Expression of Class III β-Tubulin Is Predictive of Patient Outcome in Patients with Non–Small Cell Lung Cancer Receiving Vinorelbine-Based Chemotherapy

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Abstract

Purpose: To determine the prevalence and the prognostic value of microtubule component expression in tumors of patients with locally advanced or metastatic non–small cell lung cancer (NSCLC).

Experimental Design: Expression of microtubular components was immunohistochemically examined in 93 tumor samples from untreated patients with stage III and IV NSCLC. All patients received vinorelbine-based chemotherapy. Response to chemotherapy, progression-free survival, and overall survival were correlated with the expression of microtubule proteins.

Results: The response rate was 27.3% (21 partial responses among 77 valuable patients). Although expression of microtubule components was not associated with the response rate, high class III β-tubulin expression was correlated with resistance to vinorelbine, defined as disease progression under treatment. Patients whose tumors expressed high levels of class III β-tubulin isotype had shorter progression-free survival and overall survival ($P = 0.002$ and 0.001, respectively). High Δ2 α-tubulin expression was associated with a shorter overall survival ($P = 0.018$). Tubulin II levels were not found to be correlated with patient outcome. A multivariate analysis, taking into account sex, age, histology, stage, weight loss, and class II β-tubulin, class III β-tubulin, and Δ2 α-tubulin levels, confirmed that class III β-tubulin expression was independently correlated with progression-free survival ($P = 0.04$) and overall survival ($P = 0.012$).

Conclusions: These findings suggest that a high level of expression of class III β-tubulin in tumor cells is associated with resistance to vinorelbine and a poor prognosis in patients with NSCLC receiving vinorelbine-based chemotherapy.

Epidemiologic estimates for the year 2000 showed that lung cancer was one of the most common cancers in the world, both in terms of incidence (with 1.2 million new cases corresponding to 12.3% of the world total of all cancers) and mortality (with 1.2 million deaths corresponding to 17.8% of the world total; ref. 1). Non–small cell lung cancer (NSCLC), which includes the major histologic subtypes, adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, accounts for 80% of all lung cancers. More than half of NSCLCs are advanced stage IIIB or IV at presentation, and patients with advanced NSCLC are candidates for systemic chemotherapy. The standard treatment for stage III unresectable disease (without pleural or pericardial effusion) is a combined-modality therapy with radiotherapy and chemotherapy (2). In metastatic disease, treatment is based on a combination of cisplatin [*cis*-diammine-dichloroplatinum (CDDP)] or carboplatin with one of new active agents including vinorelbine, taxanes (paclitaxel and docetaxel), gemcitabine, and irinotecan (2, 3). These new regimens have produced superior therapeutic results in comparison with single-agent CDDP alone or previous CDDP-based regimens, with a survival benefit of 8 to 10 months (4). Recent randomized studies have shown that the more recent CDDP-based combinations possess similar efficacy although their toxicity profiles may differ (5, 6).

The optimization of currently existing therapies would greatly benefit from the identification of predictive factors guiding the clinician's choice, taking into account both the genetic make-up of the patient and the biological characteristics of disease. Recently, several publications have shown that genome expression in tumor cells and genetic polymorphism of the host may predict response to drugs and patient outcome (7–12). Moreover, pharmacogenetics, the study of genes that influence drug activity and toxicity, seems an interesting way in

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offering the possibility of tailoring therapy to the specific profile of individual patients and tumors.

Antitubulin agents such as taxanes and vinorelbine are widely used in the treatment of patients with NSCLC. The best described mechanism of resistance to tubulin-binding agents is the multidrug resistance phenotype (13). The targets of these compounds, 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 microtubule-associated proteins. Microtubule-associated proteins constitute a complex family of proteins, including τ protein, microtubule-associated protein 4, and stable tubule-only polypeptide proteins, many of which have been shown to regulate tubulin polymerization and function (13). Microtubules are susceptible to posttranslational alterations, including glutamylation and phosphorylation of β-tubulin and detyrosination of α-tubulin in Δ2 α-tubulin. In humans, α-tubulin and β-tubulin exist under the form of tubulin isotypes which mainly differ in their COOH-terminal sequences (14).

Few data are currently available about the expression of microtubule components in tumor samples or in their healthy counterparts. Although the functional specificity of tubulin isotypes remains controversial, it has been shown that the expression of some of the tubulin isotypes is tissue specific and that expression levels of tubulin isotypes have often been found to be correlated with the sensitivity to antitubulin agents (15–19). We have recently reported that tubulin isotype expression level was predictive of patient outcome in breast cancer patients receiving taxanes (20). In a preliminary study, we showed by immunochemistry that high expression of class III tubulin by tumor cells in 19 NSCLC patients receiving a taxane-based regimen was associated with a poor response to chemotherapy and a shorter progression-free survival (21). Recently, Rosell et al. (7) used quantitative PCR to analyze expression of β-tubulin III and stathmin in mRNA isolated from paraffin-embedded tumor biopsies of 25 located advanced NSCLC patients treated with vinorelbine/CDDP. These authors showed that time to progression was influenced by β-tubulin III and stathmin levels.

To determine whether predictive factors could be provided by immunohistochemistry in patients with NSCLC, we conducted a retrospective study of pretreatment tumor samples of patients with advanced NSCLC treated with vinorelbine-based regimens in the Pneumology Departments of the Hospices Civils de Lyon between 2000 and 2003 and correlated these biological results with patient outcome.

Materials and Methods

Patients and samples. The analysis was done on samples from 93 patients with unresectable locally advanced (stage IIIB) or metastatic (stage IV) NSCLC treated between January 2000 and December 2003 in the Pneumology Departments of the Hospices Civils de Lyon, France. These patients had adequate tumor biopsy specimens obtained before chemotherapy. Histopathologic subtypes were determined on the basis of the WHO classification. The current International Staging System was used for clinical disease staging (22). The clinicopathologic characteristics of the patient population are listed in Table 1. Their median age at diagnosis was 59 years (range, 37-79 years). Seven of the 39 stage IIIB patients and 9 of the 54 stage IV patients were women. All the patients were treated with vinorelbine, alone or in combination with CDDP or carboplatin. The median follow-up of the 93 patients, measured from the onset of chemotherapy, was 205 days (range, 1-1,443 days). After obtaining informed consent in accordance with institutional guidelines, all of the patients underwent tumor biopsy and chemotherapy.

Table 1. Characteristics of 93 NSCLC patients

Characteristics	No. patients
Total no. patients	93
Gender	
Male	77
Female	16
Age, y	
Median	59
Range	37-79
Histology	
Squamous cell carcinoma	37
Adenocarcinoma	28
Large cell carcinoma	28
Stage	
IIIB	39
IV	54
Weight loss	
‹10%	76
›10%	17
Chemotherapeutic regimen	
Cisplatin + vinorelbine	73
Carboplatin + vinorelbine	9
Oral vinorelbine alone	5
I.v. vinorelbine alone	6

Chemotherapy. The platinum-based regimens were vinorelbine 30 mg/m^2 on days 1 and 8 plus CDDP 80 mg/m^2 on day 1 of a 21-day cycle (73 patients), and vinorelbine 30 mg/m^2 on days 1 and 8 plus carboplatin dosed with an area under the curve of 5 on day 1 of a 21-day cycle (9 patients). Single-agent vinorelbine regimens were oral vinorelbine 60 mg/m^2 once a week (5 patients) and i.v. vinorelbine 30 mg/m^2 once a week (6 patients). All of the patients who received at least two courses of chemotherapy were evaluated for response. We used the standard response criteria (23) to evaluate response to chemotherapy. Complete response was defined as the disappearance of all signs of disease both at clinical examination and on the computed tomography scan. Partial response was defined by a reduction of >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 <50% decrease or a <25% increase in tumor size. Tumor progression was defined as an increase in the size of tumor lesions by >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 valuable cases. Response was evaluated after two to four cycles of chemotherapy. Overall survival was calculated as the time between the beginning of chemotherapy and death or last follow-up. Progression-free survival was calculated as the time between the beginning of chemotherapy and the date of tumor progression or last follow-up.

Histopathologic analysis. Immunohistochemical analyses were done on paraffin-embedded sections of pathologic samples obtained before therapy. Samples were obtained by bronchoscopy in 59 cases, by node cervical or susclavicular biopsy in 8 cases, by thoracotomy in 17 cases, by mediastinoscopy in 5 cases, and metastasis biopsy in 4 cases. The antibodies used (Table 2) were directed against total β-tubulin (clone TUB2, Sigma-Aldrich, Saint-Quentin Fallavier, France), class II (clone 7B9) and class III (clone TUJ1) β-tubulin isotypes (produced by Anthony Frankfurter, Department of Biology, University of Virginia, Charlottesville, VA), α-tubulin (clone DM1A, Sigma-Aldrich), τ protein (Euromedex, Strasbourg, France), P-glycoprotein (involved in classic

Table 2. Immunohistochemistry methods

Marker	Dilution	Time (min)	Antigen retrieval
Pan β-tubulin	1/1,000	32	EDTA (pH 8), 45 min
Class II tubulin	1/1,000	32	EDTA (pH 8), 45 min
Class III tubulin	1/400	32	EDTA (pH 8), 45 min
Anti – α-tubulin	1/1,000	32	EDTA (pH 8), 45 min
Anti – τ protein	1/5	32	0.01 mol/L citrate buffer (pH 6), 45 min
Anti – P-glycoprotein	1/10	Overnight	EDTA (pH 8), 45 min
Anti – Δ2 α-tubulin	1/1,000	32	EDTA (pH 8), 45 min

multidrug resistance, JSB1 clone from Biogenex, San Ramon, CA), and Δ2 α-tubulin (produced by Didier Job, 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 done with 3,3′-diaminobenzidine. Negative controls were done by omitting the primary antibody. All of the slides were examined and scored by the same pathologist (S.I.) without knowledge of the patient data. Immunostaining was assessed and scored as 1 (<50% positive cells) or 2 (>50% positive cells).

Statistical analysis. Bivariate correlations between immunohistochemical expression, patient or tumor characteristics, and response to chemotherapy were examined using the χ^2 test (or Fisher's exact test, as appropriate). Survival curves were estimated by the Kaplan-Meier method, and differences in progression-free survival and survival between subgroups were compared using the log-rank test. The Cox proportional hazards model was used for multivariate analysis to adjust the observed value of the expression of microtubule components for the determination of predictive factors. A level of $P < 0.05$ was considered as significant. All statistical analyses were done using Statistica 5.0.

Results

Immunohistochemical data. To determine which biological variables were likely to be predictive, we first determined which of these variables had varying levels of expression among 10 samples obtained by thoracotomy or mediastinoscopy from 10 patients. All tumor samples were positive with pan β and α antibody. The percentage of positive cells positive was 100% in 7 of 10 samples for total β-tubulin and 100% in 8 of 10 samples for α-tubulin (data not shown). τ protein was not expressed in any of the tumor samples analyzed and P-glycoprotein expression was found in a single tumor sample (data not shown). All tumor samples were found to express class II and class III β-tubulin and anti – Δ2 α-tubulin. The expression patterns of these markers were highly variable with a staining of 10% to 70% of the cells for class II tubulin, 10% to 100% of the cells for class III tubulin, and 10% to 70% of the cells for anti – Δ2 α-tubulin. An example of immunohistochemical staining with anti – tubulin III antibody is shown in Fig. 1A. On the basis of these results, we then extended the study to the remaining 83 samples and did staining with antibodies directed against class II β-tubulin, class III β-tubulin, and Δ2 α-tubulin.

Results of immunostaining of tumor samples are summarized in Table 3. Immunostaining intensity varied markedly among lung cancer samples. All control slides, prepared without primary antibodies, revealed no background immunoperoxidase staining and were used as negative controls. Expression of these microtubular components was also compared with that found in nonneoplastic lung tissue (data not shown). Class II expression was weak in the epithelium and bronchial glands and strong in stroma and nerves. Class III staining was found only in certain cells of the epithelium. Class III expression was negative in alveoli but strong in stroma, endothelial cells, and nerves (Fig 1B). Δ2 α-tubulin was strongly expressed at the apical pole of the bronchial epithelium as well as in nerves and blood vessels.

Patient outcome. Response was evaluated in 77 patients. Twenty-one patients responded, yielding an overall response rate of 27.3% in patients having received at least two treatment cycles and 22.6% in the entire patient population. Twenty-eight patients had a stable disease (36.4%) on evaluation and 28 patients progressed on therapy (36.4%). Of the 35 patients with stage IIIB disease, 19 received thoracic radiotherapy after the completion of chemotherapy. The median overall survival and progression-free survival were 205 and 118 days, respectively, in the entire patient population, and 393 and 245 days, respectively, in responding patients. Eighty-three patients had died at follow-up, seven were alive, and three patients were lost to follow-up.

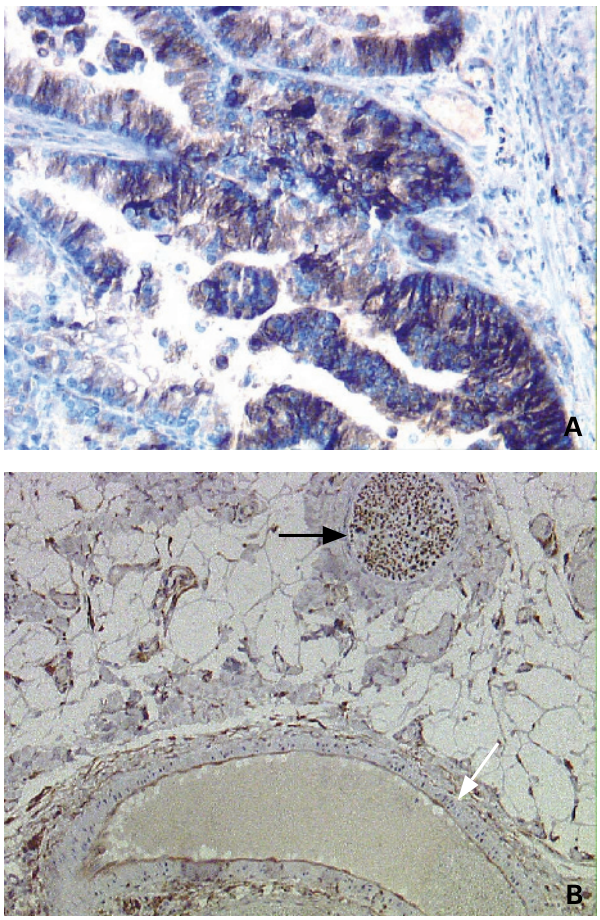

Fig. 1. *A,* adenocarcinoma of the lung strongly stained with anti – class III β-tubulin antibody. Ninety percent of tumor cells are positive for class III β-tubulin. *B,* immunostaining of tubulin III in nonneoplastic lung tissue. Class III staining was strongly expressed in nerves (*black arrow*) and endothelium (*white arrow*).

Table 3. Results of immunostaining for class II β-tubulin, class III β-tubulin, and Δ2 α-tubulin

	Class II β-tubulin (%)	Class III β-tubulin (%)	Δ2 α-tubulin (%)*
0-25% cells	39 (41.9)	23 (24.7)	25 (27.2)
26-50% cells	20 (21.5)	16 (17.2)	15 (16.3)
51-75% cells	21 (22.6)	19 (20.4)	28 (30.4)
76-100% cells	13 (14)	35 (37.6)	24 (26.1)

*Data missing for one patient.

Microtubule component expression and response to treatment. Expression levels of microtubule components were not found to be correlated with the response rate to chemotherapy (Table 4). However, we found a statistically significant up-regulation of class III β-tubulin in the resistant subset, defined as disease progression under treatment. Patients with high class III expression were more resistant to vinorelbine (47.7% progression rate in patients with >50% of stained cells versus 18.7% in patients with <50%; $P = 0.009$). We also found significant relation between high Δ2 α-tubulin expression and vinorelbine resistance (47.6% progression rate in patients with >50% of stained cells versus 21.2% in patients with <50%; $P = 0.018$). Patient characteristics (age, gender, weight loss > 10%, histologic subtype, and stage) were not found to be correlated with response to chemotherapy (data not shown).

Microtubule component expression and survival. A high level of expression (>50% versus <50% of positive cells) of class III β-tubulin protein was correlated with a shorter median progression-free survival of 89 days versus 208 days in patients with low class III expression ($P = 0.002$, Fig. 2A). Low overall survival was significantly correlated with high class III and high Δ2 α-tubulin expression. The median overall survival was 162 days in patients with high level of class III isotype as opposed to 306 days in patients with low level of class III isotype ($P = 0.001$, Fig. 2B) and 168 days in patients with high level of Δ2 α-tubulin as opposed to 243 days in patients with low level of Δ2 α-tubulin ($P = 0.045$). Class II tubulin levels were not found to be correlated with patient outcome. Among patient characteristics, stage IV disease was associated with shorter progression-free survival ($P = 0.03$) and weight loss of >10%

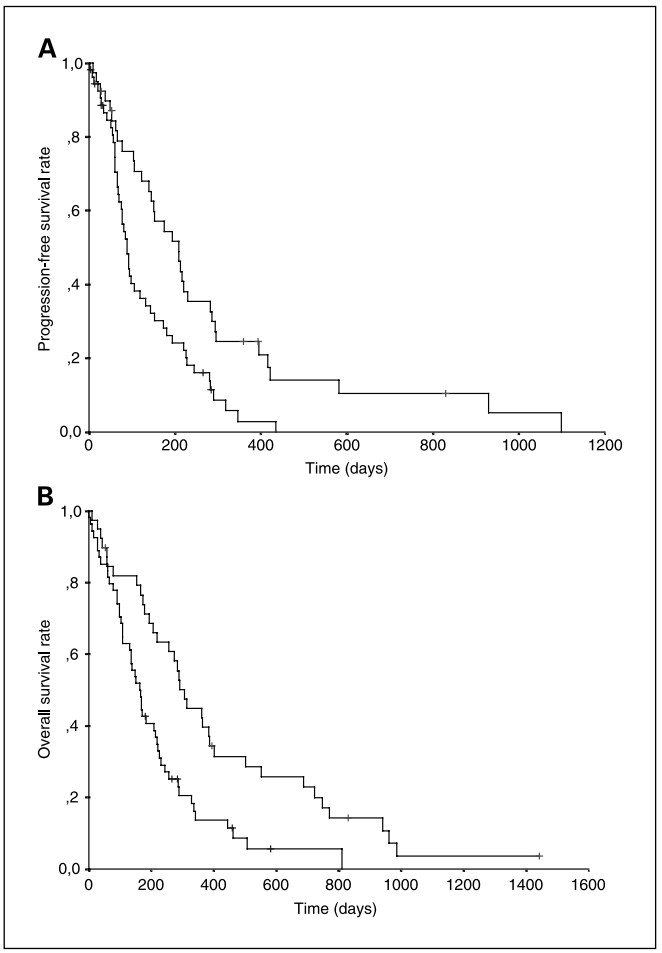

Fig. 2. *A,* the progression-free survival curve for 93 patients with advanced NSCLC, according to class III β-tubulin expression. *B,* the overall survival curves for 93 patients with advanced NSCLC, according to class III β-tubulin expression.

Table 4. Relationship between expression of microtubule components, response, progression on therapy, and survival in 93 NSCLC patients treated with a vinorelbine-based regimen

	n	Response rate (%)	*P*	Progression rate (%)	*P*	PFS (d)	*P*	OS (d)	*P*
Class II β-tubulin									
≤50%	59	28	0.9	30.0	0.163	150	0.15	226	0.12
>50%	34	26.9		46.2		85		184	
Class III β-tubulin									
≤50%	49	34.4	0.2	18.7	0.009	208		306	0.001
>50%	44	22.7		47.7		89	0.002	162	
Δ2 α-tubulin*									
≤50%	40	24.2	0.7	21.2	0.018	173	0.3	243	0.045
>50%	52	28.6		47.6		105		168	

Abbreviations: PFS, progression-free survival; Progression, progression on therapy; OS, overall survival.
*Data missing for one patient.

was associated with shorter progression-free survival ($P = 0.05$) and shorter overall survival ($P = 0.03$; data not shown).

Multivariate analysis for progression-free survival and overall survival. Multivariate analysis was done with the Cox proportional hazards model to determine whether the prognostic value of class II and class III tubulin and of Δ2 α-tubulin disappeared when other prognostic factors were considered (data not shown). The multivariate analysis that included sex, age, histology, stage, weight loss, tubulin class II, tubulin class III, and Δ2 α-tubulin showed that histology, weight loss, and high tubulin class III expression were significant independent variables that correlated with low progression-free survival ($P = 0.03$, $P = 0.014$, and $P = 0.04$, respectively). A high class III level yielded a hazard ratio of 1.75, with a 95% confidence interval ranging from 1.02 to 3.03. High class III and weight loss were independently correlated with low overall survival ($P = 0.012$ and $P = 0.043$ respectively). A high class III level yielded a hazard ratio of 2.03, with a 95% confidence interval ranging from 1.17 from 3.53.

Discussion

This is the first study showing a relationship between the level of expression of microtubular proteins in NSCLC tumor samples and patient outcome after treatment with a vinorelbine-based regimen. β-Tubulin class III isotype levels were found to be independently correlated with the rate of progression on therapy, progression-free survival, and overall survival whereas Δ2 α-tubulin levels were independently correlated with the rate of progression on therapy and overall survival. These results are in keeping with a previous report by Rossell et al. (7) who showed that class III and stathmin mRNA levels were correlated with time to progression in 25 patients treated with a vinorelbine/cisplatin combination regimen. These authors did not observe correlation between class III levels and response or overall survival, perhaps because of the limited sample size. Insofar as access to frozen lung biopsy tissue for RNA analyses is often difficult, the demonstration that immunohistochemistry provides useful predictive factors in this group of patients is likely to be useful in the choice of chemotherapy regimens.

There is ample data in the literature relating altered microtubule component expression with resistance to antitubulin agents. Kavallaris et al., Raganathan et al., and ourselves have reported overexpression of class III tubulin in models of resistance to taxanes (16, 24, 25) and vinblastine (25). We have also previously shown that class III tubulin expression was predictive of survival in NSCLC patients receiving taxane-based therapy (21). However, microtubule-based resistance to antitubulin agents is usually multifactorial and involves a number of microtubule-associated or microtubule-related proteins (26). 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 (27), or, alternatively, that the microtubule contained in the tumor cells may have different dynamic properties and thus may be less sensitive to antitubulin agents (28, 29). 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 (30). 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 (31). A possible explanation for the predictive value of Δ2 α-tubulin in our study is that tumor cells containing high levels of this protein possess relatively stable microtubules and are therefore relatively insensitive to antitubulin agents.

Interestingly, using semiquantitative real-time PCR and quantitative immunohistochemistry, a recent clinical study on 41 advanced ovarian cancer patients treated with paclitaxel by Mozzetti et al. (32) revealed a significant up-regulation of class III tubulin expression at both mRNA and protein levels in the resistant subset, defined as patients who progressed under chemotherapy. No relationship was assessed between tubulin isotype expression and clinical data, progression free survival, and overall survival. Similarly, by defining the resistance to therapy as disease progression under treatment, we found that tubulin III overexpression was closely related to response to vinorelbine therapy. Taken together, Mozzetti's studies and ours suggest that overexpression of β-tubulin III is a possible mechanism of resistance to antitubulin agent therapy both in ovarian and lung cancers.

A recurrent and essential question in the quest to determine factors predictive of response to a given type of therapy is whether one is not simply identifying factors correlated with disease aggressivity, independently of the type of therapy given to patients. This issue has been raised in the case of P-glycoprotein expression in acute myeloid leukemia patients, because it has been suggested that P-glycoprotein expression in itself is an adverse prognosis factor, including in patients who are not treated with multidrug resistance–sensitive compounds (33). The confirmation that microtubular component levels are specifically predictive of response to antitubulin-based chemotherapeutic regimens will require their analysis in patients receiving unrelated regimens. These studies are required before such predictive factors can be evaluated to help clinicians adapt chemotherapy regimens to the biological profiles of the patients' tumors.

In conclusion, our findings indicate that expressions of β-tubulin III and Δ2 α-tubulin proteins are correlated with clinical outcome in advanced NSCLC patients treated with vinorelbine. Future prospective studies evaluating the value of these markers in NSCLC patients receiving antitubulin-based regimens and unrelated regimens should be done to determine the clinical usefulness of these markers.

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