Is Class III β-Tubulin a True Predictive Marker of Sensitivity to Vinorelbine in Non-small Cell Lung Cancer? Chemosensitivity Data Evidence

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Abstract. *Aim: To clarify whether class III β-tubulin (TUBB3) is a true predictive marker for chemotherapy with vinorelbine, chemosensitivity was examined using an in vitro drug sensitivity assay. Patients and Methods: Initially, 9 specimens were obtained to analyze the dose–response curve and to measure the median effective dose 50 (ED_{50}) in the histoculture drug response assay (HDRA). Subsequently, 68 surgically resected non-small cell lung cancer (NSCLC) specimens were applied to the HDRA and H-scores were calculated by immunohistochemical staining. Results: The mean (±SD) slope factor, ED_{50} and maximal response was 8.7±5.4, 39.0±17.9 μg/ml and 85.5±5.1% respectively. The mean inhibition rate was 26.4±16.2% and the mean H-score was 1.09±1.07. The inhibition rate was significantly correlated with TUBB3 expression (r=0.27, p=0.03), and was significantly higher in TUBB3-positive specimens than in TUBB3-negative specimens (p=0.003). Conclusion: Tumors with high TUBB3 levels exhibited greater chemosensitivity to vinorelbine than tumors with low TUBB3 levels. This finding provides support for the results of the JBR.10 trial.*

Lung cancer is the most frequent cancer worldwide (1). Until a report by the International Adjuvant Lung Trial in 2003 (2), there was little convincing evidence that adjuvant chemotherapy improves the outcomes of non-small cell lung cancer (NSCLC). Since then, five randomized trials have reported that adjuvant chemotherapy improves survival in resected NSCLC (3-7). However, recent randomized trials have indicated that there are no significant differences in efficacy among combinations of cisplatin with new drugs, although they show varying toxicity profiles (8). These results suggest that the maximum efficacy has been reached in conventional treatment strategies, and that it is now necessary to treat individual patients based on the biological characteristics of their tumors.

Anti-microtubule agents, such as vinorelbine, are widely used in the treatment of NSCLC patients. However, in a subset analysis of the JBR.10 trial (9), no survival benefit was observed in patients with low levels of class III β-tubulin (TUBB3), whereas survival benefits were observed in patients with high TUBB3 levels. These findings suggest the possibility of tailored therapy for NSCLC patients by measuring their TUBB3 levels. However, Sève *et al.* (10) showed that high TUBB3 levels were correlated with resistance to treatment and poor clinical outcomes in patients with advanced NSCLC which were treated with a vinorelbine-based regimen. The reason for the discrepancies between the results from the advanced and adjuvant setting trials has remained unclear.

In vitro drug response assays (11-16) have been used for the identification of effective chemotherapy agents for individual patients with various malignancies. The histoculture drug response assay (HDRA) (14-16) is a representative *in vitro* drug response assay used for anticancer agents. Several clinical studies involving colorectal and gastric cancers have shown that the inhibition rates obtained with the HDRA can predict the clinical responses to chemotherapy. In May 1994, we instituted the HDRA for lung cancer using resected tumors. We previously reported that the HDRA is useful for predicting the response to chemotherapy for NSCLC (17).

In the present study, we examined the predictive value of TUBB3 for vinorelbine-based chemotherapy for NSCLC patients using the HDRA. First, we determined the assay conditions for vinorelbine in the HDRA. Next, using these conditions, we examined NSCLC specimens to determine the inhibition rate for vinorelbine using the HDRA.

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Key Words: Histoculture drug response assay, non-small cell lung cancer, class III β-tubulin, predictive marker, vinorelbine.

Patients and Methods

Patients. All the study protocols were approved by the Institutional Review Board for Clinical Practice of our institution, and written informed consent was obtained from each patient.

Patients and specimens for the dose-response curve of vinorelbine in the HDRA. From March 2006 to August 2007, fresh surgically resected tumor specimens obtained from 9 patients were used to analyze the dose–response curve of vinorelbine in the HDRA and to measure the median effective dose (ED_{50}). The patients comprised 6 male patients and 3 female patients who ranged in age from 57 to 77 years (mean=70 years). The specimens examined were 6 adenocarcinomas, 2 squamous cell carcinomas and 1 carcinoid.

Patients and specimens for the inhibition rate of vinorelbine in the HDRA. After the ED_{50} measurement, surgically resected fresh tumor specimens was obtained from 68 NSCLC patients at our institution from August 2007 to June 2010, and applied to the HDRA to measure the inhibition rate of vinorelbine. The patients comprised 47 male patients and 21 female patients who ranged in age from 43 to 83 years (mean=70 years). The specimens examined were 38 adenocarcinomas, 19 squamous cell carcinomas, 3 adenosquamous carcinomas, 2 pleomorphic carcinomas and 6 other histological types.

HDRA. The HDRA was used as an *in vitro* drug sensitivity test, and was carried out as described previously (17). Collagen sponge gels manufactured from pig skin were purchased from Sumitomo Pharma (Osaka, Japan). Cancerous portions of the specimens were minced into approximately 10-mg pieces and the pieces were placed on prepared collagen surfaces in 24-well microplates. The specimens were incubated in the presence of vinorelbine dissolved in RPMI-1640 medium (Sigma, St. Louis, MO, USA) containing 20% fetal calf serum (Invitrogen, Carlsbad, CA, USA) for 7 days at 37˚C under a humidified atmosphere with 5% CO_2. Vinorelbine was provided by Kyowa Hakko Kirin Co. Ltd. (Tokyo, Japan).

After completion of the histoculture, 100 μl of Hanks' balanced salt solution containing 0.1 ml of type I collagenase (0.6 mg/ml) (Sigma) and 0.1 ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (2 mg/ml) (Sigma) were added to each culture well and incubated with the specimens for 16 hours. After removal of the medium, formazan was extracted with 0.5 ml of 100% dimethylsulfoxide, and the absorbance of the solution in each well was measured at 540 nm (control: 630 nm) using a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA). The absorbance per gram of cultured tumor tissue (OD/g) was calculated from the mean absorbance of the tissue specimens in 4 culture wells and the tumor tissue weight determined prior to the culture. The efficacy of the anticancer agent was calculated according to the inhibition rate (IR) using the following formula: IR (%)=(1–mean OD/g of cultured tumor/mean OD/g of control tumor) ×100. The HDRA was regarded as evaluable when the mean absorbance of the extracted formazan at 540 nm of the control tumor was ≥15 per gram. When the IR of the drug was negative, it was regarded as 0 and indicated absolutely no chemosensitivity.

Dose–response curve analysis. Dose–response curve analysis was performed as described previously for a cytotoxic anticancer agent (18, 19). The HDRA was performed for vinorelbine at concentrations of 3.375, 6.75, 12.5, 25, 50, 100, 200 and 400 μg/ml, in order to analyze the dose–response curves for individual patients. Each dose–response curve was calculated using the following formula: $y=A\ [1–1/(1+\exp\{B\ (x–\log C)\}]$, where y is the inhibition rate, x is the logarithm of the drug concentration, A is the maximal response (IR at the maximum concentration), B is the slope factor (slope at ED_{50}) and C is the ED_{50}.

The OD/g values were directly applied to a nonlinear least squares analysis using the following formula for a simplified dose–response curve: $y=\alpha+\beta/\{1+\exp(\gamma x–\delta)\}$, where the values of α, β, γ and δ were obtained. Using these values, the dose–response curve of each specimen was reconstructed according to the following relationships: slope factor=γ, $ED_{50}=10^{(\delta/\gamma)}$ and maximal response=$100\times\beta/(\alpha+\beta)$. The validity of the acquired dose-response curve was judged using the Akaike information criterion (AIC) (20). The dose–response curve was only adopted in specimens with an AIC that was smaller than the AIC of their completely nonresponsive equation ($y=\alpha$).

Histopathologic analysis. Tissue sections were deparaffinized and rehydrated through 100%, 95% and 90% ethanol solutions. Heat-induced antigen retrieval was performed by a 40 min treatment with Target Retrieval Solution (code S2031; Dako, Tokyo, Japan) followed by a 20 min cool-down period at room temperature. The sections were immunostained using an automated staining system (AutostainerPlus; Dako) and incubated with an anti-neuronal class III β-tubulin monoclonal antibody (clone TUJ1; 1:500 dilution; code MMS-435P; Covance Inc., Berkeley, CA, USA) for 30 min at room temperature. Positive reactions were visualized by incubation with a labeled polymer (EnVision+ system; code K4001; Dako) for 30 min at room temperature, followed by incubation with 3,3'-diaminobenzidine (DAB) as a chromogen for 5 min. The sections were counterstained with hematoxylin for 5 min.

All of the sections were examined by an observer (Tatsuya Yoshimasu) who was blinded to the clinical information of the patients, and H-scores were calculated as previously reported (21). The staining intensity was graded on a scale of 0 to 3. The ratio of positively stained tumor cell cytoplasm was calculated for each specimen, and a proportion score was assigned. The proportion score was multiplied by the staining intensity to obtain a final semiquantitative H-score.

Statistical analysis. All the values were calculated as the mean±standard deviation (minimum–maximum). Dose–response relationships were calculated by a nonlinear least squares analysis using the Davidon–Fletcher–Powell algorithm. Relationships between immunohistochemical expression and the inhibition rate in the HDRA were examined using Spearman's correlation coefficient test by rank test and one-factor analyses of variance (ANOVA). Values of $p<0.05$ were considered to be significant.

Results

Dose–response curves of vinorelbine in the HDRA. The HDRA for vinorelbine was successful in all 9 specimens. Fitting of the dose–response curve was judged to be appropriate in all specimens (Figure 1). The mean values (±SD) for the slope factor, ED_{50} and maximal response were 8.7±5.4, 39.0±17.9 μg/ml and 85.5±5.1%, respectively.

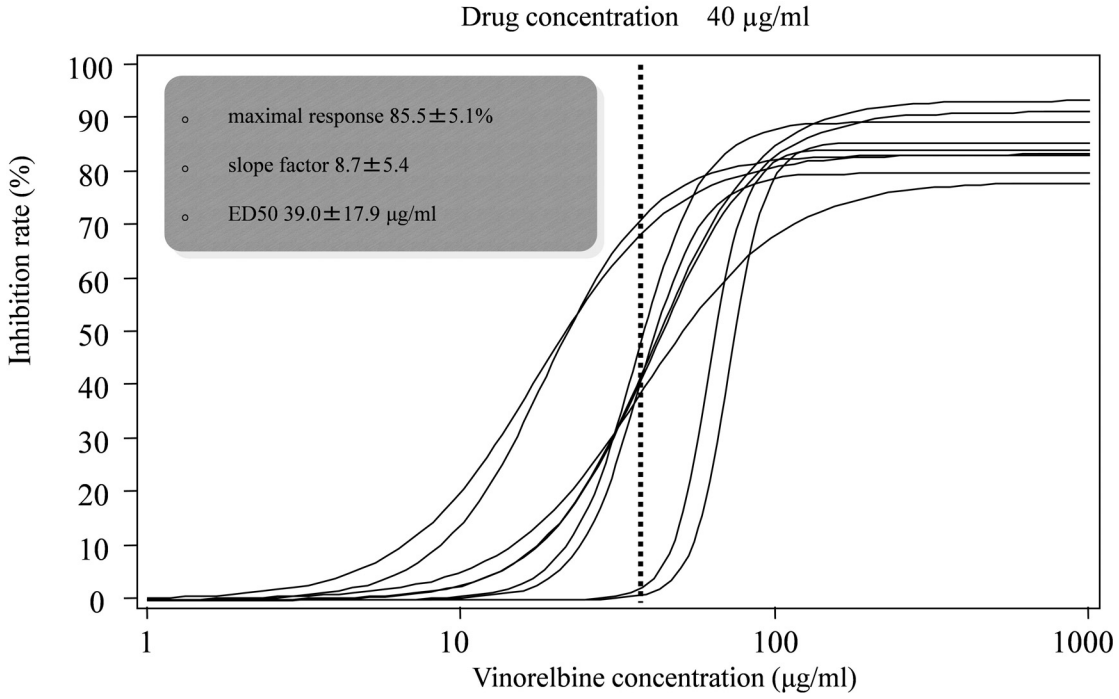

Figure 1. *Dose–response curves of vinorelbine.*

Inhibition rates of vinorelbine in the HDRA. Subsequently, the inhibition rate of vinorelbine in 68 NSCLC specimens was measured at a drug concentration of 40 μg/ml, which is adjacent to the ED_{50}. The inhibition rate of vinorelbine was 26.4±16.2%. The distribution of the inhibition rate for vinorelbine is shown in Figure 2.

To define a cut-off value for the inhibition rate, in order to determine the drug sensitivity, we selected a cut-off value of 35%, based on the response rate for vinorelbine in a historical clinical phase II trial (22, 23, 24). In that phase II trial, the positive rate for vinorelbine was 29.1%-30.2%.

Immunohistochemistry. Sections processed without a primary antibody revealed no background immunoperoxidase staining and were used as negative controls. Immunohistochemical staining of TUBB3 was successful in all specimens. A representative example is shown in Figure 3. The mean H-score was 1.09±1.07. The distribution of the H-score is shown in Figure 4. The specimens were considered to be positive when the H-score was greater than 2.4, and were classified into high and low expression groups on the basis of that criterion. The baseline characteristics of the patients in the two groups are shown in Table I. The patient characteristics were not correlated with the expression of TUBB3 in their tumors.

Expression of TUBB3 and in vitro drug sensitivity. The inhibition rate of vinorelbine was significantly correlated with the expression of TUBB3 by Spearman's correlation coefficient test (r=0.27, p=0.03). The inhibition rate was significantly higher in TUBB3-positive tumors than in TUBB3-negative tumors (p=0.003) (Figure 5).

Table I. *Relationships between TUBB3 expression and patient characteristics.*

	TUBB3		
	Positive (n=15)	Negative (n=53)	*p*-Value
Age (years)			
<70	6	33	0.21
≥70	9	20	
Gender			
Female	4	18	0.83
Male	11	35	
T stage			
1	6	32	0.27
≥2	9	21	
N stage			
0	12	36	0.56
≥1	3	17	
Histology type			
Squamous	3	16	0.63
Adenocarcinoma	10	28	
Other	2	9	

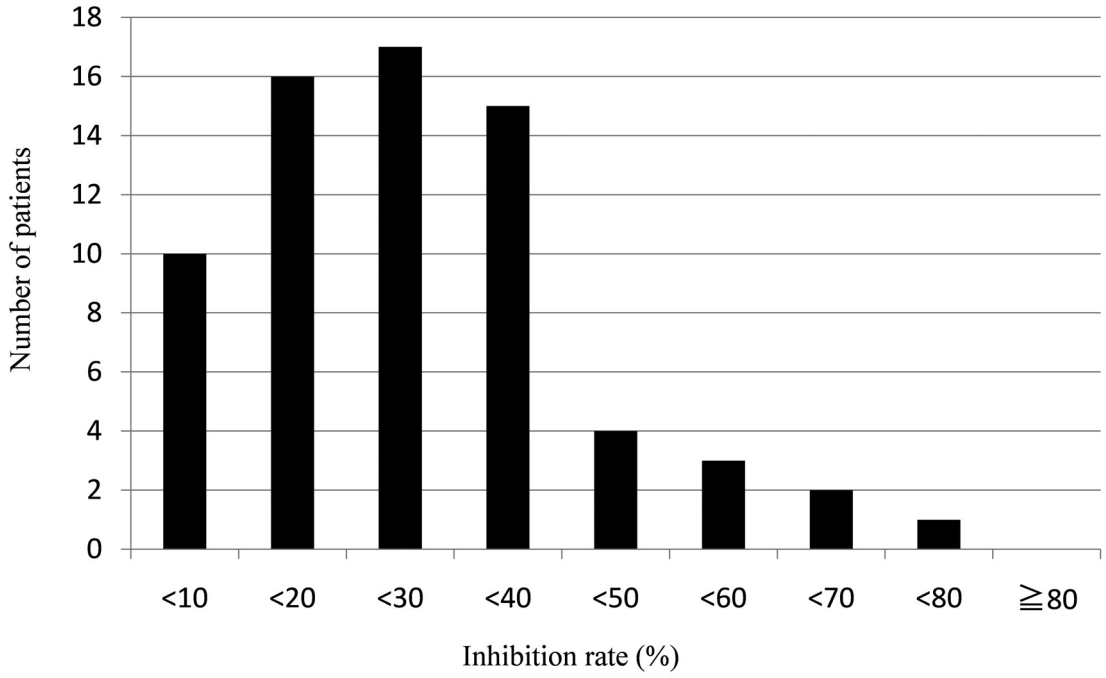

Figure 2. *Distribution of the inhibition rate of vinorelbine.*

Discussion

Vinorelbine is one of the key drugs used in chemotherapy for NSCLC (3, 5, 7). It binds to the β-subunit of tubulin dimers at a distinct region called the vinca-binding domain. Vinorelbine blocks cell division at the metaphase/anaphase junction of mitosis by interfering with the function of the mitotic spindle (25). Recent studies have shown that adjuvant chemotherapy improves survival in completely resected NSCLC.

Sève *et al*. (10) showed that the abundance of TUBB3 was correlated with resistance to treatment and poor clinical outcomes in advanced NSCLC patients treated with a vinorelbine-based regimen. On the other hand, Sève *et al*. (9) found that the recurrence-free survival and overall survival benefits of adjuvant chemotherapy were greater in patients with high TUBB3 levels than in patients with low TUBB3 levels in the JBR.10 trial. They further showed that high levels of TUBB3 expression were associated with poorer recurrence-free survival and overall survival in patients treated by surgery alone (9). These findings suggest that TUBB3 has value as both a predictive and prognostic factor. The different results observed in these clinical trials may reflect the difference between the advanced and adjuvant settings in the trials. There is an intrinsic difficulty in identifying a factor which is solely predictive in a clinical trial, because almost all biomarkers have both predictive and prognostic value.

In theory, when a possible predictive marker for chemotherapy is associated with patient prognosis, it is difficult to prove in a clinical trial whether or not the marker is truly predictive. Some negative prognostic factors are related to tumor aggressiveness, and consequently influence the prognosis of patients. A randomized clinical trial is the most suitable method for evaluating the results of certain clinical treatments. However, when we evaluate particular markers for their predictive value in chemotherapy in a clinical trial, the results of the trial must be interpreted with caution. The results of a clinical trial are based on the status comparisons of the patients between groups, and therefore has both predictive and prognostic values. In particular, when a negative prognostic value is too strong in an advanced trial setting, the clinical benefits of the chemotherapy may be masked by the strong negative influence of the prognostic factor. In this situation, the marker may be judged as a negative predictive marker even if it is actually a positive predictive marker. This may have occurred in the advanced setting trial described by Sève *et al*. (10).

To clarify whether a marker is truly predictive in a clinical trial, it is necessary to compare four groups: a non-treated marker-negative group, a non-treated marker-positive group, a treated marker-negative group and a treated marker-positive group. At present, however, it is not ethically permitted to plan an advanced clinical trial setting with a best supportive care arm because best supportive care is not a standard therapy for NSCLC.

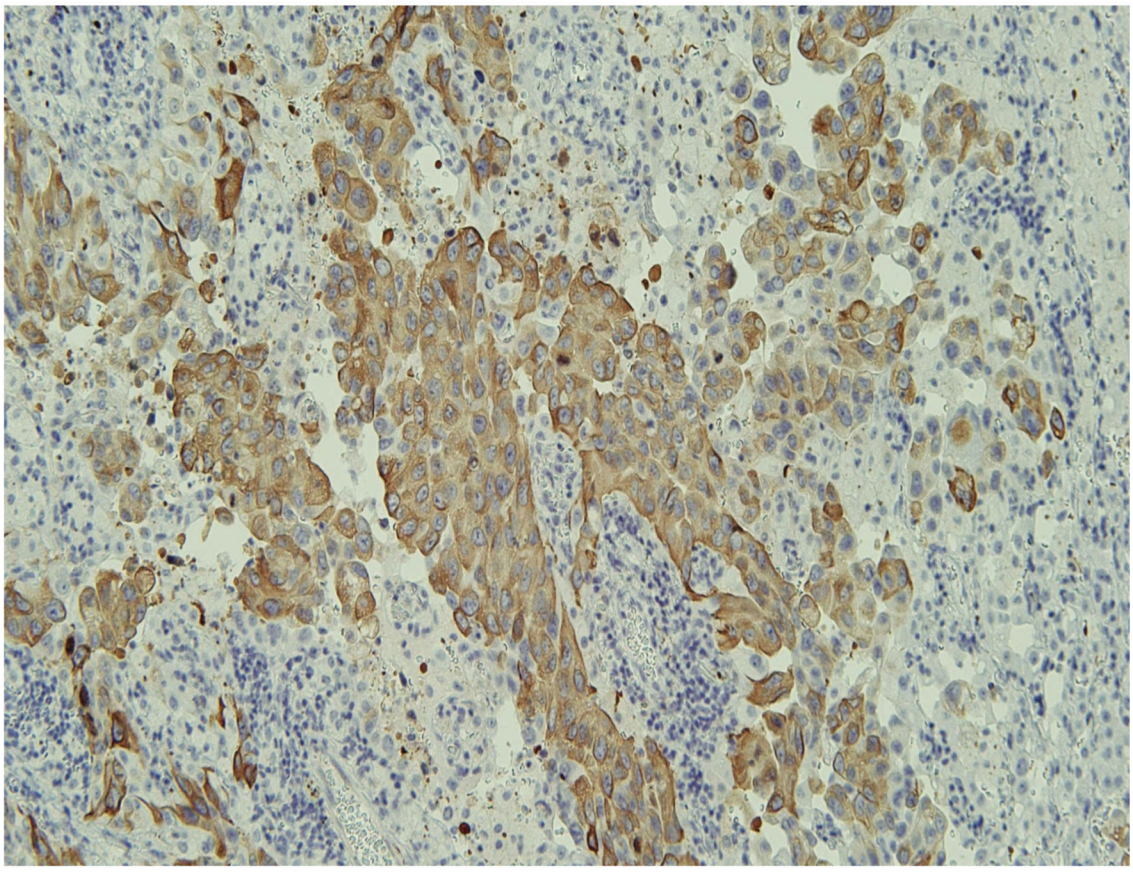

Figure 3. *Representative immunohistochemical staining of TUBB3 in a tumor.*

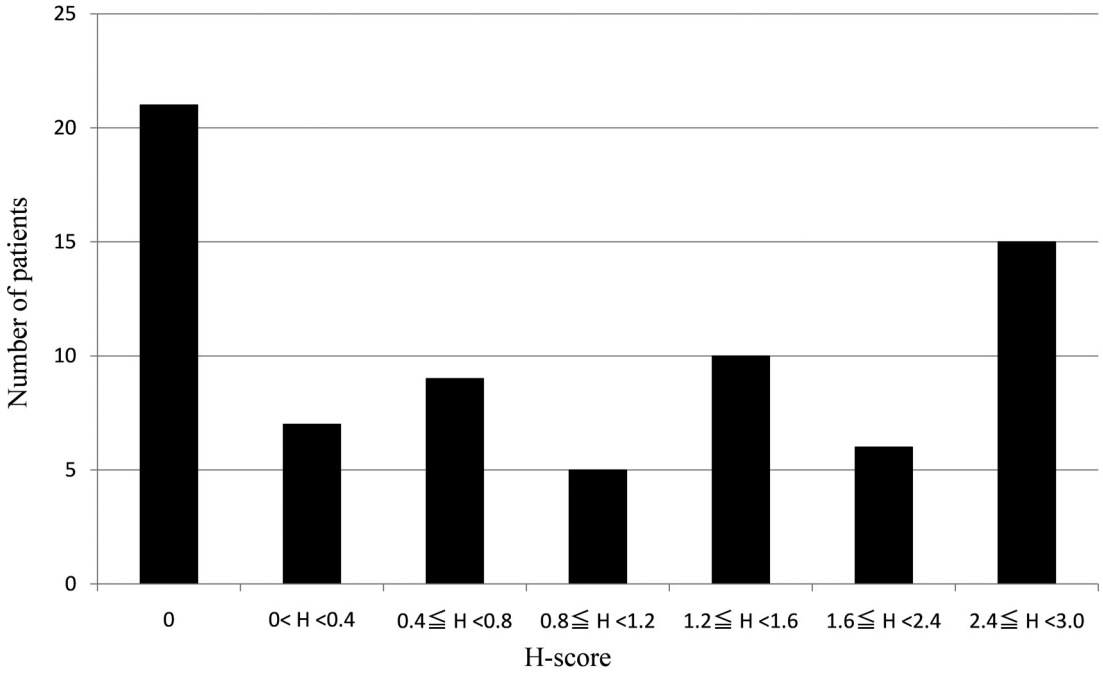

Figure 4. *Distribution of the H-score*.

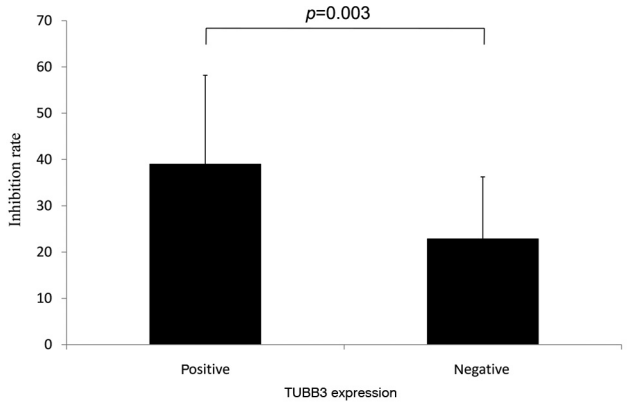

Figure 5. *Correlation between TUBB3 expression and the inhibition rate for vinorelbine in the HDRA.*

To overcome this difficulty, we applied the HDRA in order to examine the predictive value of chemotherapy only. The HDRA is an *in vitro* drug sensitivity test that uses resected specimens. Therefore, the HDRA is not influenced by any of the patient situations after surgery, and we can evaluate the drug sensitivity alone without any prognostic value. We have applied this method for resected NSCLC specimens since 1994. The HDRA shows a high evaluability rate of 97.4% and good predictabilities for clinical response, comprising a positive predictive value of 73.2%, a negative predictive value of 100% and an accuracy of 83.0% (17). We have already reported that excision repair cross-complementation 1 gene (*ERCC1*) expression is well correlated with the inhibition rate for cisplatin in the HDRA in NSCLC (26). Hayashi *et al*. (27) reported that TUBB3 expression correlated well with resistance to docetaxel using the HDRA.

In the present study, we have demonstrated that tumors with high TUBB3 levels exhibit higher chemosensitivity to vinorelbine than tumors with low TUBB3 levels using the HDRA, which can examine sensitivity alone, without any prognostic value. These findings suggest that NSCLC patients with high TUBB3 levels are very likely to benefit from chemotherapy with vinorelbine, and provide support for the results of the JBR.10 trial (9).

In conclusion, our results suggest that the high expression of TUBB3 could be a predictive marker for sensitivity to vinorelbine in patients with NSCLC.

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Received January 17, 2011
Revised February 23, 2011
Accepted February 24, 2011