 2 days after exposure.

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

Fig. 4 Expression of various drug-resistance-related genes in parent and paclitaxel-resistant cells. **A.** RT-PCR of MDR and MRP1-7 mRNA in DU145, DU145-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 1. **B.** Expression of P-glycoprotein. 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 DU145, DU145-TxR, PC-3, and PC-3-TxR cells.

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 kDa 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.

Fig. 6 Paclitaxel sensitivity in iMDR-1 transfected TxR cells. **A and C.** Forty-eight h 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 h after plating on 24-well plates. Twenty-four after transfection with 30 nM non-targeting iRNA or iMDR-1, cells were treated with 0, 1, 3, 10, 30, 100, 300, and 1000 nM paclitaxel and cultured for 48 h. 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 4.

Gene	Forward	Reverse
GAPDH	5' -GACCACAGTCCATGCCATCA-3'	5' -TCCACCACCCTGTTGCTGTA-3'
MDR-1	5' -ATGCTCTGGCCTTCTGG ATG GGA-3'	5' -ATGGCGATCCTCTGCTTCTGCCCCA C-3'
MRP-1	5' -GCATGA TCCCTGAAGACGA-3'	5' -TAGAGCTGG CCCTTGTA CTC-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' -TTGGATTGCCCCTCATAGTC-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' -ATCGTGA CTGCCTGAGAGCT-3'

Drug	DU145	DU145-TxR	Fold difference
PTX (nM)	11.3	384.2	34.0
EMP (μ M)	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 (μ M)	0.83	1.10	1.33
CDDP (μ M)	1.32	1.97	1.49

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Drug	PC-3	PC-3-TxR	Fold difference
PTX (nM)	5.00	217.1	43.4
EMP (μ M)	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	1218.2	10.0
VP-16 (μ M)	4.40	5.95	1.35
CDDP (μ M)	1.47	1.66	1.13

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%

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GeneName	SystematicName	PC-3-TxR signal	PC-3 signal	Fold Change	Description
TNS	NM_022648	704	97	7.23	tensin
ABCB1	NM_000927	5902	980	6.02	ATP-binding cassette, sub-family B (MDR/TAP)
LAMA4	NM_002290	10609	1991	5.33	laminin, alpha 4 (LAMA4)
IGSF4	NM_014333	2586	486	5.32	immunoglobulin superfamily, member 4
CD33L3	AK092746	22410	4403	5.09	cDNA FLJ35427 fis, clone SMINT2001731
MMP1	NM_002421	10946	2293	4.77	matrix metalloproteinase 1 (interstitial collagenase)
TIMP4	NM_003256	2786	644	4.33	tissue inhibitor of metalloproteinase 4
AUTS2	NM_015570	821	199	4.12	autism susceptibility candidate 2
PLA2G7	NM_005084	4353	1068	4.08	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)
ROBO4	NM_019055	2058	508	4.05	roundabout homolog 4, magic roundabout (Drosophila)
IL1RL1	NM_016232	1121	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	31443	8611	3.65	solute carrier family 35, member F2
FZD4	NM_012193	9899	2791	3.55	frizzled homolog 4 (Drosophila) (FZD4)
MGC4083	NM_032525	20402	5787	3.53	tubulin beta MGC4083
TFPI2	AK092499	21336	6056	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	2295	705	3.26	PFTAIRE protein kinase 1
BNIP3	NM_004052	26713	8310	3.21	BCL2/adenovirus E1B 19kDa interacting protein 3
C14orf149	NM_144581	25746	8094	3.18	hypothetical protein FLJ25436
H19	AK056774	2557	815	3.14	cDNA FLJ32212 fis, clone PLACE6003399, weakly similar to SPIDROIN 1
MGC2574	NM_024098	24317	7812	3.11	hypothetical protein MGC2574
LOC114990	NM_138440	1953	647	3.02	hypothetical protein BC013767

MGC10981	BC004397	776	259	2.99	hypothetical protein MGC10981
PRSS11	NM_002775	14523	5115	2.84	protease, serine, 11 (IGF binding)
DPYSL4	NM_006426	776	274	2.83	dihydropyrimidinase-like 4
GPR56	NM_005682	32764	11683	2.80	G protein-coupled receptor 56
BAG3	NM_004281	3952	1425	2.77	BCL2-associated athanogene 3
SC5DL	BC012333	4652	1685	2.76	sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, fungal)-like
C10orf125	NM_198472	3036	1101	2.76	FLJ26016 protein (FLJ26016)
MTVR1	NM_152832	8897	3302	2.69	Mouse Mammary Tumor Virus Receptor homolog 1
GPR4	NM_005282	1507	565	2.66	G protein-coupled receptor 4
FAHD1	NM_031208	6677	2517	2.65	hypothetical protein DKFZp566J2046
FLJ37078	NM_153043	395	149	2.64	hypothetical protein FLJ37078
POLA2	NM_002689	10368	3935	2.64	polymerase (DNA-directed), alpha (70kD)
LOC201194	AK022617	540	207	2.61	cDNA FLJ12555 fis
MGC16291	NM_032770	3167	1228	2.58	hypothetical protein MGC16291
ESM1	NM_007036	7643	2966	2.58	endothelial cell-specific molecule 1
CDCA5	NM_080668	15949	6219	2.56	cell division cycle associated 5
DGAT2	NM_032564	530	207	2.56	diacylglycerol O-acyltransferase homolog 2
SLC35F2	AK128062	2548	998	2.55	cDNA FLJ46182 fis
LOC201194	AK022617	488	192	2.54	cDNA FLJ12555 fis, clone NT2RM4000764.
ESM1	NM_007036	6500	2560	2.54	endothelial cell-specific molecule 1
LOC201194	AK022617	553	218	2.53	cDNA FLJ12555 fis, clone NT2RM4000764.
EHBP1L1	AL834433	2554	1008	2.53	cDNA DKFZp762C186
FANCA	NM_000135	874	348	2.51	Fanconi anemia, complementation group A
ESM1	NM_007036	10124	4042	2.50	endothelial cell-specific molecule 1 (ESM1), mRNA

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For Peer Review

GeneName	SystematicName	PC-3-TxR signal	PC-3 signal	Fold Change	Description
IL23A	NM_016584	988	16873	17.07	interleukin 23, alpha subunit p19
CALB1	NM_004929	482	7509	15.57	calbindin 1, 28kDa
CTEN	NM_032865	591	6318	10.69	C-terminal tensin-like
PLAC8	NM_016619	290	2886	9.95	placenta-specific 8
LXN	NM_020169	214	1957	9.13	latexin protein
CDH1	NM_004360	626	4957	7.92	cadherin 1, type 1, E-cadherin (epithelial)
S100A2	NM_005978	2579	17878	6.93	S100 calcium binding protein A2
KLK6	NM_002774	140	930	6.66	kallikrein 6 (neurosin, zyme)
IL6	NM_000600	1592	10288	6.46	interleukin 6 (interferon, beta 2)
LCN2	NM_005564	1277	7457	5.84	lipocalin 2 (oncogene 24p3)
IL13RA2	NM_000640	143	752	5.26	interleukin 13 receptor, alpha 2
CSF2	NM_000758	1595	8099	5.08	colony stimulating factor 2 (granulocyte-macrophage)
CD33	NM_001772	205	1037	5.07	CD33 antigen (gp67)
SERPINB4	NM_002974	708	3545	5.01	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 4
CYP1B1	NM_000104	3090	15173	4.91	cytochrome P450, family 1, subfamily B, polypeptide 1
NOX5	NM_024505	1707	7840	4.59	NADPH oxidase, EF hand calcium-binding domain 5
CRB3	NM_174882	390	1774	4.55	crumbs homolog 3 (Drosophila) (CRB3)
NMES1	NM_032413	3567	16181	4.54	normal mucosa of esophagus specific 1
PTAFR	NM_000952	297	1344	4.53	platelet-activating factor receptor
THBD	NM_000361	165	749	4.53	thrombomodulin
SAT	NM_002970	2227	10029	4.50	spermidine/spermine N1-acetyltransferase
FXYP6	NM_022003	594	2674	4.50	FXYP domain containing ion transport regulator 6
SAA1	NM_000331	410	1834	4.47	serum amyloid A1 (SAA1)
TACSTD1	NM_002354	4722	21027	4.45	tumor-associated calcium signal transducer 1
IL1F7	NM_014439	1532	6811	4.45	interleukin 1 family, member 7 (zeta)
ADMP	NM_145035	504	2213	4.39	ADMP

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3	IFI27	NM_005532	211	915	4.35	interferon, alpha-inducible protein 27
4	S100A9	NM_002965	2678	11624	4.34	S100 calcium binding protein A9 (calgranulin B)
5	AY358920	AY358920	216	931	4.31	clone DNA129549 ALGV3072 (UNQ3072)
6						
7	IFI27	NM_005532	139	594	4.27	interferon, alpha-inducible protein 27
8	ALOX5AP	NM_001629	2028	8503	4.19	arachidonate 5-lipoxygenase-activating protein
9						
10	AREG	NM_001657	1461	6122	4.19	amphiregulin (schwannoma-derived growth factor)
11	ANKRD1	NM_014391	131	545	4.16	ankyrin repeat domain 1 (cardiac muscle)
12						
13	SGNE1	NM_003020	748	3076	4.11	secretory granule, neuroendocrine protein 1 (7B2 protein)
14	IFITM1	NM_003641	698	2783	3.99	interferon induced transmembrane protein 1 (9-27)
15						
16	FCGR2C	NM_201563	198	785	3.96	Fc fragment of IgG, low affinity IIc, receptor for (CD32)
17	FLJ31204	NM_174912	150	584	3.89	hypothetical protein FLJ31204
18						
19	S100P	NM_005980	2465	9570	3.88	S100 calcium binding protein P
20	HIST1H1C	NM_005319	3943	14847	3.77	histone 1, H1c
21						
22	CD33	AY162464	500	1875	3.75	sialic acid binding immunoglobulin-like lectin 3
23	CXCL2	NM_002089	4477	16726	3.74	chemokine (C-X-C motif) ligand 2
24	CXCL3	NM_002090	4728	17552	3.71	chemokine (C-X-C motif) ligand 3
25						
26	SERPINB3	NM_006919	1206	4461	3.70	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 3
27						
28	CCL20	NM_004591	844	3109	3.68	chemokine (C-C motif) ligand 20
29	KLF5	NM_001730	2148	7908	3.68	Kruppel-like factor 5 (intestinal)
30	AMIGO2	NM_181847	852	3108	3.65	Homo sapiens amphoterin induced gene 2
31	AIM2	NM_004833	232	845	3.64	absent in melanoma 2
32						
33	THC1991570	THC1991570	1416	5074	3.58	AY140952 G-protein coupled receptor GPR110
34	SAA2	NM_030754	450	1591	3.54	serum amyloid A2
35						
36	TGFA	NM_003236	1294	4544	3.51	transforming growth factor, alpha
37	FGFBP1	NM_005130	1619	5680	3.51	heparin-binding growth factor binding protein
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Fig. 1

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