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

著者	Takeda Masashi, Mizokami Atsushi, Mamiya Kiminori, You Qiang Li, Jian Zhang, Keller Evan T., Namiki Mikio
journal or publication title	Prostate
volume	67
number	9
page range	955-967
year	2007-06-15
URL	http://hdl.handle.net/2297/9569

doi: 10.1002/pros.20581

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

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

1 Department of Integrative Cancer Therapy and Urology, Kanazawa University Graduate School of Medical Sciences, 13-1 Takara-machi, Kanazawa, Ishikawa, 920-8640 Japan

Tel: +81-76-265-2393 Fax: +81-76-222-6726

2 Department of Medicine, Division of Hematology/Oncology, Room 2E110, VA Pittsburgh Healthcare System, R&D (151-U), University Drive, Pittsburgh, PA 15240 USA

Tel: 412-527-8327

3 Unit for Laboratory Animal Medicine and Dept. of Pathology, University of Michigan, Ann Arbor, MI, USA

Tel: 757-446-7040

Corresponding author: Atsushi Mizokami, MD, PhD

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

Running title: The establishment of two paclitaxel resistant prostate cancer cell lines

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 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. Knock down of 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.

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.

However, even HRPC treated with paclitaxel-based chemotherapy also relapses as occurred using other anti-cancer 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 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 prostate cancer, 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 prostate cancer 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 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 x 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 x 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 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 x 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 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 $5x10^5$ 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 x 10^4 DU145-TxR and PC-3-TxR cells or 3 x 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 x 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, IC_{50} 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 (IC_{50}: 384.2 nM) and 43.4-fold (IC_{50}: 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 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 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 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 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, 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 h restored paclitaxel sensitivity compared to transfection with NT siRNA (Fig. 5B). IC_{50} 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. IC_{50} 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 ß6, ß2, and ß4, 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.

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. demonstrated that doxorubicin resistance rat prostate cancer cell line expressed more Id-1, MIF, and GSTpi mRNA than parent cell line (26). 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 our knowledge, this is the first report that compared gene expression profile about paclitaxel-resistance in hormone refractory prostate cancer 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. 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 βIII (9-fold) and βIVa (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

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 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.

Acknowledgements

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: a cancer journal for clinicians 2006;56(2):106-130.
2. Obasaju C, Hudes GR. Paclitaxel and docetaxel in prostate cancer. Hematology/oncology clinics of North America 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. The New England journal of medicine 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. The New England journal of medicine 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. Molecular pharmacology 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. Nature medicine 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. British journal of pharmacology 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.

Legends

Table 1 The Primers used for RT- PCR analysis.

Table 2 IC_{50} value of DU145 and DU145-TxR cells

Table 3 IC_{50} value of PC-3 and PC-3-TxR cells

Table 4 IC_{50} 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-3and 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.