Serum exosomal P-glycoprotein is a potential marker to diagnose docetaxel resistance and select a taxoid for patients with prostate cancer

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Abstract

Objectives: Docetaxel is used as the first-line chemotherapy for castration-resistant prostate cancer (CRPC), but docetaxel resistance occurs in part owing to induction of P-glycoprotein (P-gp) encoded by multidrug resistance protein 1 (MDR1) gene. A recently developed taxane—cabazitaxel—has poor affinity for P-gp and is thereby effective in docetaxel-resistant CRPC. It has been recently demonstrated that exosomes in the body fluids could serve as a diagnostic marker because they contain proteins and RNAs specific to the cells from which they are derived. In this study, we aimed to investigate if P-gp in blood exosomes could be a marker to diagnose docetaxel resistance and select a taxoid for patients with CRPC.

Methods and materials: Exosomes were isolated by differential centrifugation from docetaxel-resistant prostate cancer (PC-3) cells (PC-3R) and their parental PC-3 cells and from the serum of patients. Silencing of P-gp was performed by small interfering RNA transfection. Protein expression was examined by Western blot analysis. Viability of cells treated with docetaxel or cabazitaxel was determined by water soluble tetrazolium salt (WST) assay.

Results: The level of P-gp was higher in exosomes as well as cell lysates from PC-3R cells than in those from PC-3 cells. Cabazitaxel effectively killed PC-3R cells, and MDR1 knockdown improved the sensitivity of PC-3R cells to docetaxel but not to cabazitaxel. The P-gp level in blood exosomes was relatively higher in clinically docetaxel-resistant patients than in therapy-naïve patients.

Conclusions: Our results suggest that detection of P-gp in blood exosomes, which is involved in resistance to docetaxel but not to cabazitaxel, could be useful to diagnose docetaxel resistance and select an appropriate taxoid for patients with CRPC—docetaxel or cabazitaxel. © 2015 Elsevier Inc. All rights reserved.

Keywords: Castration-resistant prostate cancer; Docetaxel; Cabazitaxel; Exosomes; P-glycoprotein/P-gp; Multidrug resistance protein 1/MDR1

1. Introduction

Prostate cancer is one of the most common male cancers and is the second leading cause of cancer death among men in the United States [1]. Although many patients with prostate cancer have disease control after primary therapy, 34\% of them developed metastatic disease [2]. Androgen deprivation therapy is considered as the most appropriate intervention for metastatic or recurrent prostate cancer [3]. However, 10\% to 20\% of patients with prostate cancer develop castration-resistant prostate cancer (CRPC) [4]. Docetaxel that is currently used as the first-line chemotherapy for CRPC offers an overall survival benefit for
patients, but there is a finite amount of time before acquiring resistance [5].

P-glycoprotein (P-gp) encoded by multidrug resistance protein 1 (MDR1) gene is a member of the superfamily of adenosine triphosphate–binding cassette transporters, which acts as a drug efflux pump and contributes to the development of resistance against chemotherapy [6–9]. Docetaxel resistance is also caused in part by P-gp [10–12]. A novel taxane-based chemotherapeutic agent cabazitaxel is one of the second-line treatments for CRPC [13,14]. Although cabazitaxel improved overall survival among patients with docetaxel-resistant CRPC because of its poor affinity for P-gp, adverse events occurred at a higher rate. Especially, the incidence of febrile neutropenia is higher in patients treated with cabazitaxel when compared with those treated with docetaxel or mitoxantrone after docetaxel therapy [13,14]. In addition, several studies have recently shown the efficacy of docetaxel rechallenge in patients who were diagnosed as docetaxel refractory as well as in those who were previously docetaxel sensitive and in those who had an interval from the first treatment [15–17]. Although cabazitaxel chemotherapy and docetaxel rechallenge are effective therapeutic options for some patients with docetaxel-refractory CRPC, there are no available biomarkers to diagnose docetaxel resistance and select an appropriate taxoid for patients with CRPC—docetaxel or cabazitaxel.

Exosomes are microvesicles with a diameter of 40 to 150 nm that are secreted from cells [18–20]. Exosomes are present in the body fluids such as blood and urine and could serve as diagnostic markers for various diseases including cancer, because they contain proteins and RNAs specific to the cells from which they are derived [21–23]. Given that molecular information within the cells is available by examining exosomes in the body fluids, one could select appropriate treatment for patients. This is especially the case for patients with prostate cancer, because serial biopsy of the prostate is not usually performed because of its invasiveness.

In this study, we hypothesized that the P-gp level in blood exosomes could reflect that in prostate cancer cells and be used as a marker to diagnose docetaxel resistance and select a taxoid—docetaxel or cabazitaxel. We first confirmed that the P-gp level in exosomes secreted from docetaxel-resistant prostate cancer cells was higher than that from sensitive cells. We then characterized the docetaxel and cabazitaxel sensitivity of docetaxel-resistant cells and analyzed the effects of MDR1 knockdown on the sensitivity. Lastly, we demonstrated that serum exosomal P-gp level was relatively higher in patients with clinically docetaxel-resistant prostate cancer than in therapy-naïve patients.

2. Materials and methods

2.1. Reagents and antibodies

Docetaxel and cabazitaxel were purchased from Sigma-Aldrich (St. Louis, MO) and Selleck Chemicals (Houston, TX), respectively. Anti–P-gp and anti-CD9 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-GAPDH and anti–prostate-specific membrane antigen (PSMA) antibodies were from Cell Signaling Technology (Beverly, MA).

2.2. Cell culture

Human castration-resistant prostate cancer cell line PC-3 cells were obtained from the American Type Cell Collection (Manassas, VA) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO₂. PC-3 cells resistant to paclitaxel and docetaxel (PC-3R) were generated from parental PC-3 cells by stepwise increases of paclitaxel concentrations in the culture medium, as described previously [24]. In this study, PC-3R cells viable at 20 nM of paclitaxel were used.

2.3. Isolation of exosomes from cell culture medium

To isolate exosomes from the cell culture medium, cells were cultured in exosome-free fetal bovine serum prepared by ultracentrifugation at 110,000 g for 16 hours. Exosomes were isolated according to the method described in our previous report, with minor modification [25]. Briefly, 5.0 × 10⁵ cells were seeded in 75-cm² flasks. The conditioned medium was collected 72 hours after seeding. The medium was centrifuged at 1,800 g for 10 minutes to eliminate cells. The second centrifugation was at 16,500 g for 20 minutes to get rid of debris. Then, exosomes were pelleted by ultracentrifugation at 110,000 g for 70 minutes. Pellets were suspended in radioimmunoprecipitation assay buffer containing protease inhibitor cocktail (Sigma-Aldrich, MO). Samples were stored at −20°C until use.

2.4. Collection of serum from patients with prostate cancer and isolation of exosomes from serum

Serum was collected from 2 groups of patients: 6 therapy-naïve patients and 4 patients with clinically docetaxel-resistant prostate cancer. Whole blood was centrifuged at 1,800 g to collect serum. Then, serum was centrifuged at 16,500 g to eliminate cells and debris. The supernatant was stored at −80°C until use. For exosome isolation, 1 mL of serum was centrifuged at 110,000 g for 70 minutes. The pellets were suspended in radioimmunoprecipitation assay buffer containing protease inhibitor cocktail. Samples were stored at −20°C until use. This study was approved by the Bioethics Committee of Gifu University, and written informed consent was obtained from all patients.

2.5. Western blot analysis

To prepare whole cell lysates, cells were resuspended in ice-cold lysis buffer (1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA, 150-mM NaCl, and 10-mM Tris-HCl [pH = 7.4])
containing protease inhibitor cocktail. Cell lysates (10 μg), exosomal protein isolated from the cell culture medium (5 μg) or whole exosomal protein isolated from 1 ml of serum were subjected to electrophoresis on 4% to 20% sodium dodecyl sulfate-polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking in 5% skim milk, membranes were probed with a primary antibody and then with a horseradish peroxidase-linked secondary antibody. After washing, bound proteins were detected using the enhanced chemiluminescence Western blotting detection system (Amersham GE Healthcare, Piscataway, NJ).

2.6. Cell viability assay

To determine the sensitivity of cells to docetaxel and cabazitaxel, WST assay was performed. Cell proliferation reagents for WST assay were purchased from Roche (Basel, Switzerland).

2.7. Small interfering RNA transfection

For this study, 2 small interfering RNAs (siRNAs) for human MDR1 gene were purchased from Thermo Fisher Scientific (Waltham, MA). Sequences of the MDR1 siRNAs were as follows: siRNA1, sense: 5’-GAAACUGCCUCAUAAAUU-3’, antisense: 5’-AAUUUAUGAGGCAGUUU-3’ and siRNA2, sense: 5’-GACCAUAUAUGUAAGGUUU-3’, antisense: 5’-AAACCUAUAUUAGGU-3’. As a negative control siRNA, Negative Control Medium GC Duplex 2 was obtained from Invitrogen (Carlsbad, CA). siRNA was transfected at 10 nM using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific).

2.8. Statistical analysis

The data analysis was performed using the SPSS software version 11 (Armonk, NY). P < 0.05 was considered statistically significant.

3. Results

3.1. Expression of P-gp in cell lysates and exosomes derived from docetaxel-sensitive PC-3 and docetaxel-resistant PC-3R cells

We previously demonstrated that P-gp expression was higher in PC-3R cells when compared with that in parental PC-3 cells [24,26,27]. To determine if the P-gp level is also higher in exosomes, we isolated exosomes from the cell culture medium by differential centrifugation and performed Western blot analysis. As shown in Fig. 1, CD9, an exosomal marker, was detected at a similar level in PC-3 and PC-3R cells. The P-gp levels in exosomes as well as cell lysates were higher in PC-3R cells than in PC-3 cells, indicating that P-gp is present and detectable in exosomes secreted from docetaxel-resistant prostate cancer cells.

3.2. Sensitivity to taxoids in docetaxel-sensitive PC-3 and docetaxel-resistant PC-3R cells

We performed WST assay to examine the sensitivity of PC-3 and PC-3R cells to docetaxel and cabazitaxel. As
shown in Fig. 2, PC-3 cells with little or no expression of P-gp were sensitive to docetaxel, and PC-3R cells with higher P-gp expression were resistant to docetaxel at 40 to 500 nM \( (P < 0.05, \text{ Student } t \text{ test, } n = 5). \) In contrast, cabazitaxel effectively killed both PC-3 and PC-3R cells, although the sensitivity to 10 nM of cabazitaxel was statistically higher in PC-3 cells than in PC-3R cells \( (P = 0.03, \text{ Student } t \text{ test, } n = 5). \) showing that cabazitaxel is effective in docetaxel-resistant PC-3R cells.

### 3.3. Effects of MDR1 knockdown on the sensitivity to taxoids

To confirm the relationship between intracellular P-gp expression and the sensitivity to taxoids, we knocked down MDR1 by siRNA transfection in PC-3R cells. As shown in Fig. 3, both siRNA1 and siRNA2 silenced P-gp expression, but siRNA1 did more effectively than siRNA2 did \( (n = 3). \) In PC-3R cells transfected with MDR1 siRNAs, docetaxel sensitivity was improved when compared with negative control–transfected cells, and the effect was more evident in siRNA1-transfected cells than in siRNA2-transfected cells. There was a statistical difference in cell viability between siRNA1- or siRNA2-transfected cells and negative control–transfected cells at 40 and 50 nM of docetaxel \( (P < 0.05, \text{ 1-way analysis of variance following Fischer least significant difference, } n = 5). \) In contrast, MDR1 knockdown did not affect the sensitivity of PC-3R cells to cabazitaxel, as evidenced by lack of statistical significance in viability among groups at any concentration \( (P = \text{ not significant, 1-way analysis of variance following Fischer least significant difference, } n = 5). \) These findings suggested that P-gp expression was associated with docetaxel resistance but did not influence on the anticancer activity of cabazitaxel. Thus, cabazitaxel is effective in docetaxel-resistant prostate cancer cells expressing higher P-gp.

### 3.4. P-gp expression in exosomes isolated from blood of docetaxel-resistant and therapy-naïve patients with prostate cancer

We demonstrated that both the cellular and the exosomal P-gp levels were higher in PC-3R cells than in parental PC-3 cells. Based on these observations, we hypothesized that the P-gp level in blood exosomes might be higher in docetaxel-resistant patients than in therapy-naïve patients and isolated exosomes from the serum of patients by differential centrifugation. The characteristics of patients examined are shown in the Table. The exosomal P-gp levels of clinically docetaxel-resistant patients (patient No. 7-10) were relatively higher than those of therapy-naïve patients (patient No. 1-6, Fig. 4). CD9, an exosomal marker protein, was detected in exosomes isolated from all the patients, but the level was relatively lower in docetaxel-resistant patients than in therapy-naïve patients. The levels of PSMA, a marker for exosomes secreted from prostate cells [25], varied among patients, presumably reflecting the differences in exosome production from prostate cancer cells. These results indicated that exosomes isolated by differential centrifugation from the serum of docetaxel-resistant patients contained P-gp as well as CD9 and PSMA and suggested that exosomal P-gp could be a useful biomarker for diagnosing prostate cancer with docetaxel resistance conferred by P-gp.

### 4. Discussion

In prostate cancer as well as other types of cancer, resistance to docetaxel and paclitaxel has been reported to be caused in part owing to P-gp expression [7,9,10]. We have previously shown higher P-gp expression in docetaxel- and paclitaxel-resistant PC-3 cells when compared with that in parental PC-3 cells [24,26,27]. The P-gp level was
reported to be elevated in the tissues of patients with prostate cancer and correlate with higher tumor grade, stage, and prostate-specific antigen levels [28]. In this study, we demonstrated that the P-gp level was increased in exosomes isolated from docetaxel-resistant prostate cancer PC-3 cells when compared with that in parental cells, which was consistent with a previous report by Corcoran et al. [29] showing increased P-gp expression in exosomes derived from docetaxel-resistant variants of DU145 and 22Rv1 prostate cancer cells. These results suggest that the P-gp level in exosomes may reflect that in prostate cancer cells and correlate with docetaxel resistance and possibly with aggressiveness.

Cabazitaxel that has a poor affinity for P-gp was recently developed to overcome the resistance to taxanes such as docetaxel and paclitaxel and is therefore effective in patients with docetaxel-resistant CRPC [13]. Virgnaud et al. [30] demonstrated that cabazitaxel is as active as docetaxel in docetaxel-sensitive tumor models but is more potent than docetaxel in tumor models with innate or acquired resistance to taxanes and other chemotherapies. In PC-3R cells, reduction of the P-gp level by MDR1 siRNA transfection improved the docetaxel sensitivity in a manner dependent on P-gp expression, but it did not affect the cabazitaxel sensitivity. However, the sensitivity to 10 nM of cabazitaxel was statistically higher in PC-3 cells than in PC-3R cells, despite that there were no statistical differences in sensitivity at higher doses between the 2 cell lines, suggesting that cabazitaxel sensitivity may be at least in part regulated by P-gp. These results supported the notion that cabazitaxel is effective in patients with docetaxel resistance.

Although cabazitaxel chemotherapy and docetaxel rechallenge have been suggested to be effective for patients with docetaxel-refractory CRPC [13–17], there are currently no available biomarkers to diagnose docetaxel resistance and select an appropriate taxoid for patients with CRPC. It has been demonstrated that exosomes in the body fluids such as blood and urine could be useful biomarkers [21–23]. Given that P-gp expression in prostate cancer cells can be monitored by examining its level in blood exosomes, one could select docetaxel for patients with lower P-gp expression and cabazitaxel for those with higher expression. This would also help avoid adverse effects caused by cabazitaxel and select docetaxel rechallenge.

To demonstrate the clinical significance of exosomal P-gp in blood, we isolated blood exosomes by differential centrifugation from patients with prostate cancer and examined the P-gp levels. The results showed that the P-gp level in exosomes isolated from the serum was relatively higher in patients who were docetaxel-resistant than in therapy-naive patients. Consistent with our findings, Kharaziha et al. [31] have very recently reported that the P-gp level in exosomes isolated from docetaxel-resistant DU145 prostate cancer cells was higher than that from parental cells and that the serum exosomal P-gp level was higher in docetaxel-resistant patients than in sensitive patients. However, we are aware of the limitations of our study. P-gp was detected in some of the therapy-naive patients, although to a lesser extent. As P-gp is expressed in various normal tissues with excretory functions and blood-tissue barrier functions [32–35], it is likely that exosomes isolated from blood by differential centrifugation also contain those secreted from other cells than the prostate cancer cells. Thus, it would be important to distinguish P-gp–expressing exosomes secreted from prostate cancer cells from those secreted from other types of cells. In this regard, we have recently reported that exosomes derived from the prostate can be isolated from blood using anti-PSMA antibody bound to magnetic beads [25]. The quantitation of P-gp in exosomes expressing PSMA would enable more accurate diagnosis of prostate cancer with docetaxel resistance conferred by P-gp expression.

5. Conclusion

Our results suggest that detection of P-gp in blood exosomes, which is involved in resistance to docetaxel but not to cabazitaxel, could be useful to diagnose docetaxel

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**Table**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age PSA (ng/ml)</th>
<th>Gleason score</th>
<th>T</th>
<th>M</th>
<th>Status</th>
</tr>
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<tr>
<td>1</td>
<td>74 1.481</td>
<td>3 + 4</td>
<td>2a</td>
<td>0</td>
<td>Therapy naïve</td>
</tr>
<tr>
<td>2</td>
<td>78 6.212</td>
<td>4 + 4</td>
<td>3b</td>
<td>0</td>
<td>Therapy naïve</td>
</tr>
<tr>
<td>3</td>
<td>68 7.708</td>
<td>3 + 4</td>
<td>2c</td>
<td>0</td>
<td>Therapy naïve</td>
</tr>
<tr>
<td>4</td>
<td>64 7.825</td>
<td>3 + 3</td>
<td>1c</td>
<td>0</td>
<td>Therapy naïve</td>
</tr>
<tr>
<td>5</td>
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<td>3 + 3</td>
<td>2a</td>
<td>0</td>
<td>Therapy naïve</td>
</tr>
<tr>
<td>6</td>
<td>77 4.515</td>
<td>3 + 4</td>
<td>2a</td>
<td>0</td>
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</tr>
<tr>
<td>7</td>
<td>79 25313.0</td>
<td>5 + 3</td>
<td>Unknown</td>
<td>0</td>
<td>1c Docetaxel resistant</td>
</tr>
<tr>
<td>8</td>
<td>81 3615.5</td>
<td>5 + 5</td>
<td>2a</td>
<td>1</td>
<td>1b Docetaxel resistant</td>
</tr>
<tr>
<td>9</td>
<td>86 26.177</td>
<td>4 + 5</td>
<td>3b</td>
<td>0</td>
<td>1b Docetaxel resistant</td>
</tr>
<tr>
<td>10</td>
<td>78 600.0</td>
<td>Unknown 3a</td>
<td>1</td>
<td>1b</td>
<td>Docetaxel resistant</td>
</tr>
</tbody>
</table>

PSA = prostate-specific antigen.
resistance and select an appropriate taxoid for patients with CRPC—docetaxel or cabazitaxel. The development of diagnostic systems to specifically detect P-gp in exosomes derived from prostate cancer cells and their evaluation in large-scale clinical studies remains to be done.

References