Class III β-Tubulin Expression and Benefit from Adjuvant Cisplatin/Vinorelbine Chemotherapy in Operable Non–Small Cell Lung Cancer: Analysis of NCIC JBR.10

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Abstract

Purpose: High class III β-tubulin (bTubIII) expression in advanced non–small cell lung cancer is known to correlate with reduced response rates and inferior survival with anti-microtubule agents. JBR.10 showed a 12% and 15% improvement in 5-year recurrence-free survival (RFS) and overall survival (OS), respectively, with the addition of cisplatin and vinorelbine following resection of stage IB-II non–small cell lung cancer. We sought to determine the effect of bTubIII on patient outcome and benefit from adjuvant chemotherapy in the JBR.10 trial.

Experimental Design: We did a semiquantitative immunohistochemical assay for bTubIII on primary tumor tissue available from 265 of the 482 patients in JBR.10. Tumors were classified as bTubIII "low" or "high" using a validated method. We examined the prognostic effect of bTubIII in patients treated with or without chemotherapy and the survival benefit from chemotherapy in low versus high bTubIII subgroups.

Results: High bTubIII expression was associated with poorer RFS and OS in patients treated with surgery alone but not in patients treated with adjuvant chemotherapy. The RFS and OS benefits of adjuvant chemotherapy were greater in high versus low tubulin expressors. However, with Cox regression, the interaction between bTubIII status and chemotherapy treatment in predicting RFS or OS did not reach statistical significance.

Conclusions: Chemotherapy seemed to overcome the negative prognostic effect of high bTubIII expression. Greater benefit from adjuvant chemotherapy was seen in patients with high bTubIII expression. This is contrary to what has been seen in the setting of advanced disease; possible reasons for this difference are being explored.

Recent studies have shown that adjuvant chemotherapy improves survival in completely resected non–small cell lung cancer (NSCLC; refs. 1–3). Winton et al. (1) published recently the results of a phase III National Cancer Institute of Canada Clinical Trials Group (NCIC CTG) randomized trial of adjuvant vinorelbine and cisplatin compared with observation alone in completely resected stage IB and II NSCLC (NCIC JBR.10). Four hundred eighty-two patients were randomly assigned either to four cycles of cisplatin/vinorelbine chemotherapy (n = 242) or to observation (n = 240). Patients assigned to chemotherapy had a significantly higher survival rate (69% versus 54% at 5 years; P = 0.002) and a significantly higher relapse-free survival rate (61% versus 48% at 5 years; P = 0.013). These results have made the cisplatin and vinorelbine combination a widely accepted standard for adjuvant chemotherapy for NSCLC (4).

Although these results represent a clinically important benefit from adjuvant chemotherapy, it is important to recall that only an additional 5% to 15% of treated individuals ultimately benefit with improvement in their long-term survival (5). Adjuvant chemotherapy has drawbacks, including resource utilization and treatment toxicity. Therefore, new approaches are needed to individualize treatment by preselecting the subset of patients who are most likely to benefit from a given adjuvant therapy. The study of molecular factors that influence drug responsiveness is a potentially promising approach to decrease treatment toxicity and costs by avoiding the administration of ineffective therapy to patients destined not to benefit.

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Among the described mechanisms of resistance to anti-tubulin agents, class III β-tubulin (bTubIII) overexpression is of particular interest (6). Several studies have shown that the level of bTubIII (assessed by immunohistochemistry or other techniques) may be both a prognostic and a predictive factor in advanced NSCLC. Rosell et al. (7) correlated high bTubIII mRNA levels with inferior outcome in advanced NSCLC patients treated with anti-tubulin agents. It has also been shown that a high level of expression of bTubIII in tumor cells, assessed by a semiquantitative immunohistochemical assay, was associated with a lower response rate and a poor prognosis in advanced NSCLC patients receiving vinorelbine-based chemotherapy (8). In a recent study, high tumor expression of bTubIII assessed by immunohistochemistry in 47 NSCLC patients receiving a paclitaxel-based regimen was predictive of lower response to therapy and inferior survival (9).

These studies are concordant with *in vitro* studies and clinical studies in other tumor types that have shown that up-regulation of bTubIII in lung and ovarian tumor cell lines confers resistance to docetaxel/paclitaxel (10–12) and that overexpression of bTubIII in advanced ovarian, breast, and gastric cancers is associated with resistance to paclitaxel and a poor prognosis (13–15). Taken together, these *in vitro* and clinical studies suggest that overexpression of bTubIII is an adverse prognostic factor in cancer and is a mechanism of resistance to anti-tubulin chemotherapeutic agents.

To assess whether tubulin III might be a useful marker in early NSCLC patients undergoing adjuvant chemotherapy with a vinorelbine-based regimen, we assessed the level of bTubIII (tubulin III) in tumor samples from patients treated on the NCIC CTG JBR.10 study and correlated the levels with outcome in both treated and control patient groups.

Materials and Methods

NCIC JBR.10 clinical trial. JBR.10 was a North American Intergroup trial led by NCIC CTG with participation by the Eastern Cooperative Oncology Group, the Southwest Oncology Group, and the Cancer and Leukemia Group B. The full details of the trial have been reported previously (1). Briefly, 482 patients were accrued between July of 1994 and April of 2001 and were randomly assigned to receive adjuvant treatment with vinorelbine/cisplatin or observation. Overall survival (OS) was the primary end point. Study results showed that chemotherapy significantly prolonged the OS with a P value of 0.009 [hazard ratio (HR), 0.69; 95% confidence interval (95% CI), 0.52-0.91]. The median survivals were 94 months (95% CI, 73 months to not reached) for patients on chemotherapy arm and 73 months (95% CI, from 48 months to not reached) for patients on observation arm. Recurrence-free survival (RFS) was significantly prolonged by the chemotherapy as well with an estimated HR of 0.60 (95% CI, 0.45-0.79; $P < 0.001$).

Tissue microarrays from the NCIC JBR.10 clinical trial. With informed consent, a representative block of formalin-fixed and paraffin embedded tumor tissue of the available resection specimens were collected from 265 of the 482 patients. These materials were used to construct the JBR.10 tissue microarrays using the Manual Tissue Arrayer of Beecher Instruments (Silver Spring, MD). Using the H&E-stained slide of the block, 0.6-mm cores were obtained from three separate tumor areas. One core was also taken from the nonneoplastic area of the same tissue block. The 265 cases were arrayed into eight tissue microarray blocks. Serial 4-micron sections from each block were then mounted on silane-coated slides for immunostaining.

Histopathologic analysis. Immunohistochemical analyses were done on the tissue microarrays. As reported previously (8), we used monoclonal antibodies specific for the bTubIII isotype (clone TUJ1; generously provided by Anthony Frankfurter, Department of Biology, University of Virginia, Charlottesville, VA). Tubulin III was stained using an automated immunohistochemical stainer (Nexes, Ventana Medical Systems, Illkirch, France) following routine deparaffinization and rehydration. Antigen retrieval used citrate buffer (pH 6.0) in a pressure cooker (TTMega) for 20 min and a cool down for 20 min and then washed in running tap water for 5 min. Slides were then immunostained on a Nexes instrument with avidin and biotin. Chromogenic detection used 3,3′-diaminobenzidine.

All of the tissue arrays were examined and independently scored by two observers (R.L. and P.S.), blinded to the patients' treatment randomizations and outcomes. A numerical score was assigned for each core specimen, incorporating quantitative assessment of malignant cell cytoplasmic staining intensity and the proportions of tumor cell stained. Intensity scoring for bTubIII was based on relative intensities of staining of the tumor with reference to the normal alveoli (8). A score of '0' was given to samples with no stained tumor cells above background. A score of '1' was assigned to samples with low levels of staining, and a score of '2' was given to samples with strong staining.

The H score was calculated using the following formula: H score = $\Sigma (1 + I) \times PC$, where I represents cytoplasmic staining intensity and PC represents the percentage of malignant cells that stained at each intensity, respectively (16). Each sample therefore yielded an H score ranging from 100 to 300. The H scores of the two observers were said to agree when they differed by <10%. Discrepant scores of >10% were resolved by reassessment and consensus between the two observers.

As an internal control, the staining intensity seen in normal neurons and vascular endothelials cells was found to be consistently strong across samples and was assigned a score of 2. As an external control, we stained lung tumor samples from a separate tissue bank that were known to express bTubIII at both the mRNA and the protein levels.

Statistical analysis. Interobserver agreement for immunohistochemistry was calculated using the global agreement and κ test for more than two categories. Correlations between immunohistochemical expression and clinical outcomes were examined using a Cox proportional hazards model (17). Survival curves were generated by the Kaplan-Meier method, and differences in RFS and OS between groups were compared using the log-rank test. The Cox proportional hazards model was used to assess the independent value of tubulin III expression among identified prognostic factors and to test for an interaction between tubulin III expression and treatment assignment in predicting RFS and OS. All P values are two sided.

Results

Baseline patient characteristics. Baseline characteristics for the 265 patients who had tumor assessed for tubulin expression and the 216 who did not are shown in Table 1 (one patient with no baseline data was excluded). The subset of JBR.10 patients in the current study is representative of the trial population as a whole, although significantly more patients had T2 tumors in the tubulin subset ($P = 0.03$), whereas fewer patients had N1 involvement ($P = 0.07$). Among the patients assessed for tubulin levels, 140 were assigned to receive chemotherapy and 125 to observation alone.

Immunohistochemical data. The overall interobserver agreement for independently reported H scores was 97.7% ($k = 0.88$; 95% CI, 85-91). Immunostaining intensity varied markedly among tumor samples both for relative intensities of staining and for percentage of cell stained. Representative examples of negative and positive immunohistochemical staining with anti-tubulin III antibody are shown in Fig. 1.

Table 1. Comparison of baseline factors for 265 patients with tubulin data and 216 patients without tubulin data

Variable	Patients with tubulin data, n = 265	Patients without tubulin data, n = 216	P
Age (y)			
<61	127 (47.9)	114 (0.53)	0.31
≥61	138 (52.1)	102 (0.47)	
Sex			
Female	95 (35.8)	72 (33.3)	0.63
Male	170 (64.2)	144 (66.7)	
T stage			
T_1	30 (11.3)	40 (18.5)	0.03
T_2	235 (88.7)	176 (81.5)	
N stage			
N_0	131 (49.4)	88 (40.7)	0.07
N_1	134 (50.6)	128 (59.3)	
ECOG status			
0	126 (47.6)	110 (50.9)	0.46
1	139 (52.4)	106 (49.1)	
Type of surgery			
Pneumonectomy	63 (23.8)	50 (23.1)	0.45
Lobectomy	202 (76.2)	164 (75.9)	
Segmentectomy	0 (0)	1 (0.05)*	
Histology type			
Squamous	97 (36.6)	82 (38.0)	0.89
Adenocarcinoma	143 (54.0)	112 (51.9)	
Others	25 (9.4)	22 (10.2)	
Ras mutation			
Absent	198 (70.9)	134 (72)	0.44
Present	67 (29.1)	50 (27)	
Uncertain	0 (0)	1 (5)†	
Treatment arm			
Observation	125 (47.2)	114 (52.8)	0.22
Chemotherapy	140 (52.8)	102 (47.2)	

*One patient with no baseline data was excluded from the analysis.
†For 31 of these 216 cases, tumor tissue was not available for Ras mutation status assessment.

Comparison of baseline factors for patients with higher tubulin expression score and those with lower score (high versus low tubulin expressors). The 265 patients with tubulin data were dichotomized by median quantitative tubulin expression H score and thus classified as high or low tubulin expressors. As shown in Table 2, high tubulin expressors (n = 133) included more females (42% versus 30%; P = 0.04), fewer with squamous histology (25% versus 48%; P < 0.001), more with Ras mutations (31% versus 20%; P = 0.05), more patients ≤60 years old (53% versus 42%; P = 0.09), and more PS1 patients (58% versus 47%; P = 0.09) compared with the low tubulin expressors (n = 132). Gender, stage, type of lung resection, and chemotherapy treatment assignment were not related to tubulin III expression.

Comparison of RFS and OS in high versus low tubulin expressors: the prognostic value of bTubIII immunohistochemistry. The log-rank test stratified by treatment assignment was used to assess the value of tubulin expression in predicting RFS or OS. In both cases, high tubulin expression was associated with inferior outcome. The result was statistically significant for RFS (HR, 1.52; 95% CI, 1.05-2.22; P = 0.03) and a similar trend was seen for OS (HR, 1.39; 95% CI, 0.96-2.01; P = 0.08; Fig. 2).

The value of tubulin expression in predicting RFS or OS seemed to be largely confined to those patients assigned to the observation arm of JBR.10 (RFS: HR, 1.92; 95% CI, 1.16-3.18; P = 0.01; OS: HR, 1.72; 95% CI, 1.02-2.88; P = 0.04; Fig. 3A). Tubulin expression was not a statistically significant predictor of outcome in the patients assigned to receive chemotherapy (RFS: HR, 1.10; 95% CI, 0.62-1.95; P = 0.75; OS: HR, 1.11; 95% CI, 0.65-1.88; P = 0.7; Fig. 3B).

Cox regression stratified by treatment arm was used to examine the relationship between tubulin expression and RFS or OS after adjusting for other prognostic factors. A backward selection algorithm was used with a cutoff of P = 0.1 for inclusion in the model. In this type of model, high tubulin expression remained as a significant adverse prognostic factor for RFS (adjusted HR, 1.78; 95% CI, 1.06-3.00; P = 0.03). Similar results were seen in a model of OS (HR, 1.42; 95% CI, 0.97-2.09; P = 0.07).

Comparison of the benefits of adjuvant chemotherapy in high versus low tubulin expressors: the predictive value of bTubIII immunohistochemistry. The 132 low tubulin expressors included 72 assigned to chemotherapy and 60 to observation. In the

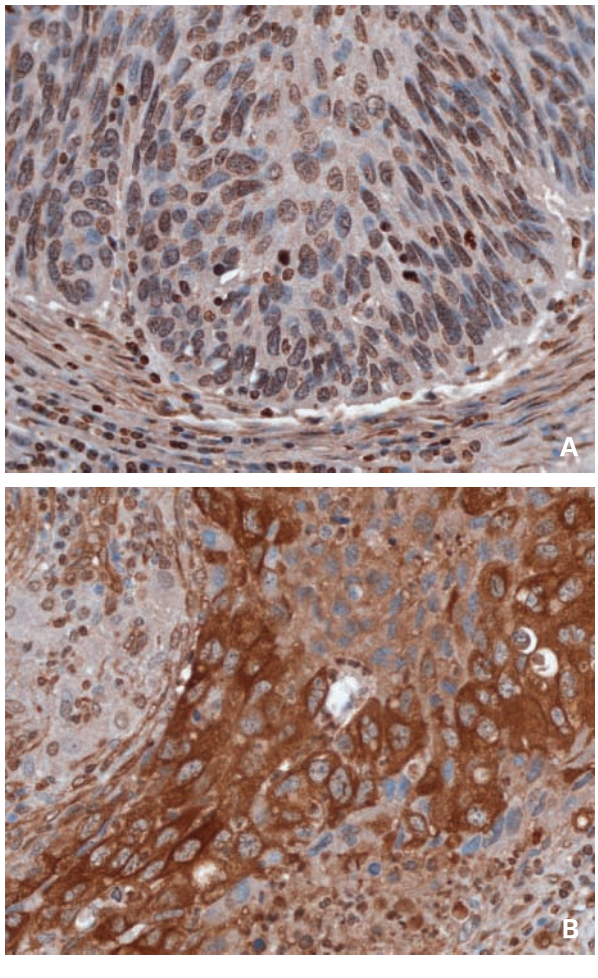

Fig. 1. *A,* squamous cell carcinoma of the lung negative for anti-bTubIII antibody. *B,* adenocarcinoma of the lung stained with anti-bTubIII antibody. Some tumor cells exhibited a low level of staining, whereas most of the cells were strongly stained.

Table 2. Comparison of baseline factors for 265 patients according to tubulin expression (high versus low)

Variable	Patients with low tubulin III expression, *n* = 132	Patients with high tubulin III expression, *n* = 133	*P*
Age (y)			
<61	56 (42.4)	71 (52.6)	0.09
≥61	76 (57.6)	62 (46.7)	
Sex			
Female	39 (29.5)	56 (42.1)	0.04
Male	93 (80.5)	77 (57.9)	
T stage			
T_1	16 (12.1)	14 (11.7)	0.70
T_2	116 (87.9)	119 (88.3)	
N stage			
N_0	62 (47)	69 (51.9)	0.46
N_1	70 (53)	64 (48.1)	
ECOG status			
0	70 (53)	56 (42.1)	0.09
1	62 (47)	77 (57.9)	
Type of surgery			
Pneumonectomy	34 (25.8)	29 (21.8)	0.45
Lobectomy	98 (74.2)	104 (75.9)	
Segmentectomy	0 (0)	0 (0)	
Histology type			
Squamous	64 (48.5)	33 (24.8)	<0.0001
Adenocarcinoma	64 (48.5)	79 (59.4)	
Other	4 (3)	21 (15.8)	
Ras mutation			
Absent	106 (80.3)	92 (69.2)	0.05
Present	26 (19.7)	41 (30.8)	
Treatment arm			
Observation	60 (45.5)	65 (48.9)	0.62
Chemotherapy	72 (54.5)	68 (51.1)	

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

low tubulin group, no significant difference in RFS (HR, 0.78; 95% CI, 0.44-1.37; $P = 0.4$) or OS (HR, 1.00; 95% CI, 0.57-1.75; $P = 0.99$) was seen in between patients assigned to chemotherapy and observation (Fig. 4A).

The 133 high tubulin expressors included 68 assigned to chemotherapy and 65 to observation. In the high tubulin group, patients receiving chemotherapy had significantly improved RFS (HR, 0.45; 95% CI, 0.27-0.75; $P = 0.002$) and a trend toward improved OS (HR, 0.64; 95% CI, 0.39-1.04; $P = 0.07$) compared with patients in the observation arm (Fig. 4B).

Cox regression with interaction between tubulin status and treatment assignment was used to test for the difference in the treatment effects between the tubulin expression levels in RFS or OS. The interaction terms were not statistically significant ($P = 0.15$ for RFS; $P = 0.25$ for OS).

Discussion

The results of this study suggest that high tubulin III expression in resected NSCLCs is associated with poorer survival in the absence of adjuvant chemotherapy treatment but not in patients who receive adjuvant chemotherapy. These findings suggest that adjuvant cisplatin/vinorelbine chemotherapy can overcome the adverse biology of cancers that express higher amounts of tubulin III. Furthermore, adjuvant chemotherapy significantly prolonged the RFS and OS in the high tubulin expressors, but its effect was not clear for the low tubulin expressors in this study.

High tubulin III expression is associated with a higher risk of relapse following surgery alone but also with a higher probability of benefit from adjuvant cisplatin plus vinorelbine chemotherapy. Apparently, tubulin III immunohistochemistry assays could eventually be used as part of the process of selecting patients for adjuvant chemotherapy. However, further study of this topic will be required before tubulin III immunohistochemistry can be introduced in the clinic. At this stage, our data can be considered hypothesis generating. The conclusions that can be drawn from our study are limited because not all tumors in the JBR.10 trial were available for analysis and a statistically significant interaction was not found between tubulin III status and treatment outcome in the Cox

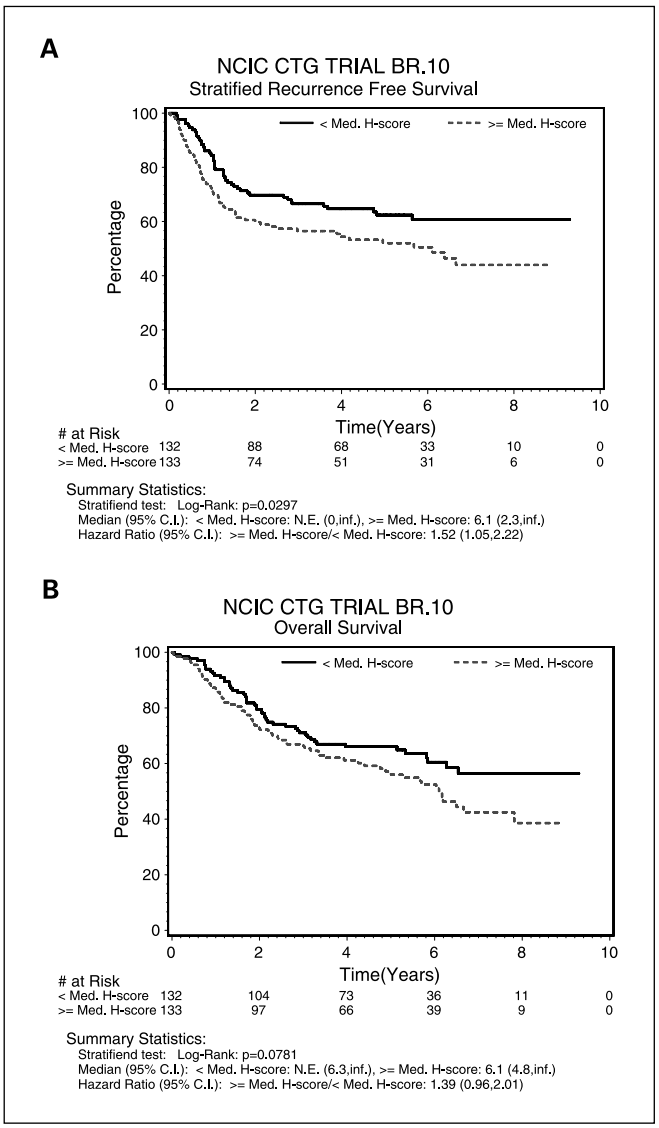

Fig. 2. The RFS and OS curves for 265 patients included in the BR-10 trial according to bTubIII expression.

regression model. A study with a larger sample size would be required to either confirm or exclude a true interaction between tubulin expression level and benefit from chemotherapy. Because our study was not powered to exclude a potential benefit from adjuvant chemotherapy in low tubulin-expressing patients, we feel that these patients should continue to be offered adjuvant treatment unless it is proven in confirmatory studies to be truly ineffective in this group.

The adverse prognostic significance of high tubulin III expression observed in this study is consistent with prior published reports in the setting of advanced NSCLC. However, this report is contrary to the data from advanced NSCLC about the value of tubulin III expression in predicting benefit from chemotherapy. In the setting of advanced disease, low tubulin III expression is associated with a higher objective response rate to chemotherapy containing the anti-microtubule agents vinorelbine or paclitaxel (7–9) but does not seem to predict response to regimens that do not target microtubules, such as gemcitabine (9). The results seen in advanced NSCLC are concordant with findings in breast, ovarian, and gastric cancer patients treated with taxanes and with preclinical studies showing that tubulin III confers paclitaxel resistance (10–15, 18). However, a recent study in unresectable advanced ovarian cancer showed that β-tubulin positivity was not associated with response to paclitaxel, whereas cases with high β-tubulin expression had a worse OS (19).

On the other hand, the results of the present study suggest that in early lung cancer, it is the patients with high tubulin III expression that are most likely to benefit from adjuvant cisplatin and vinorelbine.

It is possible that all the studies are correct and that tubulin III has differential predictive implications in early versus advanced NSCLC. This discrepancy between the metastatic and adjuvant setting is not without precedent. In colorectal cancer, the relationship between thymidylate synthase and benefit from chemotherapy differs between operable and advanced disease (20–22). The reason for the current discrepancy is as yet unexplained. We are currently exploring potential explanations, as this will be important to the further evaluation of tubulin III as a marker of chemosensitivity.

Our study reflects the intrinsic difficulty in identifying predictive factors. Currently validated predictive factors (e.g., estrogen receptor status for benefit from hormonal therapy of breast cancer and HER-2 status for benefit from trastuzumab) are mixed predictive and prognostic factors (23). Whereas prognostic assays can be readily identified and validated in nonrandomized series of patients, predictive assays can ultimately only be proven in the setting of clinical trials, in which patients are randomly allocated or not to the treatment of interest. Whereas many biomarkers may seem to hold promise as predictive assays, few have been formally validated in appropriately designed randomized studies.

In conclusion, high bTubIII expression as assessed by immunohistochemistry in our study conferred adverse prognosis but seemed to be associated with increased benefit from adjuvant cisplatin/vinorelbine chemotherapy in patients with operable NSCLC in the NCIC JBR.10 clinical trial. This result contrasts with the metastatic setting. These results are not definitive and require confirmation, and further study is

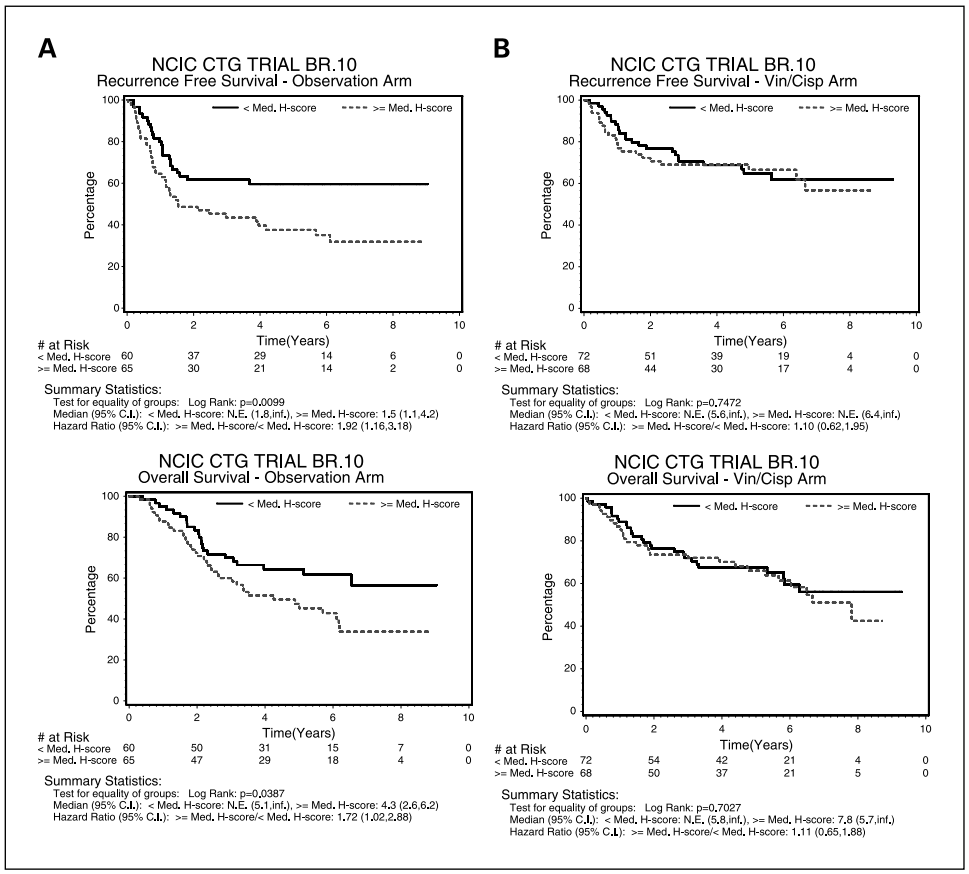

Fig. 3. *A,* the RFS and OS curves for patients assigned to observation alone according to bTubIII expression. *B,* the RFS and OS curves for patients assigned to adjuvant chemotherapy according to bTubIII expression.

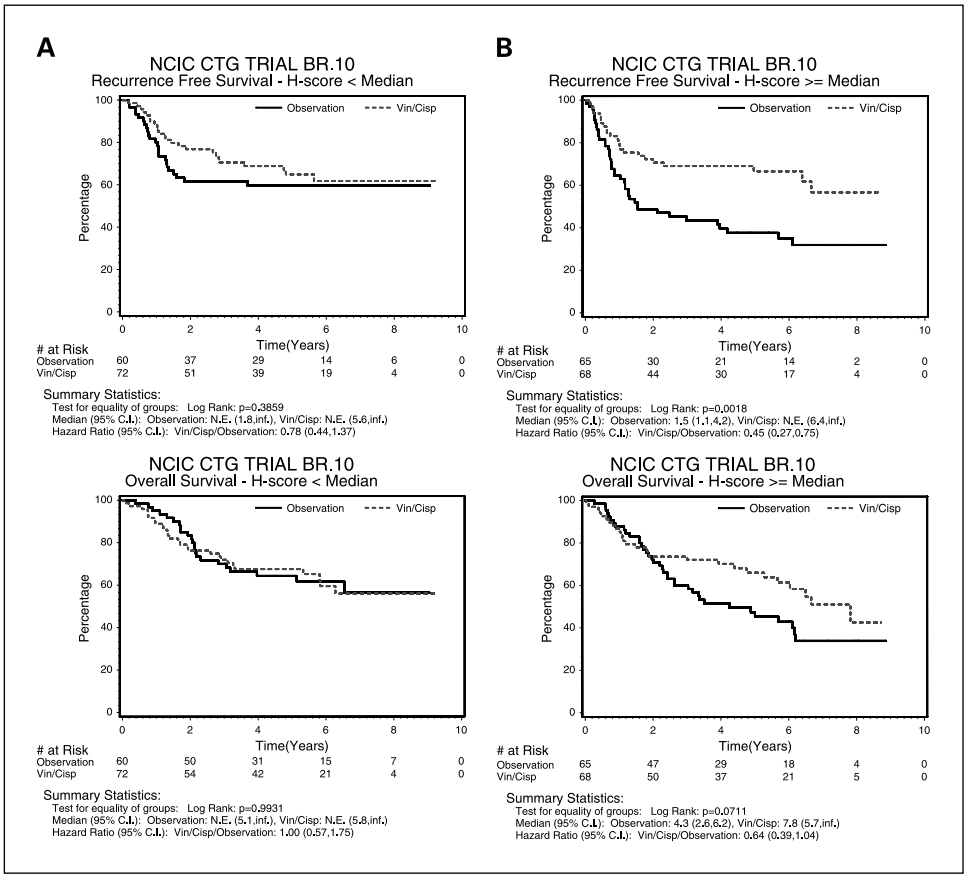

Fig. 4. *A,* the RFS and OS curves for low tubulin expressors according to treatment assignment. *B,* the RFS and OS curves for high tubulin expressors according to treatment assignment.

warranted to determine if this assay should be developed for the clinic. Possible avenues for further exploration of this topic include further preclinical work to clarify the role of tubulin III in conferring resistance or sensitivity to chemotherapy other than taxanes, confirmation of our results in tumor banks from other randomized chemotherapy trials, and new prospective studies incorporating tubulin III immunohistochemistry.

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