Protein Abundance of Class III Beta-tubulin but Not Δ2-Alpha-Tubulin or Tau is Related to Paclitaxel Response in Carcinomas of Unknown Primary Site

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Abstract. *Aim: To determine the prognostic value of microtubule component expression in tumors of patients with carcinomas of unknown primary site (CUP). Patients and Methods: Class III β-tubulin, Δ2-α-tubulin and tau protein were examined immunohistochemically in 51 CUP tumors from patients receiving paclitaxel and compared with their response to treatment. Results: The overall response rate was 18.4% among 49 evaluable patients. Δ2-α-Tubulin and tau were not correlated with response or patient outcome. High class III β-tubulin expression was correlated with both resistance to chemotherapy and shorter overall survival, while there was no relation with progression-free survival. In multivariate analysis taking into account clinical factors, class III β-tubulin expression was independently correlated with overall survival. Conclusion: These findings show that in tumor cells a high level of expression of class III β-tubulin, but not Δ2-α-tubulin or tau, is associated with resistance to paclitaxel and a poor prognosis in CUP patients receiving paclitaxel.*

Cancer of unknown primary site (CUP) represents a group of heterogeneous tumors that share the clinical characteristic of being metastatic epithelial diseases with no identifiable origin at the time of diagnosis and comprise 2-5% of all new cancer cases (1). The prognosis of most CUP is very poor, with a median survival of three to four months in registry studies (2, 3). However, several clinicopathological entities should be singled out because of their better prognosis with treatment (1, 4). These subsets include men with poorly differentiated carcinoma and "extragonadal syndrome" features, women with axillary lymph node metastases or peritoneal adeno-carcinomatosis, or patients with cervical lymph metastases, squamous carcinoma and neuroendocrine carcinomas. Although evidence-based medicine has not defined a standard for the systemic treatment of CUP that do not belong to a specific entity, chemotherapy is generally considered for patients with a good performance status (PS) (0 or 1) (4, 5). Since 1997, several prospective phase II trials have assessed the use of taxanes (paclitaxel or docetaxel) in combination with a platinum compound for the large group of patients who do not fit into any favourable subset (6-9). These studies showed promising results, with median survivals ranging between 8 and 13 months, and one-year survival ranging between 15% and 29%. These results led many authors to consider combination regimens with platinum and taxanes as the standard of care. Study of mechanisms of resistance to taxanes are therefore of particular interest in CUP.

The molecular targets of taxanes, microtubules, are complex polymers consisting of tubulin dimers (containing one α-tubulin and one β-tubulin molecule) to which these compounds bind, and a variety of tubulin-associated proteins (10). In humans, α- and β-tubulin exist in the form of tubulin isotypes, which mainly differ in their C-terminal sequences. Microtubules are susceptible to post-translational alterations, including glutamylation and phosphorylation of β-tubulin and detyrosination of α-tubulin into Δ2-α-tubulin.

Abbreviations: CUP, Carcinomas of unknown primary site; NSCLC, non-small cell lung cancer; OS: overall survival; PS, performance status; PFS, progression-free survival.

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Several recent studies suggested that class III β-tubulin overexpression, one of the seven β-tubulin isotypes, may have clinical relevance (11). High expression of class III β-tubulin has been found to be correlated with low response rates in patients treated with taxane/vinorelbine-containing regimens and with reduced survival in patients with various types of cancers (11). In previous study, we showed by immunochemistry that high expression of class III β-tubulin in tumor cells from 47 non-small cell lung cancer (NSCLC) patients receiving a taxane-based regimen was predictive of poor response to therapy and poor patient outcome (12). We recently showed by immunochemistry that high expression of class III β-tubulin in tumor cells from 40 CUP patients receiving a taxane-based regimen was predictive of poor response to therapy and poor patient outcome (13). We previously reported that high Δ2-α-tubulin expression was associated with a shorter overall survival in 93 patients receiving vinorelbine-based chemotherapy for locally advanced or metastatic NSCLC (14). However, the prognostic value disappeared when other prognostic factors were considered.

Other targets, such as tubulin-associated proteins may also be involved in taxane resistance. Microtubule associated proteins (MAP) constitute a complex family of proteins, including tau protein, MAP4 and STOP proteins, many of which have been shown to regulate tubulin polymerization and function (10). Among these proteins, tau is of particular interest. Tau protein promotes tubulin polymerization and stabilizes microtubules. Two studies have shown that an absence of tau was an independent predictor of complete pathological response in breast cancer patients (15) and clinical response in gastric cancer patients (16) receiving paclitaxel.

Accordingly, we conducted a study to assess the predictive value of microtubule component expression in tumors of CUP patients. In order to confirm the results of our previous study and to assess the value of tau protein and Δ2-α-tubulin expression, we conducted a retrospective study of pre-treatment tumor samples of 52 patients with CUP subsequently treated with taxane-based regimens.

Patients and Methods

The analysis was performed on samples from 52 patients treated with paclitaxel-based chemotherapy. Forty patients were part of a first preliminary study performed at the Medical Oncology Department at the Cross Cancer Institute in Edmonton (Canada) and 12 patients were treated between January 1999 and December 2005 at one Medical Department of the Hospices Civils de Lyon (France). Patients were excluded who had an obvious primary tumor identified at the time of their initial visit, or had an unknown primary cancer of non-epithelial origin. Patients were also excluded from the present study if they had any of the following features requiring well-defined treatments: women with adenocarcinoma that involved only axillary nodes, women with primary papillary serous carcinoma of the peritoneum and patients with cervical lymph nodes containing squamous carcinoma. All patients met entry criteria and had adequate tumor biopsy specimens obtained before chemotherapy. The characteristics of the patient population are listed in Table I. Their median age at diagnosis was 58.6 years (range, 25-80 years). All patients were treated with carboplatin and paclitaxel, alone or in combination with etoposide. The median follow-up of the 52 patients, measured from the onset of chemotherapy, was 100.5 days (range, 10-1,443 days).

Table I. *Characteristics of 52 carcinomas of patients with unknown primary site.*

Total no. of patients	52
Gender	
Male	20
Female	32
Age, years	
Median	58.5
Range	25-80
Histology	
Adenocarcinoma	30
Poorly differentiated adenocarcinoma or carcinoma	16
Undifferentiated carcinoma	5
Neuroendocrine features	1
Clinical features	
Node involvement	32
Liver involvement	21
Pleura involvement	6
Lung involvement	11
Bone involvement	18
Number of metastases	
1	17
≥2	35
Performance status	
0-1	32
≥2	20
Lactate dehydrogenase levels*	
≤1	18
>1	29
Chemotherapeutic regimen	
Carboplatin + paclitaxel	16
Carboplatin + paclitaxel + etoposide	36

*Five missing data.

Chemotherapy. The platinum-based regimen was paclitaxel 175 mg/m^2 plus carboplatin dosed with an area under the curve of 5 on day 1 of a 21-day cycle, or paclitaxel 175 mg/m^2 plus carboplatin dosed with an area under the curve of 6 on day 1 plus oral etoposide on day 1 to 10 of a 21-day cycle (6). All but two patients were evaluated for response. Standard response criteria were used to evaluate response to chemotherapy (17). Complete response was defined as the disappearance of all signs of disease both at clinical examination and on CT scan. Partial response was defined by a reduction of more than 50% in the sum of products of the largest perpendicular diameters of all tumor localizations, with no new tumor lesions. Stable disease was defined by a less than 50% decrease or a less than 25% increase in tumor size. Tumor

Table II. *Immunohistochemical methods used in this study.*

Marker	Dilution	Time	Antigen retrieval
Class III β-tubulin	1/400	32 min	EDTA, pH 8, 45 min
Anti-τ protein	1/50	32 min	EDTA, pH 8, 45 min
Anti-Δ2-α-tubulin	1/1000	32 min	EDTA, pH 8, 45 min

progression was defined as an increase in the size of tumor lesions by more than 25% or the appearance of a new lesion. The response rate was defined as the total of the complete response cases and partial response cases expressed as a percentage of all the evaluable cases. Overall survival (OS) was calculated as the time between the beginning of chemotherapy and death or last follow-up. Progression-free survival (PFS) was calculated as the time between the beginning of chemotherapy and the date of tumor progression or last follow-up.

Histopathological analysis. Immunohistochemical analyses were performed on paraffin-embedded sections of surgical samples obtained before therapy. Samples were obtained by liver biopsy in 17 cases, by node biopsy in 19, by peritoneal biopsy in 4, by bone marrow biopsy in 2, by skin biopsy in 3, by brain biopsy in 3, and one case each by bowel biopsy, pelvic mass and adrenal gland biopsy. The antibodies used (Table II) were directed against class III (clone TUJ1) tubulin isotypes (produced by Anthony Frankfurter, University of Virginia, Charlottesville, VA, USA), Tau-1 protein (clone PC1C6) (produced by Sigma, USA), and Δ2-α-tubulin (produced by Laurence Lafanéchère, CEA, Grenoble, France). All molecular markers were stained using an automated immunohistochemical stainer (NexES, Ventana Medical Systems, Illkirch, France) using 5-μm-thick tissue sections following routine deparaffination, rehydration and appropriate antigen retrieval. Chromogenic detection was performed with 3,3-diaminobenzidine (DAB). All of the slides were examined and scored by the same pathologist (SI) without knowledge of the patient data. Scoring for class III β-tubulin was based on relative intensities of staining of the tumor with reference to the normally strong class III β-tubulin nuclear or cytoplasmic staining within the endothelial cells (14). The internal references were then used as internal positive controls between slides and samples as well as for the staining procedure. Scores ranged from 0 (no staining) to 2+ (at least equal to endothelial cells). Only cells with a score of 2+ cells were considered positive. For correlations with patient outcome, samples were then scored as low expression (50% or fewer positive cells) or high expression (more than 50% positive cells). This cutoff was prospectively defined prior to any data analysis, based on that derived from our previous studies (12, 13). For Δ2-α-tubulin, nerves and blood vessels were used as control for staining and scoring. The previously reported IHC scoring was followed: 1, fewer than 50% positive cells; 2, more than 50% positive cells (14). For tau protein, sections of normal breast were used as external reference. The previously reported IHC scoring was used: 0 no staining; 1+, less staining than normal; 2+, similar to normal epithelium; 3+, uniform staining more intense than normal cells. In accordance with previous studies, cases with 0 or 1+ staining were considered tau-negative, and tumors with 2+/3+ were considered tau-positive (15, 16).

Statistical analysis. Univariate associations between immunohistochemical expression and the clinical variables were examined using the chi-square test (or Fisher's exact test, as appropriate). Survival curves were estimated by the Kaplan-Meier method, and differences in PFS and OS between groups were compared using the log-rank test. The Cox proportional hazards model was used for multivariate analysis to adjust the observed predictive value of the expression of tubulin components for the influence of various prognostic factors. All explanatory variables with p values <0.1 were eligible to enter the final model. $P<0.05$ was considered as significant. All statistical analyses were performed using SPSS.12 (Chicago, IL, USA).

Results

Immunohistochemical data. The expression patterns of these markers were highly variable with a staining of 0% to 100% of the cells for class III β-tubulin and 10% to 70% of the cells for anti-Δ2-α-tubulin. An example of immunohistochemical staining with anti-class III β-tubulin antibody is shown in Figure 1. Results of immunostaining of tumor samples are summarized in Table III. Results varied markedly among CUP samples, both for relative intensities of staining and percentage of immunoreactive cells. No significant associations were found between class III β-tubulin, tau or Δ2-α-tubulin abundance and patient characteristics (age [≥58.5 years *vs*. <58.5 years]; gender [male *vs*. female]; histological subtype [adenocarcinoma *vs*. other]; liver, node, pleura, bone and lung involvement; number of metastases [>1 *vs*. 1]; PS [PS2-4 *vs*. 0-1]; lactate dehydrogenase levels [>1 *vs*. normal]).

Patient outcome. Response was evaluable in 49 paclitaxel-treated patients. Two patients had complete responses while 7 patients had partial responses, yielding an overall response rate of 18.4% . Fifteen patients had a stable disease (30.6%) and 24 patients progressed on therapy (48.9%). Five patients received radiotherapy after the completion of chemotherapy, while 5 patients received radiotherapy before the onset of chemotherapy. The median OS and PFS were 105.5 and 81 days, respectively, in the entire patient population, and 731 days and 365 days, respectively, in responding patients. Thirty-five patients died during follow-up and 5 were lost to follow-up.

Microtubule component expression and response to treatment. Expression levels of microtubule components were not found to be associated with the response rate to chemotherapy (Table III). However, a statistically significant up-regulation of class III β-tubulin was found in the resistant subset, defined as disease progression under treatment. Patients with high class III β-tubulin expression were more resistant to chemotherapy (69.6% progression rate in patients with more than 50% of stained cells *vs*. 30.4% in patients with fewer than or equal to 50% ; p=0.007). None

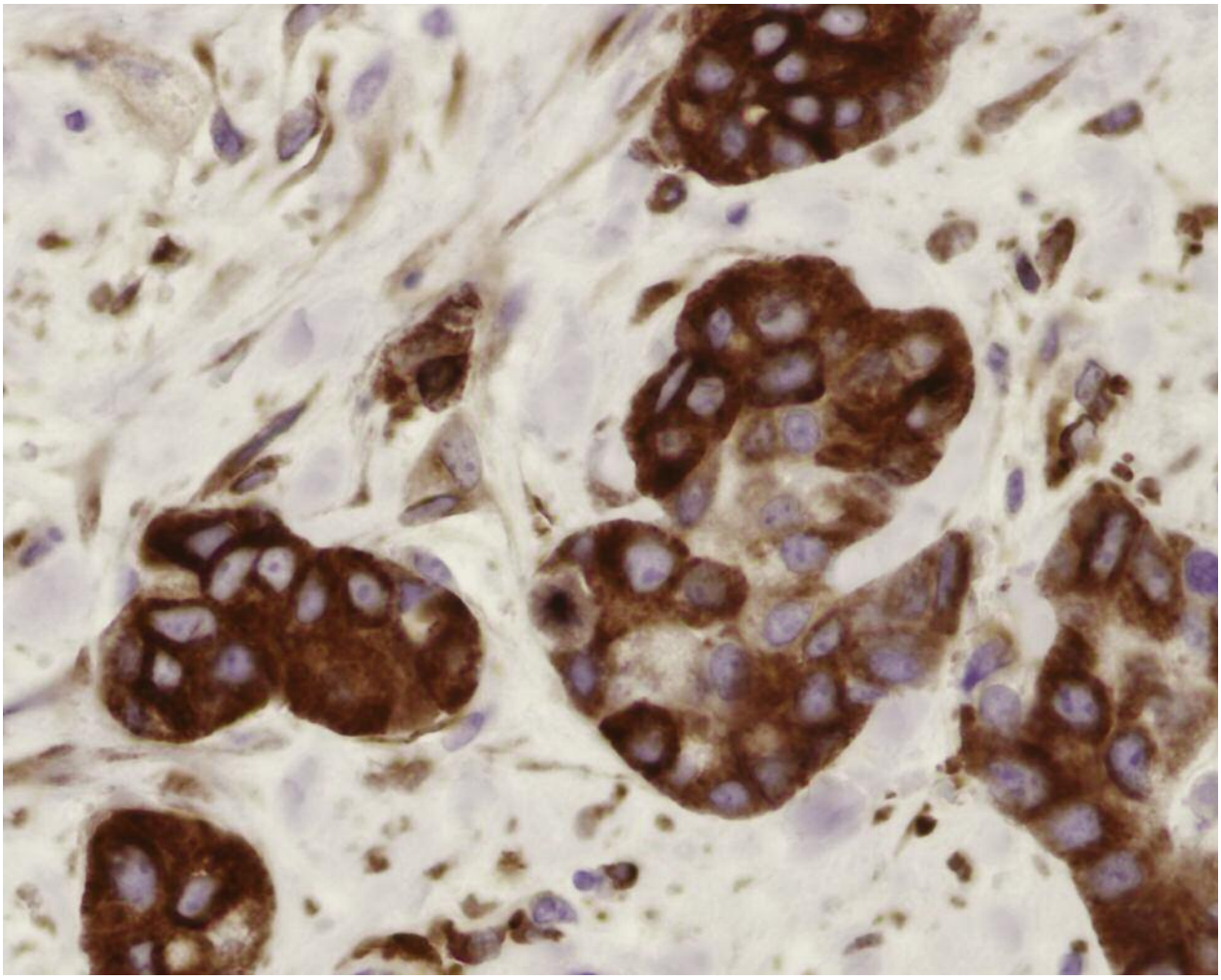

Figure 1. *CUP adenocarcinoma metastasized to a node strongly stained with anti-class III β-tubulin antibody (x10). Ninety percent of tumor cells were positive for class III β-tubulin.*

of the patient characteristics (age, gender, histological subtype, organ involvement, number of metastases, PS and lactate dehydrogenase levels) were found to correlate with response to chemotherapy (data not shown).

Microtubule component expression and survival. No relationship was found between microtubule component expression and PFS. Among patient characteristics, only poor PS (p=0.01), bone metastases (p=0.04) and high lactate dehydrogenase levels (p=0.002) were associated with shorter PFS. A high level of expression (more than 50% *vs.* fewer than or equal to 50% of positive cells) of class III β-tubulin protein was correlated with a shorter median overall-free survival of 101 days *vs.* 381 days in patients with low class III β-tubulin expression (p=0.02, Figure. 2). Δ2-α-tubulin and tau levels were not found to be associated with patient outcome. Among patient characteristics, poor PS (p=0.01), liver involvement (p=0.03) and bone involvement (p=0.004) were associated with shorter survival.

Multivariate analysis of overall survival. Multivariate analysis was performed by using the Cox proportional hazards model to determine the prognostic value of class III β-tubulin when other prognostic factors were considered. The multivariate analysis that included PS, bone involvement, liver involvement and class III β-tubulin, showed that poor PS (2-4), bone involvement and high class III β-tubulin expression were independent variables correlated with shorter OS (p=0.004, 0.017 and p=0.014, respectively). A high class III β-tubulin level yielded a hazard ratio of 2.22, with a 95% confidence interval ranging from 1.17 to 4.18.

Table III. *Relationship between expression of microtubule components, response, progression on therapy and survival in 52 CUP patients treated with a paclitaxel-based regimen.*

	N	Response rate* (%)	*p*	Progression rate (%)	*p*	PFS (days)	*p*	OS (days)	*p*
Tau†									
Low expression	21	23.8	0.28	45.8	0.28	149	0.47	218	0.97
High expression	24	21.8		54.2		125		197	
Class III β-tubulin*									
≤50%	26	26.9	0.23	30.4	0.007	234	**0.177**	381	**0.02**
>50%	23	13		69.6		94		101	
Δ2-α-tubulin*									
≤50%	32	21.9	0.72	47.1	0.69	115	0.93	186	0.98
>50%	17	17.6		52.9		177		218	

PFS: progression-free survival; Progression: progression on therapy; OS: overall survival. †Data missing for 5 patients, *2 patients.

Discussion

This is the first study to explore the predictive value of the level of protein abundance of Δ2-α-tubulin and tau in CUP tumor samples prior to treatment with a paclitaxel-based regimen. Tau protein promotes tubulin polymerization and stabilizes microtubules. Tau is able to bind to both outer and inner surfaces of microtubules, and it may bind to the same inner-surface pocket as paclitaxel (18). Rouzier *et al*. reported decreased paclitaxel binding and reduced microtubule polymerisation in breast cancer cells after preincubation of tubulin with tau protein (15). In contrast to these authors (15) and to Mimori *et al*. (16), who showed that reduced tau expression was associated with response to paclitaxel in treated breast cancer patients and in gastric cancer patients, respectively, we did not find any relationship between tau expression and response to paclitaxel or patient outcome. A possible explanation for these discrepant results is that gastric and breast cancers are very infrequent sources of CUP in necropsy series (19). Δ2-α-tubulin, resulting from the loss of the two terminal tyrosine residues of α-tubulin has been shown to be associated with a prolonged microtubule half-life *in vitro* or "stable" microtubules (20). We have previously shown that high Δ2-Tubulin expression was associated with a shorter overall survival in 93 patients receiving vinorelbine-based chemotherapy for locally advanced or metastatic NSCLC (14). However, the prognostic value disappeared when other prognostic factors were considered. In this study, we were not able to demonstrate any prognostic value for Δ2-α-tubulin expression.

In this study, we found that high class III β-tubulin expression was correlated with resistance to chemotherapy, defined as disease progression under treatment. Patients whose tumors expressed high levels of class III β-tubulin isotype had shorter overall survival while there was no relation with PFS. Multivariate analysis showed that class III β-tubulin expression was independently correlated with overall survival. Δ2-α-tubulin and tau expression were not related to patient outcome. These results confirm our previous study in 40 CUP patients receiving taxane-based regimens (13).

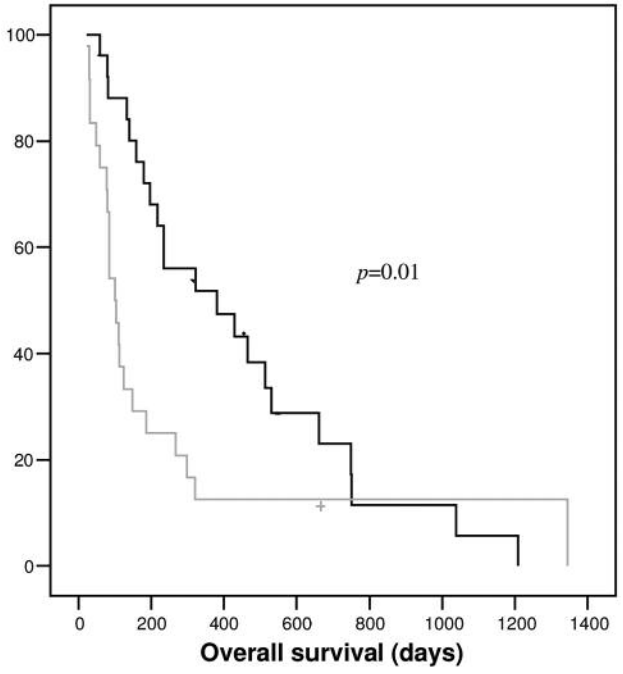

Figure 2. *The overall survival curves for 52 patients with carcinomas of unknown primary, according to β-tubulin III expression.*

Many preclinical studies have shown that high levels of class III β-tubulin expression are associated with paclitaxel resistance in human cancer cell lines (for lung cancer (21), ovarian cancer (22), prostate cancer (23) and breast cancer

(21), and with docetaxel resistance in human pancreatic cancer cell lines (24). The mechanistic involvement of these alterations in the determination of resistance remains open to debate. Current hypotheses are that these alterations may alter drug binding to the tubulin dimer (25), or, alternatively, that the microtubule contained in the tumor cells may have different intrinsic dynamic properties and thus may be less sensitive to antitubulin agents (26, 27). It has been shown, in a study comparing the dynamic properties of microtubules composed of $\alpha\beta_{II}$, $\alpha\beta_{III}$ or $\alpha\beta_{IV}$ dimers, that the dynamics of $\alpha\beta_{III}$ microtubules were less sensitive to taxanes (28). Class III β-tubulin reduces the polymerization rate of microtubules, thereby overcoming microtubule polymerization by paclitaxel (29). Using an antisense approach, Kavallaris *et al*. showed that the reduction of class III β-tubulin content allowed *in vitro* sensitization to tubulin binding agents (30). Using the RNA interference technique, the same authors recently showed that class III β-tubulin expression could mediate the response to microtubule stabilization, and for the first time, DNA damage in two NSCLC cell lines (31). The authors hypothesized that the enhancement of the effect of tubulin-binding agents and DNA-damaging agents by down-regulation of class III β-tubulin is through regulation of gene expression, *e.g*. for genes that are involved in apoptosis, DNA repair pathway, or mitosis. Recently, Kamath *et al*. showed that overexpression of class III β-tubulin induces paclitaxel resistance by reducing the ability of paclitaxel to suppress microtubule dynamics (32). Using an tetracycline-inducible expression system of class III β-tubulin, they showed that in the presence of paclitaxel dynamic instability was suppressed to a significantly lesser extent in cells overexpressing class III β-tubulin than in cells overexpressing class I β-tubulin, whereas, in the absence of paclitaxel, there were no differences in any aspect of dynamic instability in the two cell lines. Thus, fundamental studies support a pivotal role of class III β-tubulin overexpression as a mechanism of tubulin-binding agents resistance.

High expression of class III β-tubulin has been found to be correlated with both low response rates in patients treated with taxane/vinorelbine–containing regimens and with reduced survival in patients with NSCLC, breast, ovarian and gastric cancer (11). Two studies have shown that advanced NSCLC patients receiving paclitaxel whose tumors expressed high levels of class III β-tubulin had a lower response rate and shorter survival, whereas this variable was not found to be predictive in patients receiving regimens without TBA (12, 33). Conversely, analysis of samples from patients in the JBR-10 trial, which compared chemotherapy to no further therapy in operable NSCLC, showed that chemotherapy seemed to overcome the negative prognostic effect of high class III β-tubulin expression and that the greatest benefit from cisplatin/vinorelbine was observed in patients with high class III β-tubulin expression (34). Further analyses in operable and advanced NSCLC showed a relationship between high class III β-tubulin expression and baseline factors such as: age under 60 years, adenocarcinoma and large cell carcinoma histologies, and advanced stage (11). These results suggest that class III β-tubulin could be both a prognostic and a predictive factor, depending on the setting and tumor.

In conclusion, we confirm that high expression of class III β-tubulin is correlated with resistance to paclitaxel chemotherapy and poor clinical outcomes in CUP patients treated with paclitaxel, while tau and Δ2-α-tubulin are not. However, the critical outstanding question is whether class III β-tubulin is simply a *prognostic marker* correlated with disease aggression independently of the type of therapy administered to patients, or a *predictive marker* specifically relevant to those patients receiving compounds directed against microtubules. In order to address this question, we are currently conducting a study of patients with CUP treated with either taxane-based regimens or gemcitabine-based regimens. As has been shown for advanced NSCLC, we expect to demonstrate in CUP patients that expression of low class III β-tubulin correlates with better response and clinical outcome only in case of treatment with tubulin-binding agents.

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