Overexpression of Class III β-Tubulin Predicts Good Response to Taxane-Based Chemotherapy in Ovarian Clear Cell Adenocarcinoma

Daisuke Aoki,1 Yoshina Oda,3 Satoshi Hattori,8 Ken-ichi Taguchi,7 Yoshihiro Ohishi,3 Yuji Basaki,5,6 Shinji Oie,2 Nao Suzuki,10 Suminori Kono,4 Masazumi Tsoneyoshi,3 Mayumi Ono,5,6 Daisuke Aoki,1 Yoshina Oda,3 Satoshi Hattori,8 Ken-ichi Taguchi,7 Yoshihiro Ohishi,3 Yuji Basaki,5,6 Shinji Oie,2 Nao Suzuki,10 Suminori Kono,4 Masazumi Tsoneyoshi,3 Mayumi Ono,5,6 Takashi Yanagawa,8 and Michihiko Kuwano6,9

Abstract  Purpose: Of the various microtubule-associated molecules, β-tubulin III has been reported to be closely associated with the therapeutic efficacy of taxane-based chemotherapy against ovarian cancer. Stathmin and microtubule-associated protein 4 (MAP4) have been reported to play an important role in microtubule stabilization. In this study, we investigated whether expression of these microtubule-associated factors affects the therapeutic efficacy of taxane-based chemotherapy in ovarian clear cell adenocarcinoma.

Experimental Design: Drug sensitivity of paclitaxel or cisplatin was assessed in ovarian cancer cell lines treated with small interfering RNA of tubulin isoforms, MAP4, and stathmin. We examined 94 surgically resected ovarian clear cell adenocarcinoma specimens from patients treated with taxane-containing regimens (n = 44) and with taxane-free regimens (n = 50), using immunohistochemistry to detect expression of β-tubulin III, stathmin, and MAP4.

Results: Knockdown of β-tubulin III and IV specifically conferred drug resistance to paclitaxel in one ovarian cancer cell line, but not to other molecules. Estimated overall survival revealed a significant synergistic effect between taxane and β-tubulin III in patients with ovarian clear cell adenocarcinoma. Of three microtubule-related molecules, among the taxane-based chemotherapy group, cases with higher β-tubulin III expression were associated with a significantly more favorable prognosis compared with those having lower β-tubulin III expression. By contrast, there was no statistical significance in the synergistic relationships between stathmin and taxane or between MAP4 and taxane.

Conclusions: Taxane-based chemotherapy was effective for patients with ovarian clear cell adenocarcinomas who were positive for β-tubulin III but not for those who were negative for these proteins.

Microtubules are the principal target of a large and diverse group of natural-product anticancer therapeutic drugs, particularly of two major classes of antimicrotubule agents: the vinca alkaloids and the taxanes (1). Microtubules are composed of polymers of heterodimers that consist of two closely related polypeptides, α-tubulin and β-tubulin, which in turn contain α- or β-subunits and at least six isoforms encoded by different genes. Isoform composition influences the intrinsic dynamics of microtubules, and the sensitivity of microtubules to depolymerizing and polymerizing agents is related to the composition of tubulin isotypes or microtubule-associated proteins (MAP; ref. 2). MAPs, important components of the tubulin and microtubule system, can bind to the microtubule wall and stabilize microtubules (3). MAP2 and MAP-7 are abundantly expressed in mature neurons, and MAP4 is ubiquitously expressed in both proliferating and differentiated cells (4). Stathmin is also the founding member of the microtubule-stabilizing family of proteins, which regulate the dynamics of microtubule polymerization and depolymerization. Stathmin is expressed at high levels in a variety of human cancers.
Translational Relevance

It has been reported that β-tubulin III, one of microtubule-associated molecules, was expected to be a useful biomarker for the clinical efficacy of taxane-based chemotherapy against human ovarian cancer. These studies have been conducted in serous adenocarcinoma of ovarian cancer. Previous reports show, however, that ovarian clear cell adenocarcinoma constituted about 20% of ovarian adenocarcinoma in Japan, although only 2% to 5% of cases of ovarian cancer worldwide were clear cell adenocarcinoma. Clear cell adenocarcinoma, which is a rare variant in western counties, has been recognized as a chemoresistant phenotype compared with serous adenocarcinoma, which is the most widespread ovarian cancer. This study aimed to identify a predictive marker for the clinical efficacy of taxane-based chemotherapy against ovarian clear cell adenocarcinoma. In this study, we found that a taxane-based regimen was effective for patients with ovarian clear cell adenocarcinomas who were positive for stathmin or β-tubulin III.

and also plays a role in altered drug sensitivity in human cancer cells, including ovarian cancer cells (5, 6).

The antitumor drug taxane stabilizes microtubules and reduces their dynamics, promoting mitotic arrest and cell death. Paclitaxel, a representative anticancer agent of the taxanes, was initially defined by Horwitz and colleagues, and its binding sites are distinct from those of colchicine, podophyllotoxin, and the vinca alkaloids (7, 8). Paclitaxel initially received regulatory approval for the treatment of patients with ovarian cancer after failure of first-line or subsequent chemotherapy (9). In a Gynecologic Oncology Group study (GOG-111), it was thus determined to be the primary induction therapy in suboptimally debulked stage III and IV ovarian cancer, which mainly consists of serous adenocarcinoma (10). This study first compared the therapeutic efficacy of paclitaxel/cisplatin and cyclophosphamide/cisplatin in patients with ovarian cancer (10). The paclitaxel arm showed a distinct advantage in terms of progression-free survival (PFS) as well as overall survival (OS). A clinical trial by the European Organization for Research and Treatment of Cancer and the National Cancer Institute of Canada also showed that a paclitaxel/cisplatin regimen improved both PFS and OS (11). Another clinical trial study, however, reported that survival in the paclitaxel arm was similar to that seen in the control arm that received either carboplatin or cisplatin, doxorubicin, and cyclophosphamide (12). It remains unclear whether paclitaxel-cisplatin (or carboplatin) therapy is superior to cyclophosphamide/cisplatin (or carboplatin) therapy.

Of the various molecular markers related to drug sensitivity to taxanes, class III β-tubulin is expected to be a useful biomarker for the clinical efficacy of paclitaxel-based chemotherapy. Class III β-tubulin is hypothesized to counteract suppression of microtubule dynamics (13). Ferlini et al. reported that a novel taxane targeting class III β-tubulin overcame paclitaxel resistance, suggesting close involvement of this tubulin isotype in drug sensitivity to paclitaxel (14).

Mozzetti et al. reported that class III β-tubulin overexpression represented a prominent mechanism of resistance to paclitaxel-platinum treatment in ovarian cancer (15). Moreover, class III β-tubulin overexpression could be useful in identifying poor clinical outcome in patients with advanced ovarian cancer who are treated with platinum/paclitaxel, those mainly affected with serous adenocarcinoma (16). These studies have been conducted mainly in serous adenocarcinoma of ovarian cancer. It remains unknown, however, whether class III β-tubulin overexpression is also predictive of poor outcome in clear cell adenocarcinoma, which is a rare variant in western countries, where it is reported to constitute 5% to 10% of ovarian carcinomas (17–19). Clear cell adenocarcinoma has been recognized as a chemoresistant phenotype (20, 21).

Japanese investigators have reported that clear cell adenocarcinoma constitutes about 20% of ovarian carcinomas in Japan (20, 22), although clear cell adenocarcinoma of the ovary accounts for only 2% to 5% of cases enrolled in large-scale randomized trials worldwide (22, 23). Thus, it is unclear whether carboplatin/paclitaxel therapy, which was introduced broadly as a standard regimen for epithelial ovarian cancer based on the results of such trials, can be readily applied for clear cell adenocarcinoma. Development of novel treatment strategies based on molecular biological characteristics is further required for clear cell adenocarcinoma.

In the present study, we addressed whether expression of β-tubulin III, MAP4, and stathmin could affect the efficacy of taxane-based therapeutic regimens against clear cell adenocarcinoma. Using immunohistochemical analysis of surgically resected clinical samples of clear cell adenocarcinoma, we examined expression levels of the above three biomarkers. In comparison with ovarian cancer patients treated with taxane-free regimens, we observed a significant and specific association of β-tubulin III expression with therapeutic outcomes of ovarian cancer treated with taxane-based regimens. We discuss whether the expression of β-tubulin III could be a predictive marker for the clinical efficacy of taxane-based chemotherapy against ovarian clear cell adenocarcinoma.

Materials and Methods

Cells and reagents. The human ovarian cancer lines OVCAR-3 and SKOV-3, which expressed β-tubulins (I, II, III, and IV), MAP4, and stathmin, were obtained from the American Type Culture Collection. Cells were grown in Ham’s F-12 Medium (Nissui Seiyaku Co.) with 10% fetal bovine serum (FetalClone III; Hyclone), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Inc.) in a humidified atmosphere of 5% CO2 at 37°C. Paclitaxel (Taxol injection) and cisplatin (Briplatin injection) purchased from Bristol-Myers Squibb were clinically used. The polyclonal antistathmin was obtained from Calbiochem. The monoclonal class III β-tubulin antibody (clone 5G8) was obtained from Promega. The monoclonal MAP4 antibody (clone 18) was purchased from BD Transduction Laboratories.

Silencing of β-tubulins (I, II, III, IV), MAP4, and stathmin genes. To reduce the expression of some genes, we used Stealth RNAi (Invitrogen Life Technologies) to knock down the expression of β-tubulin I (NM_030773_stealth_706), β-tubulin II (NM_001069_stealth_1444), β-tubulin III (NM_006086_stealth_233), β-tubulin IV (NM_006087_stealth_352), MAP4 (NM_002375_stealth_2042), and stathmin (STM1-HSS142799). Subconfluent human ovarian cells were cultured overnight in Opti-MEM I medium (Invitrogen Life
Technologies) without antibiotics, then 40 nmol/L small interfering RNA (siRNA) and Lipofectamine RNAiMax (Invitrogen) were applied according to the manufacturer's instructions. After 32 h, cells were detached from the culture plates and seeded into 96-well plates in F-12 medium with 10% fetal bovine serum. After a further 16-h incubation, paclitaxel or cisplatin was applied and cells were cultured for 3 d more. The numbers of cells were estimated by WST-8. The IC50 value was estimated from the regression line of log-logit plots of T/C (%) value versus drug concentration. The assays were carried out in quadruplicate.

Quantitative real-time PCR. RNA was reverse-transcribed from random hexamers using AMV reverse transcriptase (Promega). Real-time quantitative PCR was done using the Real-Time PCR system 7300 (Applied Biosystems). In brief, the PCR amplification reaction mixtures (20 μL) contained CDNA, primer pairs, the dual-labeled fluorogenic probe, and TaqMan Universal PCR Master Mix (Applied Biosystems). The thermal cycle conditions included maintaining the reactions at 50°C for 2 min and at 95°C for 10 min, and then alternating for 40 cycles between 95°C for 15 s and 60°C for 1 min. The primer pairs and probes were obtained from Applied Biosystems. The relative gene expression for each sample was determined using the formula $2^{(-\Delta\Delta Ct)} = 2^{(Ct_{GAPDH}-Ct_{target})}$, which reflected the target gene expression normalized to GAPDH levels.

Patients. Ninety-four patients with primary ovarian clear cell adenocarcinoma, who had undergone debulking surgery at Keio University Hospital from 1983 to 2005, were examined. The histopathologic diagnoses of the all cases were confirmed according to the most recent WHO classification (WHO 2003). Patients were staged according to the International Federation of Obstetrics and Gynecology (FIGO) classification (24). Forty-four patients underwent chemotherapy using regimens containing taxanes [paclitaxel plus carboplatin ($n = 39$), paclitaxel plus cisplatin ($n = 3$), docetaxel plus cisplatin ($n = 2$); paclitaxel, 180 mg/m2 body surface/day 1, docetaxel, 70 mg/m2 body surface/day 1, cisplatin, 60 mg/m2 body surface/day 1, and carboplatin, area under the curve 6/day 1]; Fifty patients received taxane-free regimens [CAP groups ($n = 36$): cisplatin (60 mg/m2 body surface/day 1), epirubicin (50 mg/m2 body surface/day 1), and cyclophosphamide (500 mg/m2 body surface/day 1); CAP plus fluorouracil ($n = 1$), CAP plus tegafur-uracil ($n = 2$), cisplatin plus cyclophosphamide ($n = 11$)]. The doses of carboplatin were calculated using Calvert’s formula.

The effect of chemotherapy was evaluated approximately every 6 mo by computed tomography after 6 cycles of administration of chemotherapy. After chemotherapy, all patients were followed up every 2 mo for the first year, every 3 to 4 mo for the next 2 y, and every 6 mo

Fig. 1. Drug sensitivity to paclitaxel or cisplatin in human ovarian cancer cells treated with siRNA for β-tubulin isoforms, MAP4, and stathmin. A, mRNA expression of β-tubulin isoforms (I, II, III, IV), MAP4, and stathmin after treatment with respective siRNA for 48 h were determined by real-time PCR analysis. The expression of β-tubulin IV mRNA in OVCAR-3 cells was not detected. B, cells treated with respective siRNA were seeded into 96-well plates at 2 × 103 cells/0.1 mL/well and incubated overnight. On the following day, a 100-μL aliquot containing paclitaxel or cisplatin was added to the wells and cultured for a further 3 d. The number of viable cells was estimated using the WST-8 assay. The assays were carried out in quadruplicate.
thereafter. Clinical outcome was measured by PFS and OS. PFS was defined as the interval from the date of first treatment (laparotomy or the first administration of neoadjuvant chemotherapy) to the date of the diagnosis of progression. We obtained informed consent from all patients, and personal information was removed from all samples before analysis.

**Immunohistochemistry.** Surgically resected specimens were fixed with 10% formalin and embedded in paraffin. Sections 4-μm thick on silane-coated slides were stained using the streptavidin-biotin-peroxidase method with a Histofine SAB-PO kit (Nichirei) according to the manufacturer’s instructions. At least one representative section without degenerative change or necrosis was examined in each tumor. After deparaffinization, rehydration, and inhibition of endogenous peroxidase, sections were exposed to the primary antibodies at 4°C overnight. The dilutions of the primary antibody were as follows: MAP4, 1:1500; stathmin, 1:1000; and β-tubulin III, 1:200. After incubation of the secondary antibody and the streptavidin-biotin complex at room temperature, the sections were then incubated in 3,3′-diaminobenzidine, counterstained with hematoxylin, and mounted. For all antibody staining, sections were pretreated with microwave irradiation for antigen retrieval.

Immunohistochemical results were evaluated and scored by three pathologists (Y. Oda, K. Taguchi, and Y. Ohishi) without knowledge of patient clinical data. MAP4 and stathmin immunoreactivity was scored by estimating the percentage of labeled tumor cells. When >80% of the tumor cells showed immunoreactivity for MAP4, we judged the case to be positive. For stathmin expression, the cutoff value was 15%, based on a previous study (25). For class III β-tubulin expression, we evaluated the proportion and intensity of the immunoreactive cells following the protocol used to evaluate estrogen/progesterone receptors in breast cancer, proposed by Allred et al. (26, 27). Cases with a total score of ≥7 were regarded as positive.

**Statistical analysis.** Statistical analysis was conducted for OS and PFS to examine the effects of MAP4, stathmin, and β-tubulin III on taxane efficacy. Product-limit estimators of survival functions were obtained, respectively, relative to positivity and negativity of each marker in the patients to investigate the relationship between regimens and markers. To adjust for possible confounding factors, Cox proportional hazards models were applied. The covariates considered were a treatment indicator (0, taxane-free regimen; 1, taxane-based regimen), marker (0, negative; 1, positive), their interaction, age, two dummy variables representing FIGO stage and peritoneal cytodiagnosis (FIGO stage I-II with peritoneal cytodiagnosis negative, FIGO stage I-II with peritoneal cytodiagnosis positive, and FIGO stage III-IV) and size of residual tumor (0, <1 cm; 1, ≥1 cm).

Taking into account the size of the dataset, the latter four covariates were summarized into a propensity score (28, 29) by fitting logistic regression models with those variables to the data. The primary interest

**Table 1.** Correlation between positive or negative expression of MAP4, stathmin, and β-tubulin III and tumor stage or residual tumor

| FIGO stage | Residual tumor |
|------------|---------------|
| I/II (n = 67) | III/IV (n = 27) | No (n = 74) | Yes (n = 20) |
| No. of patients (%) | No. of patients (%) | No. of patients (%) | No. of patients (%) |
| MAP4 (-) | 36 (54) | 12 (44) | 39 (53) | 9 (45) |
| MAP4 (+) | 31 (46) | 15 (56) | 35 (47) | 11 (55) |
| Stathmin (-) | 29 (43) | 11 (41) | 33 (45) | 7 (35) |
| Stathmin (+) | 38 (57) | 16 (59) | 41 (55) | 13 (65) |
| β-tubulin III (-) | 30 (45) | 11 (41) | 33 (45) | 8 (40) |
| β-tubulin III (+) | 37 (45) | 16 (59) | 41 (55) | 12 (60) |
was the effect of the interaction between treatment and marker. With the supposition that the effect of a taxane-based regimen for marker-negative patients equals $A$ and that of the taxane-free regimen for positive patients equals $B$, the significance of the interaction shows that the effect of the taxane-based regimen for the marker-positive patients is greater than $A + B$ (i.e., it is synergistic). Evidence of a synergistic effect indicates that the effect of taxane is dependent on the status of the marker, showing the marker plays an important role in the effect of taxane. The cutoff points that determined positive and negative for each marker were chosen by the Akaike’s information criterion so that the Cox model fitted best to the data (30).

**Results**

Effects of reducing expression of β-tubulin isoforms, MAP4, and stathmin on drug sensitivity to paclitaxel and cisplatin in ovarian cancer cells. We first examined whether gene silencing of
β-tubulin isoforms, MAP4, and stathmin could affect drug sensitivity to paclitaxel and cisplatin in the cultured human ovarian cancer cell lines SKOV-3 and OVCAR-3. Cellular mRNA expression levels of these genes in two human ovarian cancer cell lines were all markedly down-regulated when treated with respective siRNA (Fig. 1A). We then examined the drug sensitivities of paclitaxel or cisplatin in ovarian cancer cells treated with siRNA of the tubulin isoforms, MAP4, and stathmin (Fig. 1B). When β-tubulin III or β-tubulin IV was silenced, the IC50 values of paclitaxel increased to 16.8 nmol/L and 14.3 nmol/L, respectively, from the control IC50 value of 3.9 nmol/L in SKOV-3 cells (Fig. 1B). By contrast, down-regulation of β-tubulins I and II, MAP4, and stathmin did not influence the sensitivity to paclitaxel in SKOV-3 cells. Down-regulation of β-tubulins I, II, III, and IV, MAP4, and stathmin did not influence sensitivity to cisplatin in either cell line (Fig. 1B). Two independent experiments consistently showed the acquisition of drug resistance to paclitaxel in SKOV-3 by knockdown of β-tubulins III and IV.

**Immunohistochemistry of MAP4, stathmin, and β-tubulin III in human ovarian clear cell adenocarcinomas.** Clinical and pathologic characteristics at diagnosis are summarized in Supplementary Table S1. The median age of the patients was 52 years (range, 29-74 years). Sixty tumors were considered to be stage I, 7 stage II, 20 stage III, and 7 stage IV. Sixteen patients who had residual tumors more than 1 cm in maximum diameter were classified into the suboptimal group, whereas 78 patients were placed in the optimal group with a residual tumor ≤1 cm, including 74 complete resections. The median follow-up for PFS for all 94 patients was 749 days (range, 23-8,318 days), whereas the median follow-up for OS was 995 days (range, 23-8,318 days). The median follow-up of those patients who are currently progression-free is 2,399 days (range, 212-8,318 days).

The cytoplasmic positive expression of MAP4 was detected in 46 tumors (49%). Positive immunostaining for stathmin was found in 54 tumors (57%), predominantly as cytoplasmic staining (Fig. 2A). β-Tubulin III immunostaining was positive in 53 (56%) tumors with total scores of 7 or 8 (Fig. 2C). Positive MAP4 and β-tubulin III expression was frequent in tumors treated with taxane-containing regimens, compared with tumors treated with taxane-free regimens (Supplementary Table S1). Stathmin-positive tumors were also more frequent in patients with the taxane-based regimen, although the difference failed to reach statistical significance. There were no measurable differences in immunoreactivities for these proteins with respect to either tumor stage or residual tumor (Table 1).

**Effects of β-tubulin III expression on survival in human ovarian clear cell adenocarcinomas.** In Fig. 3A, the product-limit estimators for OS of patients administered the taxane-free and taxane-based regimens are shown for the MAP4-negative group (left panel) and for the MAP4-positive group (right panel). The survival outcome seemed to be less favorable for the taxane-based regimen than for the taxane-free regimen in the MAP4-negative group, although the difference was not statistically significant ($P = 0.23$); there was no difference in survival between the two regimens in the MAP4-positive group ($P = 0.38$). Paclitaxel treatment was also associated with a poorer survival in the stathmin-negative patients ($P = 0.03$); there was a trend to a better survival in the group of stathmin-positive patients ($P = 0.12$), as shown in Fig. 3B.

Survival associated with paclitaxel treatment was more evidently differential based on β-tubulin III status. In the absence of β-tubulin III expression, survival was significantly shorter in patients with the taxane-based regimen compared with those with the taxane-free regimen ($P = 0.04$), and the opposite was the case in the presence of β-tubulin III expression ($P = 0.09$; Fig. 3C). Table 2 gives the estimates, confidence intervals, and $P$ values for the hazard ratios of the interaction. The table shows that for β-tubulin III, $P$ values were 0.026 for OS and 0.030 for PFS. Thus, β-tubulin III seems to determine the efficacy of the taxane-based regimen. Table 2 also shows that for stathmin, $P$ was 0.135 and the hazard ratio was 0.25 (95% confidence interval, 0.04-1.53) for OS, and 0.288 and 0.43, respectively (95% confidence interval, 0.09-2.06), for PFS.

### Table 2. Summary of interaction terms for the Cox regression

| Marker       | MAP4   | Stathmin | β-tubulin III |
|--------------|--------|----------|---------------|
| OS           | -0.77  | -1.37    | -1.68         |
| Regression coefficient (95% CI) | (-2.50 to 0.96) | (-3.17 to 0.43) | (-3.16 to 0.21) |
| $P$           | 0.383  | 0.135    | 0.026         |
| PFS          | -0.40  | -0.85    | -1.52         |
| Regression coefficient (95% CI) | (-1.91 to 1.12) | (-2.43 to 0.72) | (-2.90 to -0.15) |
| $P$           | 0.608  | 0.288    | 0.030         |

Abbreviation: 95% CI, 95% confidence interval.

### Table 3. Hazard ratios (marker positive-marker negative) for subpopulations of taxane-based regimen and taxane-free regimen by the Cox proportional hazards model; two-tailed 95% confidence intervals are given in parenthesis

| Marker hazard ratio (95% CI) | Taxane-based therapy | Taxane-free therapy | $P$ |
|-----------------------------|----------------------|---------------------|-----|
| Overall survival             |                      |                     |     |
| MAP4                        | 0.42 (0.11-1.66)     | 0.91 (0.35-2.40)    | 0.383|
| Stathmin                    | 0.96 (0.26-3.53)     | 3.78 (1.07-13.34)   | 0.135|
| β-tubulin III               | 0.72 (0.22-2.44)     | 3.91 (1.49-10.23)   | 0.026|
| Progression-free survival   |                      |                     |     |
| MAP4                        | 0.53 (0.17-1.69)     | 0.79 (0.31-2.02)    | 0.608|
| Stathmin                    | 1.11 (0.36-3.41)     | 2.60 (0.85-7.96)    | 0.288|
| β-tubulin III               | 0.77 (0.26-2.31)     | 3.52 (1.37-9.01)    | 0.030|

NOTE: Age, FIGO stage, peritoneal cytodiagnosis, and size of residual tumor were adjusted by the propensity scores representing the four covariates.

* $P$ values are based on Wald tests for interaction of taxane with the marker.
Thus, stathmin may also determine the efficacy of the taxane-based regimen, but the effect was not statistically significant. Furthermore, Table 2 shows that for MAP4, the estimated hazard ratios were far from 1 but were not statistically significant (0.383 for OS and 0.673 for PFS).

The statistical significance of the interaction of taxane with β-tubulin III shown in Table 2 indicates that the efficacy of taxane depends on β-tubulin III positivity or negativity. To interpret this interaction precisely, we give the hazard ratio of the taxane-based regimen relative to the taxane-free regimen separately for β-tubulin III–positive and -negative patients. Table 3 gives the hazard ratios for patients who were positive for β-tubulin III relative to patients who were negative; these ratios are given separately for the taxane-based and taxane-free regimens. The table shows that the hazard ratio for OS was 3.91 for the taxane-free regimen but was 0.72 for the taxane-based regimen. This outcome indicates that being positive for β-tubulin III is related to a poor prognosis in the taxane-free regimen group, but that the taxane-based regimen may prolong OS for patients who are β-tubulin III–positive.

**Discussion**

Class III β-tubulin overexpression has been reported to be a marker of poor clinical outcome in patients with advanced ovarian cancer mainly containing serous type adenocarcinoma. With treatment using platinum/paclitaxel therapy (16), expression of class III β-tubulin also predicts response and outcome in patients with non–small cell lung cancer and in those with breast cancer who are treated with taxane-based chemotherapy (31, 32). In this study, we investigated which targets could be responsible for the therapeutic efficacy of taxane-based chemotherapy against ovarian clear cell adenocarcinoma patients when treated with either cisplatin/cyclophosphamide or cisplatin/paclitaxel. Immunohistochemical staining was done for the surgically resected specimens using antibodies against class III β-tubulin, MAP4, and stathmin. Of these three targeting molecules, expression of class III β-tubulin was significantly associated with therapeutic efficacy of taxane-based chemotherapy, but not with taxane-free chemotherapy. Moreover, our present study showed that increased expression of class III β-tubulin significantly affected outcome for patients with ovarian clear cell adenocarcinoma in the taxane-treated patient group.

Our present finding is not consistent with those of previous studies identifying a close association of class III β-tubulin overexpression with poor therapeutic efficacy of taxane-based chemotherapy against ovarian cancers, including most non–small cell adenocarcinomas (14–16). Of β-tubulin isoforms, microtubules containing tubulin III or IV were more dynamic and less stable than microtubules containing other tubulin types (13, 33), suggesting that cellular expression of β-tubulin isotype III or IV plays a critical role in drug sensitivity to paclitaxel in vitro. Paclitaxel-selected drug-resistant cancer cell lines derived from human lung, breast, pancreas, and prostate cancers and glioblastoma often exhibit enhanced expression of β-tubulin III (34). Kavallaris et al. have previously reported increased mRNA expression of β-tubulins III and IV in taxane-treated ovarian tumor samples as compared with primary untreated ovarian tumors (35). However, Nicolletti et al. have reported no correlation between tubulin expression and paclitaxel sensitivity in mouse xenografts of human ovarian carcinomas (36).

In our present study, knockdown of class III and IV β-tubulin genes but not of other tubulin isoforms specifically decreased drug sensitivity to paclitaxel in one ovarian cancer cell line, indicating the possible involvement of these tubulin isoforms in the dynamics of microtubules. At present, it remains unclear why decreased expression of type III β-tubulin differentially modulates drug sensitivity to paclitaxel among various cancer cell lines in vitro, and this finding requires further study. A complex network system among microtubule-related factors, including tubulin isoforms, operates in limiting drug sensitivity to taxanes; however, the results of our present study together with those of previous reports could present a novel notion that expression levels of class III β-tubulin might thus predict the therapeutic efficacy of taxane-based therapy. This effect would depend on differences in pathologic subtype between serous adenocarcinoma and clear cell adenocarcinoma.

We also found that OS of patients with lower expression of MAP4, stathmin, and β-tubulin indicated better therapeutic efficacy with non–taxane-based chemotherapy compared with taxane-based treatment. In patients with higher expression of stathmin and MAP4, these relationships were reversed but not statistically significant. Although these appeared during follow-up periods of the taxane-based therapy group for as long as 3,000 days, low expression of these three targeting molecules might predict poor prognosis for patients with ovarian clear cell adenocarcinoma.

Altered expression of proteins that regulate microtubule dynamics also mediates paclitaxel resistance in cancer cells in vitro through interaction with tubulin dimers or polymerizing microtubules. These proteins include stathmin, a microtubule destabilizer, and MAP4, a microtubule stabilizer (34). Altered expression of stathmin (5, 6) and MAP-4 (37) induces marked changes in drug sensitivity of cancer cells to taxanes. Further study is required to understand whether the above mechanisms in vitro underlie the poor therapeutic efficacy of taxane-based chemotherapy for patients with low expression of stathmin and MAP4, as well as β-tubulin. On the other hand, increased expression of stathmin also was associated (but not significantly) with an improved therapeutic efficacy of taxane-based chemotherapy in comparison with that of taxane-free therapy. Further study with a larger number of patients as well as longer follow-up periods may predict whether stathmin can be a marker for therapeutic efficacy of taxane-based therapy against ovarian clear cell adenocarcinoma.

In conclusion, our present study showed that overexpression of type III β-tubulin was a predictive marker of better prognosis for patients with ovarian clear cell adenocarcinoma when they are treated with taxane-based chemotherapy. This finding is not consistent with those involving patients with other serous type carcinoma treated by taxane-based chemotherapy, suggesting that association of β-tubulin expression with therapeutic efficacy by taxane-based chemotherapy depends on the pathologic characteristics of ovarian cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
References

1. Jordan MA. Mechanism of action of antitumor drugs that interact with microtubules and tubulin. Curr Med Chem Anti-Canc Agents 2002;2:1–17.

2. Druker S, Kavallaris M. Microtubule alterations and resistance to tubulin-binding agents. Int J Oncol 2002; 21:621–8.

3. Maccioni RB, Cambiazo V. Role of microtubule-associated proteins in the control of microtubule assembly. Physiol Rev 1995;75:835–64.

4. Chapin SJ, Lué CM, Yu MT, Bulinski JC. Differential expression of alternatively spliced forms of MAP4: a repertoire of structurally different microtubule-binding domains. Biochemistry 1995;34:2289–301.

5. Balachandran R, Welsh MJ, Day BW. Altered levels and regulation of stathmin in paclitaxel-resistant ovarian cancer cells. Oncogene 2003;22:8924–30.

6. Ali E, Bash-Babula J, Yang JM, Haid WN. Effect of stathmin on the sensitivity to antimitotubule drugs in human breast cancer. Cancer Res 2002;62:6864–9.

7. Schi阚 PB, Fant J, Horwitz SB. Promotion of microtubule assembly in vitro by taxol. Nature 1979;277:665–7.

8. Manfredi JJ, Parness J, Horwitz SB. Taxol binds to cellular microtubules. J Cell Biol 1982;94:688–96.

9. Równisyky EK, Donehower RC. Paclitaxel (taxol). N Engl J Med 1995;332:1004–14.

10. McGuire WP, Hoskins WJ, Brady MF, et al. Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer. N Engl J Med 1996;334:1–6.

11. Piccart MJ, Bertelsen K, James K, et al. Randomized intergroup trial of cisplatin-paclitaxel versus cisplatin-cyclophosphamide in women with advanced epithelial ovarian cancer: three-year results. J Natl Cancer Inst 2000;92:699–708.

12. International Collaborative Ovarian Neoplasm Group. Paclitaxel plus carboplatin versus standard chemotherapy with either single-agent carboplatin or cyclophosphamide, doxorubicin, and cisplatin in women with ovarian cancer: the ICON3 randomised trial. Lancet 2002;360:505–15.

13. Derry WB, Wilson L, Khan IA, Luduena RF, Jordan MA. Taxol differentially modulates the dynamics of microtubules assembled from fractionated and purified β-tubulin isoforms. Biochemistry 1997;36:3554–62.

14. Felini C, Raspaglio G, Mozzetti S, et al. The secotaxane IDN5390 is able to target class III β-tubulin and to overcome paclitaxel resistance. Cancer Res 2005;65:2397–405.

15. Mozzetti S, Felini C, Concilio P, et al. Class III β-tubulin overexpression is a prominent mechanism of paclitaxel resistance in ovarian cancer patients. Clin Cancer Res 2005;11:298–305.

16. Ferrandina G, Zannoni GF, Martinelli E, et al. Class III β-tubulin overexpression is a marker of poor clinical outcome in advanced ovarian cancer patients. Clin Cancer Res 2006;12:2774–9.

17. Scully RE. Tumors of the ovary and maldeveloped gonads. 3rd series. Washington (DC): Armed Forces Institute of Pathology; 1996. p. 141.

18. Seidman JD, Russell P, Kurman RJ. Surface epithelial tumors of the ovary. In: Blaustein A, Kurman RJ. Blaustein's pathology of the female genital tract. 5th ed. New York: Springer-Verlag; 2002. p. 873.

19. Shimizu M, Nakaio T, Toki T, Shiozawa T, Fuji S. Clear cell carcinoma has an expression pattern of cell cycle regulatory molecules that is unique among ovarian adenocarcinomas. Cancer 1998;85:669–77.

20. Ozols RF, Bundy BN, Greer BE, et al. Phase III trial of carboplatin and paclitaxel compared with cisplatin and paclitaxel in patients with optimally resected stage III ovarian cancer: a Gynecologic Oncology Group study. J Clin Oncol 2003;21:3194–200.

21. Sugiyama T, Kamura T, Kigawa J, et al. Clinical characteristics of clear cell carcinoma of the ovary. Cancer 2000;88:2584–9.

22. Pectasides D, Fountzilas G, Aravantinos G, et al. Expression of alternatively spliced forms of MAP4: a repertoire of structurally different microtubule-binding domains. Biochemistry 1995;34:2289–301.

23. Nicoletti MI, Valoti G, Giannakakou P, et al. Advanced stage clear-cell epithelial ovarian cancer: the Hellenic Cooperative Oncology Group experience. Gynecol Oncol 2006;102:285–91.

24. du Bois A, Luck HJ, Meier W, et al. A randomized clinical trial of cisplatin/paclitaxel versus carboplatin/paclitaxel as first-line treatment of ovarian cancer. J Natl Cancer Inst 2003;95:1320–9.

25. International Federation of Gynecology and Obstetrics. Changes in definitions of clinical staging for cancer of the cervix and ovary. Am J Obstet Gynecol 1987;156:236–41.

26. Zhang CC, Yang JM, Bash-Babula J, et al. DNA damage increases sensitivity to vincsa alkaloids and decreases sensitivity to taxanes through p53-dependent repression of microtubule-associated protein 4. Cancer Res 1999;59:3663–70.