Prediction of Response to Docetaxel by Quantitative Analysis of Class I and III β-Tubulin Isotype mRNA Expression in Human Breast Cancers

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ABSTRACT

Purpose: The relationship of intratumoral mRNA levels of class I and III β-tubulin isotypes with clinical response to docetaxel has been studied in breast cancer patients.

Experimental Design: Expression of class I and class III β-tubulin mRNA levels was determined by a real-time PCR in breast cancer tissues obtained from 39 patients with locally advanced breast cancers (n = 26) or locally recurrent breast cancers (n = 13) before docetaxel treatment.

Results: Class I β-tubulin mRNA levels of responders [6.58 ± 1.43 (×10^2), mean ± SE] were significantly (P = 0.002) lower than those of nonresponders [14.97 ± 2.95 (×10^2)], and class III β-tubulin mRNA levels of responders (1.38 ± 0.40) were also significantly (P = 0.003) lower than those of nonresponders (7.43 ± 2.77). Breast cancers were divided into four groups according to the expression status of class I and class III β-tubulin isotype mRNA, i.e., the class I-high/class III-high group (n = 13), the class I-high/class III-low group (n = 7), the class I-low/class III-high group (n = 7), and the class I-low/class III-low group (n = 12). The class I-high/class III-high group showed a very low response rate (15%), whereas the class I-low/class III-low group showed a very high response rate (75%). The class I-high/class III-low group and the class I-low/class III-high group showed intermediate response rates of 57% and 43%, respectively.

Conclusions: These results demonstrate that high expression of class I and class III β-tubulin isotype mRNA is significantly associated with docetaxel resistance, and determination of class I and class III β-tubulin isotype mRNA levels would be useful in the prediction of response to docetaxel.

INTRODUCTION

Taxane including paclitaxel and docetaxel is one of the most active antitumor agents in the treatment of breast cancer. Taxane binds to β-tubulin, which is one of the major components of microtubule, and exerts its growth-inhibitory effects through the stabilization of microtubule, resulting in the growth arrest of tumor cells at the G2-M phase (1). Because an increasing number of breast cancer patients have been treated with taxane, development of taxane resistance is becoming a clinically important problem associated with chemotherapy. Elucidation of the resistance mechanism appears to be of pivotal importance for the development of a new strategy for overcoming resistance as well as for the development of a clinically useful predictor of response to taxane.

Although the resistance mechanism to taxane is still far from clear, it has been reported that acceleration of drug efflux via the overexpressed P-glycoprotein (MDR1) can be one of the resistance mechanisms in vitro because taxane is a substrate of P-glycoprotein. We have studied the relationship between the expression of P-glycoprotein and response to docetaxel in human breast cancers but failed to demonstrate a significant correlation (2). Monzo et al. (3) reported that somatic mutation of class I β-tubulin is observed in as high as 33% of human non-small cell lung cancers and that it plays a significant role in the acquisition of paclitaxel resistance. Thus, we have recently conducted mutational analysis of class I β-tubulin in human breast cancers and have found that somatic mutation of this gene is very rare (1 of 62; 1.6%), and, thus, the acquisition of taxane resistance is unlikely to be explained by somatic mutation of class I β-tubulin in most breast cancers (4).

Another possible mechanism for taxane resistance is altered expression of β-tubulin isotypes. In humans, at least six distinct β-tubulin isotypes (classes I, II, III, IVa, IVb, and VI) have been reported, and their expression profile is different among the tissues (5–9). Of the six β-tubulin isotypes, class III β-tubulin seems to be unique in that it destabilizes the microtubule (1). Thus, it is speculated that the antitumor action of taxane can be modified by the expression level of class III β-tubulin because class III β-tubulin might counteract the stabilizing effect of microtubule by taxane. In fact, it is reported that the high class III β-tubulin expression is associated with paclitaxel resistance in several human cancer cell lines [lung cancer, ovarian cancer, prostate cancer, and breast cancer (1)], and this association was also demonstrated clinically in ovarian cancer (10). In addition to class III β-tubulin, several reports have shown the association of high class I β-tubulin expression...
with paclitaxel resistance in human non-small cell lung cancer cell lines and ovarian tumors (10), whereas class I β-tubulin, unlike class III β-tubulin, does not destabilize the microtubule.

These results indicate the possibility that the expression level of class I and III β-tubulin can be a clinically useful predictor of response to taxane in human breast cancer, whereas this possibility has not been studied previously. Although the relationship between taxane resistance and β-tubulin isotype mRNA expression has been studied mostly on paclitaxel, recent studies have revealed similar results on docetaxel [i.e., high expression of class III β-tubulin is associated with docetaxel resistance in human pancreatic cancer cell lines (11, 12)]. Therefore, in the present study, we have studied whether or not the class I and III β-tubulin isotype mRNA levels in breast cancer tissues can be a clinically useful predictor of response to docetaxel.

MATERIALS AND METHODS

Patients. Thirty-nine female patients with locally advanced breast cancer (n = 26) or locally recurrent breast cancer (n = 13) were recruited in this study. Patient characteristics are shown in Table 1. All of the patients underwent biopsy of breast tumors or locally recurrent tumors (incisional biopsy or vacuum-assisted core needle biopsy) before chemotherapy. The standard curves for class I, class III, and β-glucuronidase transcripts as the quantitative control, and each sample was normalized on the basis of its β-glucuronidase transcript content. The primer probe mixture for β-glucuronidase was purchased from Perkin-Elmer Applied Biosystems, and the method of PCR was followed according to the manufacturer’s protocol. Briefly, 50 μl of reaction mixture containing 1 μl of cDNA template, 25 μl of TaqMan Universal PCR Master Mix (Perkin-Elmer Applied Biosystems), 0.1 μM probe, and 0.3 μM of each primer. The PCR conditions for class I and class III were set up as follows: after incubation at 50°C for 2 min and denaturing at 96°C for 10 min, 45 cycles of 95°C for 30 s and 62°C for 1 min were performed. To quantify transcripts of the genes precisely, we monitored β-glucuronidase transcripts as the quantitative control, and each sample was normalized on the basis of its β-glucuronidase transcript content. The primer probe mixture for β-glucuronidase was purchased from Perkin-Elmer Applied Biosystems, and the method of PCR was followed according to the manufacturer’s protocol. Briefly, 50 μl of reaction mixture containing 1 μl of cDNA template, 25 μl of TaqMan Universal PCR Master Mix, and 2.5 μl of primer probe mixture were amplified by the program as follows: after incubation at 50°C for 2 min and denaturing at 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min were performed.

The standard curves for class I, class III, and β-glucuronidase mRNA were generated using serially diluted solutions of plasmid clones inserted by either class I, class III, or β-glucuronidase cDNA as templates (Fig. 3A). Ct was designed as the fractional cycle number at which the fluorescence signal resulting from cleavage of the probe exceeded the threshold level. The amount of target gene expression was calculated from the standard curve (Fig. 3B), and quantitative normalization of cDNA in each sample was performed using the expression of β-glucuronidase gene as an internal control. Finally, class I and class III mRNA levels were shown as ratios to β-glucuronidase mRNA levels. Real-time PCR assays were conducted in duplicate for each sample, and the mean value was used for calculation of the mRNA expression levels.

Primer Specificity. Breast cancer-derived cDNA (1 μl), plasmid containing class I β-tubulin isotype cDNA (10^{-4} μg), plasmid containing class III β-tubulin isotype cDNA (10^{-6} μg), or normal breast tissue-derived genomic DNA (0.1 μg) was amplified with the primer sets for class I or class III β-tubulin isotype (0.5 μM for each primer) in a reaction volume of 20 μl. The PCR conditions were set up as follows: after incubation at 95°C for 10 min, 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 1 min were performed. The standard curves for class I, class III, and β-glucuronidase mRNA were generated using serially diluted solutions of plasmid clones inserted by either class I, class III, or β-glucuronidase cDNA as templates (Fig. 3A). Ct was designed as the fractional cycle number at which the fluorescence signal resulting from cleavage of the probe exceeded the threshold level. The amount of target gene expression was calculated from the standard curve (Fig. 3B), and quantitative normalization of cDNA in each sample was performed using the expression of β-glucuronidase gene as an internal control. Finally, class I and class III mRNA levels were shown as ratios to β-glucuronidase mRNA levels. Real-time PCR assays were conducted in duplicate for each sample, and the mean value was used for calculation of the mRNA expression levels.

Primer Specificity. Breast cancer-derived cDNA (1 μl), plasmid containing class I β-tubulin isotype cDNA (10^{-4} μg), plasmid containing class III β-tubulin isotype cDNA (10^{-6} μg), or normal breast tissue-derived genomic DNA (0.1 μg) was amplified with the primer sets for class I or class III β-tubulin isotype (0.5 μM for each primer) in a reaction volume of 20 μl. The PCR conditions were set up as follows: after incubation at 95°C for 10 min, 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 1 min were performed.
72°C for 30 s were performed. PCR products were subjected to 3% agarose gel electrophoresis (100 V, 45 min).

**Evaluation of Chemotherapy Response.** Chemotherapeutic response was clinically evaluated by measuring the change in tumor size in the breast or recurrent region as follows: (a) **CR**, disappearance of all known disease; (b) **PR**, ≥50% decrease in tumor size; (c) no change, <50% decrease or <25% increase in tumor size; and (d) progressive disease, ≥25% increase in tumor size or appearance of new lesions. In this study, CR and PR were defined as responders, and no change and progressive disease were defined as nonresponders.

**Statistical Methods.** Class I and class III β-tubulin mRNA expression levels were compared between responders and nonresponders by Mann-Whitney test. The relationship between β-tubulin isotype mRNA expression levels and response to docetaxel was analyzed by χ² test. Statistical significance was assumed for P < 0.05.

**RESULTS**

**Specificity of PCR Assay for Class I and Class III β-Tubulin Isotypes.** We studied the primer specificity, i.e., whether or not primer sets for class I β-tubulin isotype and class III β-tubulin isotype selectively amplify only the individual isotypes (Fig. 4). We showed that the PCR product obtained from amplification of breast tumor-derived cDNA using primer sets for class I (Lanes A–C) or class III (Lanes G–I) β-tubulin isotype was a single band of the correct size (291 bp for class I isotype and 160 bp for class III isotype) in the electrophoresis, and the direct sequencing of these PCR products confirmed the specific amplification of class I or class III β-tubulin isotype (data not shown). We also showed that there was amplification when the plasmid containing the class I β-tubulin isotype cDNA was amplified with the primer set for the class I β-tubulin isotype (Fig. 4, Lane D), and when the plasmid containing class III β-tubulin isotype cDNA was amplified with the primer set...
The primer set for class I isotype. These results demonstrate that the primer sets for the different dilutions (a, 10^{-3} μg; b, 10^{-4} μg; c, 10^{-6} μg; d, 10^{-7} μg) of standard plasmids for class I β-tubulin isotype were subjected to real-time PCR. Cycle number was plotted versus change in normalized reporter signal (ΔRn). For each reaction tube, the fluorescence signal of the reporter dye [dye for class I was 6-carboxyfluorescein, and dye for β-glucuronidase was VIC (Perkin-Elmer Applied Biosystems)] was divided by the fluorescence signal of the passive reference dye (6-carboxy-N,N',N'-tetramethylrhodamine) to obtain a ratio defined as the normalized reporter signal (Rn). ΔRn represents the normalized reporter signal (Rn) minus the baseline signal established in the first 15 PCR cycles. ΔRn increases during PCR as class I β-tubulin isotype PCR product copy number increases until the reaction reaches a plateau. Ct represents the fractional cycle number at which a significant increase in Rn above a baseline signal (horizontal black line) can first be detected. Two replicates for each standard curve point sample (a–d) were performed, but the data for only one are shown here. B, standard curve plotting log starting copy number versus Ct.

for the class III β-tubulin isotype (Fig. 4, Lane K), but there was no amplification when the plasmid containing class I β-tubulin isotype cDNA was amplified with the primer set for class III β-tubulin isotype (Fig. 4, Lane J), or when the plasmid containing class III β-tubulin isotype cDNA was amplified with the primer set for class I β-tubulin isotype (Fig. 4, Lane E). There was no amplification when genomic DNA was amplified with the primer set for class I β-tubulin isotype or class III β-tubulin isotype. These results demonstrate that the primer sets for the class I β-tubulin isotype and class III β-tubulin isotype have no cross-reactivity with other isotypes and pseudogenes.

Class I and Class III β-Tubulin Isotype mRNA Expression and Clinical Response to Docetaxel Treatment. Expression of class I and class III β-tubulin isotype mRNA levels was determined by a real-time PCR in breast cancer tissues obtained from 39 patients before docetaxel treatment. The expression of class I and class III β-tubulin isotype mRNA was detected in all of the samples. The levels of class I β-tubulin isotype mRNA [11.10 ± 1.83 (×10^2), mean ± SE] were much higher than those of class III β-tubulin isotype mRNA (4.64 ± 1.56).

Of these 39 patients, 18 showed a response (CR + PR) to docetaxel with a response rate of 46%. Class I β-tubulin isotype mRNA levels of responders to docetaxel [6.58 ± 1.43 (×10^2)] were significantly (P = 0.002) lower than those of nonresponders [14.97 ± 2.95 (×10^2)], and class III β-tubulin isotype mRNA levels of responders to docetaxel (1.38 ± 0.40) were also significantly (P = 0.003) lower than those of nonresponders [7.43 ± 2.77 (Fig. 5)]. On the other hand, the class III class I β-tubulin isotype mRNA level ratios did not differ significantly between responders and nonresponders (Fig. 5).

Prediction of Response to Docetaxel Treatment by Class I and Class III β-Tubulin Isotype mRNA Expression Levels. Breast cancers were dichotomized into the high class I β-tubulin isotype mRNA expression (class I-high) group and the low class I β-tubulin isotype mRNA expression (class I-low) group using a median value of 7.25 × 10^2 as a cutoff value, and they were dichotomized into the high class III β-tubulin isotype mRNA expression (class III-high) group and the low class III β-tubulin isotype mRNA expression (class III-low) group using a median value of 1.36 as a cutoff value. Tumors in the class I-high group showed a significantly (P < 0.05) lower response rate (30%) than those in the class I-low group (63%; Table 2). Similarly, tumors in the class III-high group showed a significantly (P < 0.01) lower response rate (25%) than those in the class III-low group (68%). Positive predictive value, negative predictive value, and diagnostic accuracy of class I β-tubulin isotype mRNA determination in the prediction of response to docetaxel were 63%, 70%, and 67%, respectively, and those of class III β-tubulin isotype mRNA determination in the prediction of response to docetaxel were 68%, 75%, and 72%, respectively.

Breast cancers were further divided into four groups according to the expression status of class I and class III β-tubulin isotype mRNA levels, i.e., the class I-high/class III-high group (n = 13), the class I-high/class III-low group (n = 7), the class I-low/class III-high group (n = 7), and the class I-low/class III-low group (n = 12; Table 3). The class I-high/class III-high group showed a very low response rate (15%), whereas the class I-low/class III-low group showed a very high response rate (75%). The class I-high/class III-low group and the class I-low/class III-high group showed intermediate response rates of 57% and 43%, respectively.

The relationship between patient characteristics and response to docetaxel treatment is shown in Table 4. There was no significant association between response and menopausal status, stage, or ER status.

DISCUSSION

Consistent with previous reports that were based mostly on in vitro studies (1), we have shown that not only high expression of class III β-tubulin isotype mRNA but also high expression of class I β-tubulin isotype mRNA is significantly associated with poor response to docetaxel in human breast cancer. The fact that the class III class I β-tubulin isotype mRNA level ratios are not significantly associated with response to docetaxel indicates that absolute mRNA expression levels of class I or class III β-tubulin isotype, but not a relative increase in class III class I β-tubulin isotype mRNA level, are important in the acquisition of
docetaxel resistance. Interestingly, tumors in the class I-high/class III-high group showed a poorer response rate (15%) than tumors in the class I-high/class III-low group and the class I-low/class III-high group (57% and 43%, respectively), and tumors in the class I-high/class III-low group and the class I-low/class III-high group showed a poorer response rate than those in the class I-low/class III-low group (75%). These results demonstrate that both class I and class III β-tubulin isotypes play a significant role in the acquisition of docetaxel resistance in breast cancer, whereas more emphasis has been put on class III β-tubulin isotype in previous studies using various human cancer cell lines, including human breast cancer cell line MCF-7 (13). Kavallaris et al. (10) also reported that both class I and class III β-tubulin isotype mRNA are up-regulated in human ovarian tumors resistant to paclitaxel. Thus, it is speculated that the role of class I β-tubulin isotype in the acquisition of taxane resistance might be different in vitro and in vivo.

Our present observation on the relationship between β-tubulin isotype mRNA expression and response to docetaxel treatment.
seems to be clinically useful because, at present, there is no reliable predictor of response to docetaxel and an increasing number of patients have been treated recently with docetaxel not only in the metastatic setting but also in the adjuvant setting. We have been able to show that tumors with class I-high/class III-high β-tubulin isotype mRNA levels are very unlikely to respond (response rate, 15%), and, on the other hand, tumors with class I-low/class III-low β-tubulin isotype mRNA levels are very likely to respond (response rate, 75%), and tumors with class I-high/class III-low or class I-low/class III-high β-tubulin isotype mRNA levels show an intermediate response rate of about 50%. This information is valuable when indication for chemotherapy with docetaxel is considered. The cutoff values for high and low expression of class I and class III β-tubulin isotype mRNA levels used in the present study are tentative ones and thus need to be determined in a future study including a large number of patients.

Although docetaxel and paclitaxel are not totally cross-resistant (14), their action mechanism is essentially identical in the acquisition of resistance to docetaxel. This issue needs to be studied in the future. In addition, it still remains to be established whether or not the mRNA levels of β-tubulin isotypes are correlated with their protein levels. In conclusion, we have shown that high expression of class I and class III β-tubulin isotype mRNA is significantly associated with docetaxel resistance, and determination of their mRNA levels would be useful in the prediction of response to docetaxel. Recently, we have shown the association of high expression of CYP3A4 mRNA in breast cancer with docetaxel resistance (16). Thus, it is expected that the combination of class I and class III β-tubulin isotype mRNA levels with CYP3A4 mRNA levels would be more useful in the prediction of response to docetaxel. This possibility seems to deserve a future study including a larger number of patients.

**REFERENCES**


**Table 4** Relationship between patient characteristics and response to docetaxel treatment

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* ER status of the breast tumors for locally advanced cases and the recurrent tumors for locally recurrent cases.
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