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ORIGINAL ARTICLE

Class III β-tubulin is a marker of paclitaxel resistance in carcinomas of unknown primary site

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Abstract
Purpose In this study, we determine the prevalence and the prognostic value of the class III β-tubulin microtubule protein examined immunohistochemically, in tumors of 40 patients with carcinomas of unknown primary site treated with paclitaxel-based chemotherapy.
Methods Immunohistochemical intensity of staining and percentage of cells were quantified. Clinical characteristics, response to chemotherapy, progression-free survival, and overall survival were assessed for relationships with the expression of class III β-tubulin.
Results The response rate was 17.9% (seven partial responses among 39 valuable patients), while eleven patients had a stable disease (28.2%) and 21 patients progressed on therapy (53.8%). Patients with high class III β-tubulin expression were more resistant to taxane-based chemotherapy, defined as progression under treatment, while patient characteristics were not found to be correlated with response to chemotherapy. Patients whose tumors expressed high levels of class III β-tubulin isotype had shorter overall survival, while there was a trend for an association with progression free survival. Multivariate analysis showed that class III β-tubulin expression was independently correlated with progression free survival and overall survival.
Conclusions These findings suggest that a high level of expression of class III β-tubulin in tumor cells is associated with resistance to paclitaxel and decreased survival in patients with carcinomas of unknown primary receiving paclitaxel-based chemotherapy.

Keywords Cancer of unknown primary · Chemoresistance · Paclitaxel · Tubulin · Tubulin III

Abbreviations

CUP	Carcinomas of unknown primary
OS	Overall survival
PS	Performance status
PFS	Progression-free survival

Introduction

Carcinomas of unknown primary site (CUP) are metastatic malignant epithelial tumours whose primary site cannot be identified during pretreatment assessment [26]. They are characterized by their slow local development and their high metastatic potential. The primary site remains unknown in 20–50% of the patients,

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but the results from autopsies show that the primary tumors are most often located in the lung and pancreas [2, 21].

Prognosis of CUP is poor. Early reports of chemotherapy in these patients documented low rates of response and no effect on the dismal median survival of 3–4 months, thereby strengthening arguments for a nihilist approach [16]. At the end of the 1980s, Greco et al. [14] demonstrated that patients with poorly differentiated carcinoma and "extragonadal syndrome" subsets could benefit from platinum therapy. Since then, several other clinicopathological entities have been identified as determinants of better prognosis and benefit from chemotherapy treatment. These sub-sets of patients include: women with axillary-lymph-node metastasis, women with serous peritoneal adenocarcinomatosis, cervical lymph nodes containing squamous carcinoma and carcinomas with neuroendocrine features. Since 1997, the use of taxane (paclitaxel or docetaxel), in combination with a platinum compound has provided an additional improved treatment option for the large group of patients who do not fit into any favourable sub-set [3, 11, 12, 15]. These studies showed promising results with median survivals ranging from 8 to 13 months and 1 year survival from 29 to 15%. These survival times and rates appear similar to results seen in other patients who have a number of metastatic neoplasms and who are receiving chemotherapy, including visceral dominant advanced breast carcinoma, Stage IIIB and IV non-small cell lung carcinoma, extensive stage small-cell lung carcinoma, advanced soft tissue carcinomas, and advanced pancreatic and gastric carcinomas, among others [12]. 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 and 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 [8]. In humans, α tubulin and β tubulin exist under the form of tubulin isotypes, which mainly differ in their C-terminal sequences. Several recent studies suggest that β-tubulin III overexpression, one of the seven β-tubulin isotypes, may have clinical relevance. First, a small study by Rosell et al. [28], has correlated high class III β-tubulin mRNA levels with poor outcome in NSCLC patients treated with vinorelbine or paclitaxel. In a recent 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 [30]. We also demonstrated that high expression of β tubulin III correlated with poor clinical outcomes in advanced NSCLC patients treated with vinorelbine [29]. Paradiso et al. [25] showed that high class III-(-tubulin immunohistochemical analysis was a relevant tumor biomarker for paclitaxel resistance in 70 advanced breast cancer patients. Using semiquantitative real-time PCR and quantitative immunohistochemistry, Mozzetti et al. [24] studied 41 advanced ovarian cancer patients treated with paclitaxel, and revealed a significant up-regulation of class III tubulin expression at both mRNA and protein levels in those patients who progressed during chemotherapy. Interestingly, a recent clinical study on 62 consecutive patients with unresectable ovarian cancer treated with paclitaxel revealed that cases with high beta III tubulin expression had a worse overall survival than women with low beta III tubulin expression. Multivariate analysis confirmed that high content of beta III tubulin was independently associated with a worse prognosis [9]. An immunohistochemical study of 20 advanced gastric patients treated with docetaxel by Urano et al. [31] showed a significantly higher response rate in patients whose tumors expressed low levels of class III β-tubulin. Together, all of these fundamental and clinical studies suggest that overexpression of β-tubulin III is a possible mechanism of resistance to antitubulin agent therapy in ovarian, breast, gastric and lung cancers.

Accordingly, we conducted a study to assess the predictive value of class III β-tubulin in CUP. In order to confirm the results of the above studies, we conducted a retrospective study of pre-treatment tumor samples of patients with CUP treated with taxane-based regimens in the Medical Oncology department at the Cross Cancer Institute between 1999 and 2003. Using immunohistochemistry, we assessed protein abundance of class III β-tubulin in each of the 40 patients treated with taxanes, and correlated these biological results with patient outcomes.

Patients and methods

The local institutional review board approved this study on samples from patients with CUP, treated with taxanes at the Cross Cancer Institute from January 1, 1999 to December 31, 2003. We identified patients from the Northern Alberta Cancer Registry. We then excluded from further analysis those 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. Fourty patients met all entry criteria and had adequate tumor biopsy specimens obtained before chemotherapy. The clinicopathological characteristics of the patient population are listed in Table 1. Their median age at diagnosis was 59.5 years (range 25–79 years). All the patients were treated with carboplatin and paclitaxel, alone or in combination with etoposide. The median follow-up of the 40 patients, measured from the onset of chemotherapy, was 100.5 days (range 10–1,443 days).

Chemotherapy

The platinum-based regimen was paclitaxel 175 mg/m^2 plus carboplatin dosed with an area under the curve of five on day one of a 21-day cycle, or paclitaxel 175 mg/m^2 plus carboplatin dosed with an area under the curve of six on day one plus oral etoposide on day one to ten of a 21-day cycle [15]. All of the patients were evaluated for response. We used the standard response criteria to evaluate response to chemotherapy [23]. 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 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.

Table 1 Characteristics of carcinoma of 40 unknown primary patients

Total no. of patients	40
Gender	
Male	16
Female	24
Age, years	
Median	59.5
Range	25–79
Histology	
Adenocarcinoma	19
Poorly differentiated adenocarcinoma or carcinoma	16
Undifferentiated carcinoma	4
Neuroendocrine features	1
Clinical features	
Node involvement	25
Liver involvement	18
Pleura involvement	4
Lung involvement	7
Bone involvement	14
Number of metastasis	
1	10
≥2	30
Performance status	
0–1	25
≥2	15
Lactate dehydrogenases levels[a]	
≤1	15
>1	24
Chemotherapeutic regimen	
Carboplatin + paclitaxel	7
Carboplatin + paclitaxel + etoposide	33

[a] One missing data

Histopathological analysis

Immunohistochemical analyses were performed on paraffin-embedded sections of surgical samples obtained before therapy. Samples were obtained by liver biopsy in 14 cases, by node biopsy in 14 cases, by peritoneal biopsy in four cases, by bone marrow biopsy in two cases, by skin biopsy in three cases, by bowel biopsy in one case, by brain biopsy in one case and pelvic mass biopsy in one case. The antibody used was the β-tubulin III isotype (produced by Anthony Frankfurter, Department of Biology, University of Virginia, Charlottesville, VA, USA). Tubulin III was stained using an automated immunohistochemical stainer (NexES, Ventana Medical Systems, Illkirch, France) on 4–5-μm-thick tissue sections following routine deparaffinization, rehydration as previously reported [29]. Antigen retrieval was done on all slides with citrate buffer pH 6.0 in a pressure cooker in amicrowave for 20 min and a cool down for 20 min, then washed in running tap water for 5 min. Slides were then immunostained on a NexES instrument with Avidin and Biotin. Complex method, chromogenic detection was done with 3,3-diaminobenzidine (DAB). A single pathologist (R. Lai), blinded to clinical characteristics and outcomes, assessed and scored the class III β-tubulin immunostaining intensities on a 0–2 + scale and percentage of cells. 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 [29]. 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 less positive cells) or high expression (more than 50% positive cells). This cutoff was prospective defined prior to any data analysis, based on a cutpoint derived from a previous analysis of lung cancer therapy [30].

Statistical analysis

Univariate correlations 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 III for the influence of various prognostic factors. All of explanatory variables with P value <0.1 were eligible to enter the final model. $P < 0.05$ was considered as significant. All statistical analysis was performed using SPSS.12.

Results

Immunohistochemical data

Results of immunostaining of tumor samples are summarized in Table 2. Results varied markedly among CUP samples, both for relative intensities of staining and percentage of immuoreactive cells. No significant correlations were found between class III β-tubulin abundance and patient characteristics (age ≥ 59.5 vs. < 59.5 years; gender male vs. female; histological subtype adenocarcinoma vs. others; liver, node, pleura, bone and lung involvement; number of metastases >1 vs. 1; Performance Status (PS) = 2–4 vs. 0–1; lactate dehydrogenase levels >1 vs. normal). Thirty four tumors had only cytoplamic staining while six tumors had both cytoplasmic and nuclear staining. Characteristic immunohistochemical staining with anti-tubulin III antibody is illustrated in Fig. 1. All negative control slides, prepared without the primary antibody, revealed no appreciable background staining. Tubulin III staining was not found in benign hepatocytes or in mantle zone cells, but was observed at strong levels in reactive germinal center cells (Fig. 2), bile duct cells, endothelial cells (Fig. 3) and nerves.

Table 2 Expression levels of tubulin III by immunohistochemistry according to the intensity score

Patient number	Score 0	Score 1	Score 2	Score
1	100	0	0	Low
2	0	100	0	Low
3	0	0	100	High
4	0	50	50	Low
5	0	90	10	Low
6	0	95	5	Low
7	0	95	5	Low
8	0	0	100	High
9	0	90	10	Low
10	95	0	5	Low
11	0	0	100	High
12	90	0	10	Low
13	0	10	90	High
14	100	0	0	Low
15	0	0	100	High
16	0	40	60	High
17	0	0	100	High
18	0	0	100	High
19	0	0	100	High
20	0	0	100	High
21	0	50	50	Low
22	0	0	100	High
23	0	0	100	High
24	0	100	0	Low
25	0	0	100	High
26	0	0	100	High
27	100	0	0	Low
28	0	20	80	High
29	0	0	100	High
30	0	95	5	Low
31	0	50	50	Low
32	75	20	0	Low
33	0	0	100	High
34	0	100	0	Low
35	0	0	100	High
36	0	0	100	High
37	0	20	80	High
38	0	0	100	High
39	0	40	60	High
40	100	0	0	Low

Results are the percentage of positive cells. Only cells with a score of 2 + cells were considered as positive. For correlations with patient outcome, samples were then scored as low expression (50% or less positive cells) or high expression (more than 50% positive cells)

Patient outcome

Response was evaluable in 39 paclitaxel-treated patients. Seven patients had partial responses, yielding an overall response rate of 17.9%. Eleven patients had a stable disease (28.2%) and 21 patients progressed on therapy (53.8%). Five patients received radiotherapy after the completion of chemotherapy, while five patients received radiotherapy before the onset of

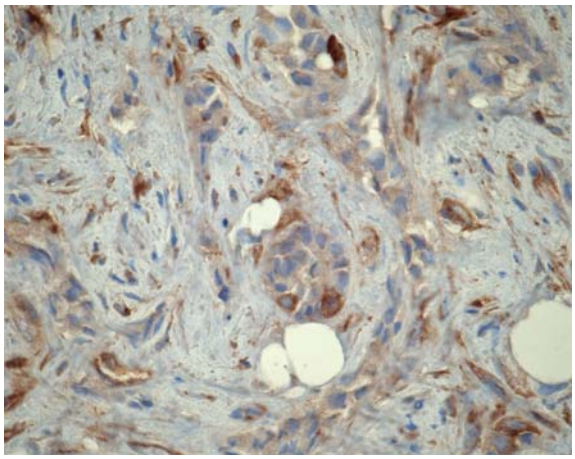

Fig. 1 Adenocarcinoma metastasized to the bone stained with anti-class III β tubulin antibody.Only a few tumor cells were positive (*red arrow*) whereas most of the cells are negative (*black arrow*)

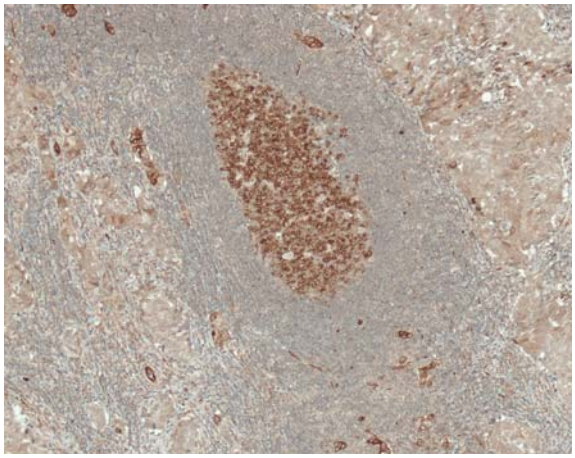

Fig. 2 A lymph node involved by metastatic CUP. Class III tubulin was strongly expressed in the reactive germinal centers (*red arrow*) and in the metastatic tumor cell clusters (*red dash dot arrow*), while benign mantle zone cells were negative (*black arrow*). Most of surrounding tumor cells were negative (*black dash dot arrow*)

chemotherapy. The median OS and PFS were 105.5 and 81 days, respectively, in the entire patient population, and 731 and 365 days, respectively, in responding patients. Thirty-five patients had died during follow-up and five were lost to follow-up (Fig. 4).

Tubulin III expression and response to treatment

When class III tubulin expression was correlated with chemotherapy outcomes, we found a relationship between tubulin III expression and response to treatment, and progression during chemotherapy in patients treated with a paclitaxel-based regimen.

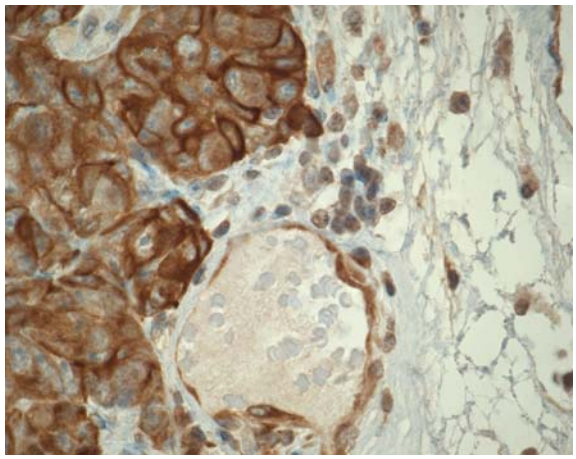

Fig. 3 Poorly differentiated adenocarcinoma metastasized to the peritoneum stained with anti-class III β tubulin antibody (*black arrow*). Class III staining was strongly expressed in endothelial cells which serve as internal positive control (*red arrow*)

Patients whose tumors showed low β-tubulin III expression (≤ 50% vs (50% of positive cells) displayed a higher response rate (33 vs 4.8%; $P = 0.03$). Moreover, we found a statistically significant up-regulation of class III β-tubulin in the primary resistant subset, defined as those who experienced disease progression during treatment. Patients with high class III β-tubulin expression were demonstrated higher rates of primary resistance to taxane-based chemotherapy (81% progression rate in 21 patients with high class III β-tubulin expression vs. 22.2% in 18 patients with low class III β-tubulin expression; $P < 0.001$). Patient characteristics

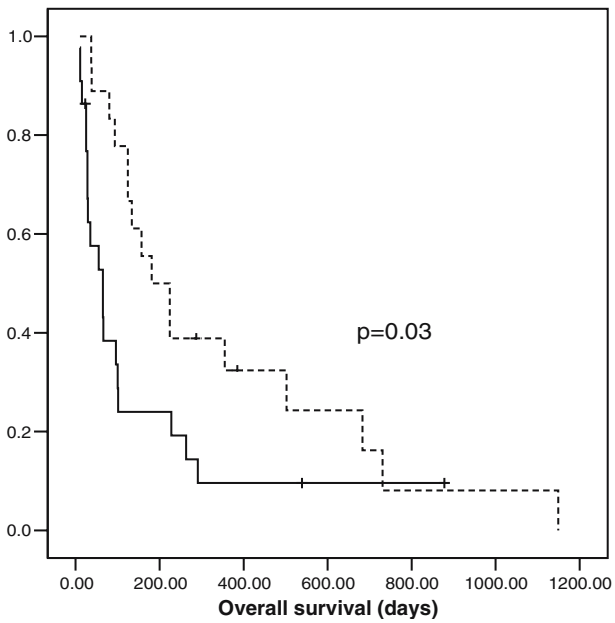

Fig.4 The overall survival curve for 40 patients with carcinomas of unknown primary,according to β-tubulin III expression

(age, gender, histological subtype, organ involvement, number of metastases and lactate dehydrogenase levels) did not correlate with response to chemotherapy (data not shown). However, we found a trend for a relationship between poor PS and resistance to taxane-based chemotherapy (71.3% progression rate in 14 patients with PS 2–4 vs. 44% in 25 patients with PS 0–1; $P = 0.09$).

Microtubule component expression and survival

We found a trend for a relationship between a high class III β-tubulin expression and a shorter PFS. The median PFS was 65 days in patients with high level of class III isotype as opposed to 224 days in patients with low level of class III isotype ($P = 0.06$). Among all patient characteristics only poor PS ($P = 0.035$) and high lactate dehydrogenase levels ($P = 0.046$) were associated with shorter PFS.

Class III β-tubulin expression was significantly associated with OS. The median OS was 202.5 days in patients with low level of class III β-tubulin isotype as opposed to 65 days in patients with high level of class III β-tubulin isotype ($P = 0.043$, Fig. 3). Among all patient characteristics, only a poor PS ($P = 0.005$) was associated with a shorter OS. There was a trend for an association between liver involvement ($P = 0.07$) and bone involvement ($P = 0.07$) with short survival.

Multivariate analysis for progression free survival and overall survival

Multivariate analysis was performed by using the Cox proportional hazards model to determine the predictive and prognostic value of class III β-tubulin when other prognostic factors were considered. The multivariate analysis that included PS, lactate dehydrogenase levels and tubulin class III, showed that high lactate deshydrogenases levels and high level of class β-tubulin isotype were significant independent variables correlated with low PFS ($P = 0.014$ and $P = 0.018$, respectively). A high class III β-tubulin level yielded a hazard ratio of 2.57, with a 95% confidence interval ranging from 1.17 to 5.63. The multivariate analysis that included PS, bone involvement, liver involvement and tubulin class III, showed that poor PS (2–4) and high tubulin class III expression were significant independent variables correlated with low OS ($P = 0.007$ and $P = 0.032$, respectively). A high class III β-tubulin level yielded a hazard ratio of 2.15, with a 95% confidence interval ranging from 1.07 to 4.33.

Discussion

This is the first study to explore the predictive value of the level of protein abundance of β-tubulin class III in CUP tumor samples prior to treatment with a paclitaxel-based regimen. After quantitative analysis of immunohistochemical staining, we found that high class III isotype levels were independently associated with response to treatment and progression of disease during chemotherapy. Moreover, we observed a significant relationship between β-tubulin class III protein abundance and PFS and OS. Because of the limited sample size and the retrospective nature of our study, these results must be interpreted cautiously. Nonetheless, these data suggest that β-tubulin III abundance may relate to important outcomes in CUP patients receiving taxane-based therapy.

These results confirm our earlier study which showed that β-tubulin III abundance protein was predictive of response to therapy and patient outcome in patients with NSCLC receiving paclitaxel-based chemotherapy [30]. Additionally, our results are consistent with other studies identifying an association between resistance to paclitaxel and high class III β-tubulin expression in ovarian cancer [9, 24], breast cancer [25], gastric cancer [31] and in preclinical models of lung cancer [5], ovarian cancer [20], prostate cancer [27], and breast cancer [5]. 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 dimmer [10], or, alternatively, that the microtubule contained in the tumor cells may have different dynamic properties and thus may be less sensitive to antitubulin agents [1, 22]. 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 [7]. Class III β-tubulin reduces the polymerization rate of microtubules, thereby overcoming microtubule polymerization by paclitaxel [17]. Using an antisense approach, Kavallaris et al. [28] showed that the reduction of class III tubulin content allowed in vitro sensitization to tubulin binding agents. Recently, Kamath et al. [18] showed that overexpression of β-tubulin III induces paclitaxel resistance by reducing paclitaxel's ability to suppress microtubule dynamics. Using an expression system of class III β-tubulin upon the control of tetracycline regulatory element, they showed that, in the presence of paclitaxel, dynamic instability was suppressed to a significantly lesser extent in cells overexpressing β-tubulin III than in cells overexpressing β-tubulin I, whereas, in the absence of

paclitaxel, there were no differences in any aspect of dynamic instability in the two cell lines. Thus, both these fundamental and clinical studies support a pivotal role of class III β-tubulin overexpression in paclitaxel resistance.

Evidence-based medicine does not define a standard systemic treatment for CUP site not belonging to a specific clinical entity [4]. Chemotherapy is only considered for patients with a good general health status (WHO PS of 0 or 1) [4]. Since 1997, several studies have shown that a combination of a platinum compound and a taxane, gemcitabine or irinotecan may improve the prognosis of these patients [3, 11–13, 15]. These non-randomized studies with platinum and taxane regimens show median survivals ranging from 8 to 13 months and 1 year survivals from 15 to 29%. Given these data, some authors consider combination regimens with platinum and taxanes the treatment of choice for CUP [26]. Briasoulis et al. [3] showed that carboplatin plus paclitaxel combination is effective in patients with predominantly nodal/pleural metastases of CUP while it offers only limited benefit in patients with liver, bone, or multiple organ involvement.

Our study confirmed the prognostic value of several factors in CUP, where bone involvement and liver metastases, poor performance status and high lactate dehydrogenase levels were independent adverse variables [6]. Most interestingly, our data showed a high association between high expression level of class III β-tubulin and; (1) response to treatment, and (2) resistance to chemotherapy defined as progression under treatment. These relationships were independent of clinical characteristics. Moreover, we found a significant and independent relationship between tubulin III, PFS and OS, underscoring the clinical relevance of this marker for chemotherapy resistance.

In conclusion, we found high expression of β-tubulin III is correlated with of resistance to paclitaxel chemotherapy and poor clinical outcomes in CUP patients treated with paclitaxel. These data suggest that class III β-tubulin could be an independent predictive marker to assist in the selection of appropriate patients and appropriate drugs in the chemotherapeutic management of CUP. Given the widespread ability for clinical laboratories to perform immunohistochemical assessment of formalin-fixed clinical samples, this assay is feasible and practical. Nonetheless, further prospective studies are now warranted to validate this result before β-tubulin III immunohistochemistry can routinely used to guide chemotherapy treatment decisions in patients with CUP.

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