Predictive Biomarkers and Personalized Medicine

Class III β-Tubulin in Advanced NSCLC of Adenocarcinoma Subtype Predicts Superior Outcome in a Randomized Trial

Adam Christian Vilmar\(^1\), Eric Santoni-Rugiu\(^2\), and Jens Benn Sørensen\(^1\)

Abstract

**Purpose:** Platinum-based doublets are the cornerstone of treatment in advanced non–small-cell lung cancer (NSCLC) and often include vinorelbine or taxanes. A predictive biomarker is greatly needed to select chemotherapy-sensitive patients for these microtubule-interfering agents. Class III β-tubulin (TUBB3) has been shown of value in NSCLC, but evidence is not uniform. Accordingly, we explored the predictive role of TUBB3 in advanced NSCLC.

**Experimental Design:** Four hundred forty-three patients with advanced NSCLC were enrolled in a phase III trial and randomized to vinorelbine- or paclitaxel-containing chemotherapy. Immunohistochemical evaluation of TUBB3 status was mainly done on bioptic material and correlated to response rates, progression-free survival (PFS), overall survival (OS), quality of life (QOL), and toxicity.

**Results:** Two hundred sixty-one (58.9%) patients had representative tissue samples for TUBB3 evaluation. Patients with TUBB3-negative adenocarcinomas had a significantly prolonged PFS and OS when compared with the opposite subgroup (7.87 vs. 6.83 months, \(P = 0.035\) and 14.17 vs. 11.17 months, \(P = 0.018\), respectively). Multivariate analyses revealed an HR of 1.55 (95% CI, 1.04–2.31, \(P = 0.032\)) for TUBB3-positive adenocarcinoma patients. TUBB3-negative adenocarcinoma patients showed a mean QOL decline of −18.25 points (95% CI, −4.28 to −32.22, \(P = 0.013\)) as compared with −3.86 (95% CI, −7.0 to 15.52, \(P = 0.5\)).

**Conclusion:** TUBB3 was of predictive value in adenocarcinoma patients in the largest, randomized advanced NSCLC population published to date. It may be clinically useful in conjunction with other biomarkers, but QOL information should be recorded during validation, as prophylactic intervention may be needed in specific subgroups at risk of toxicity. Clin Cancer Res; 17(15); 5205–14. ©2011 AACR.

Introduction

Lung cancer has traditionally been divided into small-cell and non–small-cell lung cancer (NSCLC), but today’s individualized approach has modified this perspective, resulting in new disease entities. Novel insight into the biology of NSCLC and pharmacogenomics have improved outcome for patients with EGFR mutation–positive tumors (1) and subgroups carrying the EML-4ALK fusion protein (2). However, these patients constitute only a small fraction of the entire population with advanced NSCLC. The remaining vast majority has a life expectancy below 1 year (3).

Platinum-based doublet (PBD) chemotherapy is the cornerstone of treatment in advanced NSCLC, and the emergence of valid biomarkers predicting sensitivity to different treatment options would possibly have a great impact on the overall prognosis. Furthermore, it may potentially spare patients from unnecessary side effects and deterioration in quality of life (QOL). Overall, the various PBDs differ insignificantly concerning response rates (RR), progression-free survival (PFS), and overall survival (OS) but vary more in terms of toxicity (3) and perhaps QOL.

Excision cross-complementation group 1 (ERCC1) is involved in DNA repair as the rate-limiting enzyme of the nucleotide excision repair process and is likely to be used as a predictive marker for cisplatin (4, 5) and carboplatin (6). These compounds are often combined with microtubule-interfering agents (MIA) such as a taxane and vinorelbine. MIs seem to work by suppressing spindle-microtubule dynamics, which induces apoptosis by slowing or blocking the transition from metaphase to anaphase in the mitosis (7). The microtubules are complex polymers consisting of tubulin dimers (one α-tubulin and one β-tubulin) and a variety of microtubule-associated proteins (8).

Upregulation of the spindle-microtubule dynamics during carcinogenesis may lead to a cellular state resistant to MIA-induced apoptosis. Previous studies in lung cancer have suggested that high expression level of the...
brain-specific class III β-tubulin (TUBB3) are associated with paclitaxel resistance both in a preclinical setting (9) and in patients with advanced NSCLC (10). Similar results have been suggested regarding vinorelbine-based regimens (8). In contrast, Séve and colleagues observed a significant better outcome in surgically treated NSCLC patients who were receiving adjuvant cisplatin and vinorelbine expressing high levels of TUBB3 (11).

Other research groups have also reported discordant results (12, 13). The majority of studies have small, heterogeneous patient populations, using different treatment regimens and methodologies for biomarker evaluation. These issues could explain the inconsistency about the predictive value of TUBB3, but another important point may also be lack of consensus on methodology. Both immunohistochemistry (IHC) and quantitative real-time reverse transcriptase PCR (qRT-PCR) are commonly used. Each has different properties, but IHC may possibly discriminate more effectively than qRT-PCR when evaluating biomarker expression in formalin-fixed, paraffin-embedded (FFPE) archival tissue.

Another issue remaining relatively unexplored is the correlation between biomarker expression, toxicity, and QOL. We have previously shown that patient-reported QOL deteriorated significantly among survival-favorable, ERCC1-negative patients, possibly due to increased toxicity (14). Recently, it has been shown that efficacy of same chemotherapy regimens is likely to depend on histopathologic subtype (15) and likewise our group found a significant prediction of efficiency by ERCC1 expression in adenocarcinomas but not in squamous cell carcinomas (5). However, the influence of histopathologic subtypes on TUBB3 impact remains to be explored in advanced NSCLC, though this feature has been suggested in ovarian cancer treated with taxane-based chemotherapy (12, 16).

Taken together, there may theoretically be a predictive value of TUBB3 expression in NSCLC but this issue is not yet firmly elucidated. Accordingly, we used IHC to investigate the impact of TUBB3 expression on clinical endpoints including a possible influence of histopathologic subtypes in a large, homogeneous population of advanced NSCLC patients participating in a randomized chemotherapy trial. Furthermore, we correlated TUBB3 expression to toxicity and QOL.

Materials and Methods

Patients and treatment
A total of 443 chemotherapy-naive patients aged 18 to 75 years with histologically verified inoperable NSCLC, performance status 0 to 2, and normal organ function were included in the study (LU2007) and randomized to regimen A (paclitaxel 180 mg/m² day 1 with gemcitabine 1,000 mg/m² days 1 and 8 every 3 weeks) or regimen B (cisplatin 100 mg/m² day 1 every 3 weeks and weekly i.v. vinorelbine for a maximum of 6 cycles) to examine for superiority in the intensive regimen. Antiemetic therapy was administered according to national guidelines before, during, and after treatment. Patients with brain metastasis were excluded. In total, 428 patients were needed to detect a 30% median survival rate increase with 80% power and 2-sided type 1 error of 5%. Two hundred sixty-one of these patients had sufficient histologic material to be included in the retrospective TUBB3 tumor marker study that was planned during the course of LU2007. Patients gave informed written consent.

The TUBB3 tumor marker study and LU2007 were approved by the Danish National Committee on Biomedical Research Ethics and the Danish Data Protection Agency. Six patients with significant tumor shrinkage also received additional radiotherapy with curative intent following chemotherapy, and one patient had surgery.

Patients were enrolled in LU2007 from December 2000 until June 2007 and were censored as of December 2008. Clinical endpoints in the TUBB3 tumor marker study were RR [according to Response Evaluation Criteria in Solid Tumors (RECIST)], PFS, and OS. All toxicity variables were graded according to the National Cancer Institute Common Toxicity Criteria (version 2.0). Toxicity evaluation was done on day 1 of every cycle until the end of treatment. The following variables were recorded: leukopenia, thrombocytopenia, nausea/vomiting, nephrotoxicity, neurotoxicity, worst other toxicity (e.g., fatigue, alopecia, myalgia), number of febrile episodes, and number of bleeding episodes.

QOL evaluation
QOL was assessed with the European Organization for the Research and Treatment of Cancer (EORTC) core Quality of Life Questionnaire (QLQ-C30) and with the Lung Cancer Questionnaire module QLQ-LC13 that is a trial-specific symptom checklist. Both questionnaires have been validated (17, 18), are widely used, and were...
completed by the patients at baseline (before random assignment) and before each chemotherapy cycle. Only fully completed questionnaires were included. These were compared between baseline and before cycle 3, 4, 5, or 6 depending on availability, as the number of completed questionnaires was limited.

**Tissue samples**
Archival paraffin blocks containing formalin-fixed NSCLC tissue from the 443 patients enrolled in LU2007 were mainly obtained from the Departments of Pathology at the University Hospitals of Copenhagen, Odense, and Aalborg. Two hundred sixty-one (58.9%) patients had sufficient biopsy material for TUBB3 evaluation. The histologic samples consisted of 37 surgical resections, 195 biopsies (117 endoscopic, 57 mediastinoscopic, and 21 transthoracic biopsies), and 27 miscellaneous (local biopsies from metastatic lesions including 6 clot preparations of cytologic specimens (pleural/pericardial effusions, fine needle aspirations)). Information on sample type was unavailable for 2 patients. Tissue samples were obtained from the primary lesion in 158 patients, from pulmonary, bronchial, or mediastinal lymph nodes in 20 patients and from distant metastatic lesions in 35 patients. The remaining 48 patients had combinations of these 3 categories.

**Immunohistochemical preparation of tissue samples**
Thick FFPE sections (4 μm) were cut and mounted on coated glass slides. From each tissue specimen, sections stained with hematoxylin–eosin were histologically evaluated for verification of diagnosis and eligibility for immunohistochemical analysis.

Tissue sections for TUBB3 immunostainings were antigen-retrieved with DAKO Target Retrieval Solution (code S1700) high pH for 20 minutes at 97°C, using a DAKO PT link machine according to the manufacturer’s instructions (Tubulin DAKO Target Retrieval high pH 9).

The sections were then processed with the Envision Flex+ kit (DAKO K 8002; DAKO) blocking endogenous peroxidase activity for 5 minutes and then incubating for 20 minutes with the antibody (Covalab catalogue no. mab0054-1, clone TUJ-1/TubIII/4G3; Covalab; diluted 1:400) against human TUBB3. The reactions were visualized by incubation with Envision Linker (mouse or rabbit) for 15 minutes, followed by Envision Flex+ horseradish peroxidase for 20 minutes, and finally diaminobenzidine for 10 minutes. The sections were counterstained with Mayer’s hematoxylin for 1 minute.

**Immunohistochemical evaluation for TUBB3 expression**
TUBB3 cytoplasmatic expression was analyzed as previously described (4, 5). Briefly, 2 observers (A.C. Vilmar and E. Santoni-Rugiu) blinded to the clinical data independently evaluated TUBB3 immunostaining of the eligible tissue samples under a light microscope at a magnification of 400×. A semiquantitative H-score for each tissue sample was calculated multiplying the cytoplasmatic staining intensity in cells (0, no expression; 1, weak expression; 2, moderate expression; 3, strong expression) by a proportion score based on the percentage of stained cells (0 if 0%, 0.1 if 1%–9%, 0.5 if 10%–49%, and 1.0 if ≥50%). Brain tissue was used as a positive control (corresponding to an intensity of 3) and similarly normal nerves or neuronal cells in the sections were used as an internal control (corresponding to intensity 2) on the tissue sections. Omission and substitution of TUBB3 Ab-2 with unspecific immunoglobulin were used as negative controls. The proportion score was determined by counting at least 100 tumor cells per sample. In the event of discordance between the observers, the tissue section was reevaluated to reach consensus.

The cutoff point was chosen a priori as the median value of all the observed H-scores to separate TUBB3-positive (H-score > median) from TUBB3-negative (H-score ≤ median) tumors. The highest TUBB3 value was used when more than one tissue sample per patient were available.

**Statistical analyses**
All statistical analyses were done with the use of SPSS software (SPSS version 18.0). Associations between categorical variables were compared by χ² test or Fisher's exact test. Survival curves are shown as Kaplan–Meier plots and compared by log-rank analyses. Exclusion of independent prognostic variables was analyzed by Cox regression that yielded HRs.

The EORTC QOL 30-item QLQ-C30 questionnaire and the lung cancer–specific 13-item LC-13 questionnaire were scored according to the EORTC guidelines (19). Higher scores indicated better functioning in functional domains, whereas higher scores in the symptom scales indicated deterioration. A change in score of 10 or more points from baseline was defined as clinically significant (19). To compare means, Student’s t test for paired samples was used. P values below 0.05 were considered statistically significant.

**Results**

**Characteristics of the population**
A total of 443 patients were randomized in the chemotherapy trial (LU2007) without statistical significant survival difference between the triplet regimen and the standard doublet regimen. Two hundred sixty-one (58.9%) of the 443 patients originally randomized to the 2 treatment arms could be immunohistochemically evaluated for TUBB3 expression. The remaining 41.1% of patients could not be evaluated for TUBB3 because of unavailable tissue samples or lack of tumor tissue left in the blocks (Fig. 1).

As shown in Table 1, a significant difference in TUBB3 expression was observed between the 2 treatment arms, with a larger fraction of patients having TUBB3-positive tumors when treated with the intensive regimen A (62.5% positive vs. 47% negative), in contrast to what was observed in patients receiving regimen B (37.5% positive vs. 53% negative; P = 0.013). Also, male patients...
dominated the population with TUBB3-negative tumors (68.5% vs. 50%, \(P = 0.003\)). The majority of the patients (90.4%) were in performance status (PS) 0 or 1. Furthermore, a significant difference in TUBB3 expression was observed between the histopathologic subtypes of NSCLC, and squamous cell carcinomas tended to be TUBB3 negative (41.1% vs. 11.6% positive, \(P = 0.000\); Table 1) whereas most adenocarcinomas were TUBB3 positive (56.3% vs. 37.6% negative).

**Immunohistochemical evaluation for TUBB3 expression**

A median H-score value of 1 was observed that separated the population into 149 (57.1%) TUBB3-negative patients (H-score ≤ 1) and 112 (42.9%) TUBB3-positive patients (H-score > 1). Considerable variation in the intratumoral immunostaining intensity and frequency of positive cells was observed (Fig. 2).

**Outcome in the general population (n = 443)**

Overall, RRs were 50.5%, overall PFS was 6.3 months (95% CI, 5.8–6.7), and median OS 11.1 months (95% CI, 10.0–12.2).

**Outcome in the TUBB3 tumor marker study population (n = 261)**

No difference in RRs was found according to TUBB3 expression (\(P = 0.667\)). Concerning PFS and OS, no significant difference was observed when comparing patients with TUBB3-negative tumors with the subgroup with positive tumors (\(P = 0.229\) and 0.336, respectively; Fig. 3).

**Outcome in patients with adenocarcinomas according to TUBB3 expression (n = 119)**

There was no significant difference in RR (\(P = 0.092\)). Patients with TUBB3-negative adenocarcinomas had a significantly prolonged PFS and OS when compared with the opposite subgroup (7.87 vs. 6.83 months, \(P = 0.035\); 14.17 vs. 11.17 months, \(P = 0.018\), respectively; Table 2 and Fig. 3). OS in regimen B (cisplatin and vinorelbine) was 18.76 months in TUBB3-negative adenocarcinoma patients and 11.43 months in TUBB3-positive adenocarcinoma patients (\(P = 0.069\)). In regimen A (cisplatin, gemcitabine, and taxol), the corresponding OS was 12.43 and 11.0, respectively (\(P = 0.249\)).

**Survival according to histopathology and TUBB3 expression (n = 261)**

When patients with TUBB3-negative tumors were divided into adenocarcinomas, squamous cell carcinomas, and other subtypes [large-cell carcinomas, not otherwise specified (NOS) and adenosquamous carcinomas combined], a significant difference in OS was found (14.17, 11.20, and 6.10 months, respectively; \(P = 0.000\)), whereas this was not the case for patients with TUBB3-positive tumors (11.17, 6.73, and 11.67, respectively; \(P = 0.764\); Fig. 3).

**Multivariate analyses of survival in patients with adenocarcinomas (n = 119)**

A Cox proportional hazard model was fitted to test specific variables in multivariate analyses. Together with PS, TUBB3-positive tumors emerged as the only other significant prognostic variable with an HR of 1.55 (95% CI, 1.04–2.31, \(P = 0.032\)).

**Toxicity and QOL in patients with adenocarcinomas (n = 45)**

Patients with TUBB3-negative adenocarcinomas suffered significantly more episodes of febrile leukocytopenia than patients with TUBB3-positive adenocarcinomas (13 vs. 3 patients experiencing 1–2 episodes, respectively; \(P = 0.010\)).
Patients with TUBB3-negative adenocarcinomas \((n = 18)\) showed a mean decline in QOL from baseline of \((-18.25 \pm 4.28, P = 0.013)\) as compared with \((-3.86 \pm 7.0, P = 0.5)\) for patients in the opposite subgroup \((n = 27; \text{Fig. 4})\).

**Discussion**

Individualizing antineoplastic treatment in advanced NSCLC is becoming daily practice by the use of predictive biomarkers. Patients with nonsquamous histology, prone to express low levels of thymidylate synthase, will often benefit from pemetrexed-based regimen (15), whereas patients with *EGFR* mutation-positive tumors should be treated upfront with gefitinib (1). The backbone of chemotherapy is PBDs, often including vinorelbine, taxol, or paclitaxel, and a reliable biomarker for these compounds is thus greatly needed. Evidence about TUBB3 has been promising in some studies (8, 10) but not in others (11, 13). Accordingly, we explored its predictive value in the largest patient population with advanced NSCLC published to date, randomized to MIA-containing chemotherapy with either vinorelbine or paclitaxel.

**TUBB3 was found to be of predictive value in our population. However, this feature was restricted to specific histopathologic subgroups and reached significance only in patients with adenocarcinomas.**

<table>
<thead>
<tr>
<th>Table 1. Characteristics of the population in the TUBB3 tumor marker study</th>
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<tr>
<td>(n = 149)</td>
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<td>Treatment</td>
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<td>Regimen A</td>
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<td>Regimen B</td>
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<td>Lactate dehydrogenase levels, units/L</td>
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<td>Performance expression (WHO)</td>
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<tr>
<td>Histologic subtype (WHO)</td>
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<tr>
<td>Adenocarcinoma</td>
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<td>Squamous cell carcinoma</td>
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<td>Large-cell carcinoma</td>
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<td>NOSa</td>
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<td>Adenosquamous carcinoma</td>
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<td>Stage</td>
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<td>IIIA/N2 (inoperable)</td>
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<td>IIIA/T3 (inoperable)</td>
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<td>IIIB (dry)</td>
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*The histologic subtype of NSCLC could not be classified on the basis of the available bioptic material.*

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histopathologic subtype proved to be of great significance in patients with TUBB3-negative tumors. To the best of our knowledge, the fact that the predictive role of TUBB3 depends on histopathology represents a novel observation in NSCLC but is not without precedent.

Ferrandina and colleagues found a significantly worse OS ($P = 0.002$) in 62 patients with ovarian serous adenocarcinomas expressing high TUBB3 protein levels and treated with platinum/paclitaxel chemotherapy (16). In a similar setting, Aoki and colleagues showed a significantly improved prognosis in their population of 44 patients with ovarian clear cell adenocarcinomas and high TUBB3 protein expression (12).

To our knowledge, the impact of histopathology has not been explored in advanced NSCLC and could explain some of the inconsistencies observed in the reported results and conclusions. A significant poorer outcome ($HR = 1.75, P = 0.012$) was found by Seve and colleagues in 93 patients with tumors expressing high levels of TUBB3 and treated with vinorelbine-based chemotherapy. These patients had a strikingly low PFS and OS (3 and 5.4 months, respectively), possibly due to a predominance of patients with squamous cell and large-cell carcinomas (8). The group reproduced the results among 47 patients treated with paclitaxel-based chemotherapy (10). In this study, the IHC evaluation technique had been modified but did not include the intensity of staining used by other groups on promising biomarkers such as ERCC1 (4–6, 20).

However, this method (H-score) was applied when the same group analyzed the prognostic and predictive values of TUBB3 in an adjuvant NSCLC setting of 265 patients (11). One hundred forty patients received cisplatin/vinorelbine, and the subgroup with tumors expressing high TUBB3 protein levels surprisingly showed significantly improved recurrence-free survival (RFS; $P = 0.002$) and a trend toward improved OS ($P = 0.07$), when compared with the observation arm. However, Cox regression analysis was not significant for interaction between TUBB3 expression and chemotherapy. No information on the histopathologic subtypes in the population of the chemotherapy arm was available, which may explain this observed lack of predictive value. Furthermore, the prognostic value (in the group randomized to observation alone) of high-level TUBB3 expression yielded HRs for RFS and OS of 1.78 ($P = 0.03$) and 1.42 ($P = 0.07$), respectively (11), findings confirmed by Koh and colleagues (21) and Reiman and colleagues (22).

This last mentioned cross-validation study of 1,149 patients could not prove TUBB3 of predictive value in the adjuvant chemotherapy group in accordance with Seve and coworkers (11). The observation could be due to the histopathologic constitution of their population characterized by squamous cell carcinomas (47%) and accordingly male predominance (80%; ref. 22) compared with our population (28% and 61%, respectively). Indeed, squamous cell carcinoma patients are prone to a poor prognosis despite TUBB3 status according to our results (discussed in detail later). Azuma and colleagues also failed to show TUBB3 of predictive value in a small group of patients ($n = 34$), with locally advanced NSCLC, treated with a combination of cisplatin/docetaxel and concurrent thoracic irradiation (13).

Taken together, there are a number of issues that could explain the discordance observed in the literature in addition to the potential impact of histopathology. The patient populations are generally small aside from Reiman and colleagues, increasing the risk of random observations and widening the CIs. The available tissues are often small FFPE biopsy samples that always raise the unavoidable possibility of tumor heterogeneity and thus of variation in IHC coloration (Fig. 2). Different scoring techniques are used. Today, there is growing consensus on the H-score and other methods that take into account both the intensity and frequency of immunostaining. Furthermore, the observed differences in OS in the adenocarcinoma subtype according to TUBB3 expression in our study could mainly be ascribed to patients receiving
the vinorelbine-containing regimen B whereas the taxol-containing regimen B was without major impact. This observation may suggest that TUBB3 expression is a less suited biomarker for the prediction of taxane resistance. Indeed, the majority of studies exploring these compounds have reported varying results (12, 13, 16).

Figure 3. PFS and OS in months for the entire patient population (top) of patients with advanced NSCLC and patients with adenocarcinomas (middle) stratified according to the TUBB3 tumor marker expression. OS in months for the 149 TUBB3-negative (bottom left) and 112 TUBB3-positive (bottom right) patients with advanced NSCLC according to histopathologic subtype. Neg, negative; Pos, positive; Adeno, adenocarcinomas; Other, other carcinomas (NOS, large-cell, and adenosquamous carcinomas); Squamous, squamous cell carcinomas.

Table 2. Treatment outcome in the TUBB3 tumor marker study with adenocarcinoma subtype

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<th>TUBB3 negative</th>
<th>TUBB3 positive</th>
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<tr>
<td></td>
<td>n</td>
<td>%</td>
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<tr>
<td>Response according to RECIST (n = 99)</td>
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</tr>
<tr>
<td>Progressive disease</td>
<td>4</td>
<td>8</td>
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<tr>
<td>No change</td>
<td>28</td>
<td>56</td>
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<tr>
<td>Partial response</td>
<td>18</td>
<td>36</td>
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<tr>
<td>Complete response</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Median DFS (n = 119), mo</td>
<td>7.87</td>
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<tr>
<td>Median OS (n = 119), mo</td>
<td>14.17</td>
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Alteration in self-reported QOL among 45 patients with advanced NSCLC (adenocarcinoma subtype) stratified according to the TUBB3 tumor marker expression.

Another important area of clinical research in advanced NSCLC is the maintenance of a stable QOL during treatment. Improvement of outcome through the use of biomarkers is not possible if QOL is compromised in subgroups of fragile patients often burdened with comorbidity. In accordance with our previous study addressing this area (14), we have shown that the survival-favorable, TUBB3-negative adenocarcinoma patients suffered a larger deterioration in self-reported QOL, perhaps due to more treatment-induced toxicity, than the TUBB3-positive adenocarcinoma patients. It has previously been shown that increased chemotherapy-induced hematologic toxicity and superior outcome are correlated until a certain threshold (23, 24). If our findings are reproducible, then more focus should be addressed to prophylactic intervention in the subgroups at risk, that is, adenocarcinoma patients having TUBB3-negative tumors. This could be achieved by randomizing biomarker panel-stratified patients to intensive prophylactic regimen or standard of care. In addition, it could prove to be of great value to correlate TUBB3 status in normal and tumor tissue from these patients, which may explain the enhanced tendency toward toxicity.

The major limitation of our QOL analyses is the large number of unavailable questionnaires. Concerning the biomarker study, some other issues should be addressed warranting cautious interpretation of our findings. These include relatively high number of unavailable tissue samples, the fact that the TUBB3 tumor marker study was not preplanned in the randomized treatment trial, and the uneven distribution of patients in terms of treatment regimens and gender. Furthermore, the prognostic value of TUBB3 could influence our results.

One may question the clinical relevance of the applied cisplatin-based regimen. However, our results point toward the usefulness of these compounds in the future management of advanced NSCLC when customized chemotherapy is introduced. Indeed, cisplatin, combined with third-generation cytotoxics such as paclitaxel and vinorelbine, has proven superior in terms of survival compared with carboplatin, especially in patients with nonsquamous histology (3). Our results suggest that these cisplatin combinations may be even more effective in patients selected according to TUBB3 status. Finally, we observed a high number of responders among patients with TUBB3-positive adenocarcinomas (Table 2). However, 11 more patients in this subgroup were treated with the intensive regimen A, perhaps inducing an increased response, in comparison with the patients with TUBB3-negative tumors.

The occurrence of the many responders among patients with TUBB3-positive tumors is likely to have diminished the HR of 1.55 for TUBB3-positive expression, but it has also raised a different issue. As mentioned, we have shown increased PFS and OS in the TUBB3-negative subgroup with adenocarcinomas but no difference in RR, which is the evaluation method of choice to determine chemotherapy efficacy. Additional prognostic features of TUBB3 downregulation, apart from predictive capabilities, could explain this observation. McCarroll and colleagues have recently shown that RNA interference knockdown of TUBB3 decreases anchorage-independent growth of NSCLC cells, a hallmark property of tumorigenic potential of the cancer cell, and, in addition, leads to sensitivity to cisplatin, carboplatin, and paclitaxel (25). Taken together, the net effect of the TUBB3 expression in our patient population is likely to be a combination of increased chemotherapy sensitivity and less aggressive behavior of the tumors.

As shown in Figure 3, there is a significant variation in survival among patients with TUBB3-negative tumors when divided into the 3 main histopathologic subtypes. The patients with TUBB3-negative adenocarcinomas seem to benefit the most from chemotherapy as compared with patients with squamous cell carcinomas or other subtypes (predominantly NOS).

Most adenocarcinomas are located in the periphery of the lung and believed to arise from bronchioloalveolar epithelium. A smaller fraction are centrally placed and believed to arise either from the epithelium of large bronchi or from peribronchial glands. These central adenocarcinomas tend to be more aggressive and their pathogenesis remains to be elucidated. In contrast, there are 2 main pathways proposed for the development of the peripheral adenocarcinoma (26).

One is the EGFR-mutated/amplified pathway, often found in nonsmokers, whereas the other is seen in smokers and has the following features. These tumors often harbor K-ras and p53 mutations and DNA methylation of the tumor suppressor gene p16<sup>INK4A/CDK2A</sup>, as compared with patients with squamous cell carcinomas, carrying a huge sequential accumulation of genetic and molecular abnormalities such as 9p and 3p losses, VEGF overexpression, p16

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**Figure 4.** Alteration in self-reported QOL among 45 patients with advanced NSCLC (adenocarcinoma subtype) stratified according to the TUBB3 tumor marker expression.
and p53 inactivation, as well as D1 and Bcl-2 overexpression among others (26). The relatively few number of pathogenetically significant hits (so-called driver mutations) in the adenocarcinomas genome may explain its relative chemotherapy-sensitive phenotype as opposed to the resistant squamous cell carcinomas with multiple loss and gain of functions, allowing it to evade apoptosis.

Apoptosis is the main effector triggered by chemotherapy, regulated by different proteins such as caspase 9 and Bcl-2, which hypothetically could be almost obsolete in the last subgroup of patients, primarily with NOS TUBB3-negative carcinomas. These tumors, by definition, are most often undifferentiated and likely to carry multiple mutations in both tumor suppressor genes and oncogenes. Such features may be responsible for the treatment resistance and a poor prognosis often characterizing NOS tumors, despite its otherwise survival-favorable, TUBB3-negative expression (Fig. 3), a notion supported by similar results concerning ERCC1 (5). In contrast, no difference in outcome is observed when looking at the 3 TUBB3-positive histopathologic subtypes in the same figure. This observation supports the aforementioned notion by McCarroll and colleagues (25), stating that TUBB3-positive tumors carry a dismal prognosis that possibly overwrites the potential predictive influence of histopathologic differences.

In conclusion, we have shown the predictive value of TUBB3 in advanced NSCLC patients having adenocarcinoma subtype when treated with MIAs, although an effect of TUBB3-negative expression is questionable in other histopathologic subgroups. TUBB3 may add to the combined predictive power of a biomarker panel, consisting of a number of different markers. QOL information should be recorded during prospective validation of such markers, as prophylactic intervention may be needed in certain subgroups possibly due to increased toxicity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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