Original Article

Class III β-tubulin in advanced NSCLC of adenocarcinoma subtype predicts superior outcome in a randomized trial

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Statement of translational relevance

Our group confirms the predictive role of betatubulin III (TUBB3) in the largest randomized chemotherapy trial published to date in advanced non-small cell lung cancer (NSCLC). However, the predictive influence varies with histopathology and reaches significance in adenocarcinoma patients only. Furthermore, our results suggest that this subgroup seems to suffer a significant deterioration in quality of life, possibly due to more chemotherapy induced toxicity.

These novel observations point towards careful patient selection based on histopathology during prospective validation of TUBB3 and careful observation since prophylactic intervention may be necessary in these survival favourable but toxicity vulnerable subgroups.

TUBB3 may be clinically useful in conjunction with other biomarkers like Excision cross complementation group 1 (ERCC1) that potentially could improve outcome in this devastating disease.
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 demonstrated of value in NSCLC but evidence is not uniform. Accordingly, we explored the predictive role of TUBB3 in advanced NSCLC.

Experimental Design
443 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 performed on bioptic material and correlated to response rates, progression-free survival (PFS), overall survival (OS), quality of life (QOL) and toxicity.

Results
261 (58.9%) patients had representative tissue samples for TUBB3 evaluation. Patients with TUBB3-negative (neg) adenocarcinomas had a significantly prolonged PFS and OS when compared to 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 a hazard ratio of 1.55 (95% CI 1.04-2.31, P = 0.032) for TUBB3-pos adenocarcinoma patients. TUBB3-neg adenocarcinoma patients showed a mean QOL decline of -18.25 points (95% CI - 4.28 - 32.22, p = 0.013) as compared to -3.86 (95% CI -7.0-15.52, P = 0.5).

Conclusion
TUBB3 was of predictive value in adenocarcinoma patients in the largest randomized advanced NSCLC population published to date. TUBB3 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.
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 only constitute a small fraction of the entire population with advanced NSCLC. The remaining vast majority has a life expectancy below one 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 (NER) 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 (MIAs) like taxane and vinorelbine. MIAs 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 alfa-tubulin and one beta-tubulin) and a variety of microtubule-associated proteins (8).
Up-regulation 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, Sève et al. observed a significant better outcome in surgically treated NSCLC patients expressing high levels of TUBB3 receiving adjuvant cisplatin and vinorelbine (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 regarding 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 polymerase chain reaction (qRT-PCR) are commonly used. Each one 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 demonstrated that patient-reported QOL deteriorated significantly among survival favourable 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 histopathological 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 histopathological 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 histopathological 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 & treatment

A total of 443 chemotherapy-naive patients aged 18-75 years with histologically verified inoperable NSCLC, performance expression 0-2 and normal organ function where included in the study (LU2007) and randomized to regimen A (paclitaxel 180 mg/m² and cisplatin 100 mg/m² day 1 with gemcitabine 1000 mg/m² day 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 prior, during and after treatment. Patients with brain metastasis were excluded. Totally 428 patients were needed to detect a 30% median survival increase with 80% power and two-sided type-1 error of 5%. 261 of these patients had sufficient histological 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 RECIST-criteria), PFS and OS. All toxicity variables were graded according to the National Cancer Institute Common Toxicity Criteria (CTC) (Version 2.0). Toxicity evaluation was performed at day 1 of every cycle until the end of treatment. The
following variables were recorded: Leukocytopenia, thrombocytopenia, nausea/vomiting, nephrotoxicity, neurotoxicity, worst other toxicity (e.g., fatigue, alopecia, myalgia etc), 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 of 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. 261 patients (58.9%) had sufficient biopsy material for TUBB3 evaluation. The histological samples consisted of 37 surgical resections, 195 biopsies (117 endoscopical, 57 mediastinoscopical and 21 transthoracic biopsies), and 27 miscellaneous (local biopsies from metastatic lesions including 6 clot-preparations of cytological specimens (pleural/pericardial effusions, fine needle aspirations)). Information on sample type was unavailable for two patients. Tissue samples were obtained from the primary lesion in 158 patients, from pulmonal-,
or mediastinal lymph nodes in 20 patients and from distant metastatic lesions in 35 patients. The remaining 48 patients had combinations of these three categories.

**Immunohistochemical preparation of tissue samples**

4 μm-thick FFPE sections were cut and mounted on coated glass slides. From each tissue specimen, sections stained with hematoxylin-eosin (HE) were histologically evaluated for verification of diagnosis and eligibility for IHC 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 manufacturer’s instructions (Tubulin Dako Target retrieval high pH 9).

The tissue sections were then processed with the Envision Flex + kit (DAKO K8002, DAKO, Denmark) blocking endogenous peroxidase activity for 5 minutes (min) and then incubating for 20 min with the antibody (Covalab cat. mab0054-1, Clone TUJ-1/TubIII/4G3, Covalab, U.K) (diluted 1:400) against human TUBB3. The reactions were visualized by incubation with Envision Linker (Mouse or Rabbit) for 15 min followed by Envision Flex+ horseradish peroxidase for 20 min and finally diaminobenzidine for 10 min. The sections were counterstained with Mayer’s hematoxylin for 1 min.
**Immunohistochemical evaluation for TUBB3-expression**

TUBB3 cytoplasmatic expression was analyzed as previously described (4;5). Briefly, two observers (A.V., E.S.-R.) blinded to the clinical data independently evaluated TUBB3 immunostaining of the eligible tissue samples under a light microscope at a magnification of 400x. 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% to 9%, 0.5 if 10% to 49%, and 1.0 if 50% or more). Brain tissue was used as positive control (corresponding to an intensity of 3) and similarly normal nerves or neuronal cells in the sections were used as internal control (corresponding to intensity 2) on the tissue sections. Omission and substitution of TUBB3 Ab-2 with unspecific immunoglobulin were used as negative control. 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 re-evaluated to reach consensus.

The cut-off point was chosen *a priori* as the median value of all the observed H-scores to separate TUBB3-positive (pos) (H-score > median) from TUBB3-negative (neg) (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 performed with the use of SPSS-software (SPSS version 18.0). Associations between categorical variables were compared by Chi-square test or Fisher’s exact test. Survival curves are shown as Kaplan-Meier plots and compared by log-rank analyses. Examination for independent prognostic variables was analysed by Cox regression that yielded hazard ratios.

The EORTC quality of life 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 while higher scores in the symptom scales indicated deterioration. A change in score of ≥10 points from baseline was defined as clinically significant (19). To compare means the 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. 261 patients (58.9%) of the 443 patients originally randomized to the two treatment arms could be immunohistochemically evaluated for TUBB3-expression. The remaining 41.1% of patients could not be evaluated for TUBB3 due to unavailable tissue samples or lack of tumor tissue left in the blocks (Figure 1).

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

Immunohistochemical evaluation for TUBB3-expression

A median H-score value of 1 was observed that separated the population into 149 (57.1%) TUBB3-neg patients (H-score ≤ 1) and 112 (42.9%) TUBB3-pos patients (H-score > 1). Considerable variation of the intratumoral immunostaining intensity and frequency of positive cells were observed (Figure 2).
**Outcome in the general population (n = 443)**

Overall RR were 50.5%, overall PFS was 6.3 months (95% Confidence interval (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 RR was found according to TUBB3 expression (P = 0.667). Concerning PFS and OS no significant difference was observed when comparing patients with TUBB3-neg tumors to the subgroup with positive tumors (P = 0.229 and 0.336, respectively) (Figure 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-neg adenocarcinomas had a significantly prolonged PFS and OS when compared to the opposite subgroup (7.87 vs 6.83 months, P = 0.035 and 14.17 vs 11.17 months, P = 0.018, respectively) (Table 2 and Figure 3). OS in regimen B (cisplatin and vinorelbine) was 18.76 months in TUBB3-neg adenocarcinoma patients and 11.43 months in TUBB3-pos adenocarcinoma patients (P=0.069). In regimen A (cisplatin, gemcitabine and taxol) the corresponding OS were 12.43 and 11.0, respectively (P=0.249).

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

When patients with TUBB3-neg 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), while this was not the case for patients with TUBB3-pos tumors (11.17, 6.73 and 11.67, respectively (P = 0.764) (Figure 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 performance status, TUBB3-pos tumors emerged as the only other significant prognostic variable with a hazard ratio (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-neg adenocarcinomas suffered significantly more episodes of febrile leucocytopenia when compared to patients with TUBB3-pos adenocarcinomas (13 vs 3 patients experiencing 1-2 episodes, respectively (P= 0.010). Patients with TUBB3-neg adenocarcinomas (n = 18) showed a mean decline in QOL from baseline of -18.25 points (95% CI - 4.28 - -32.22, p = 0.013) as compared to -3.86 (95% CI -7.0-15.52, P = 0.5) for patients in the opposite subgroup (n = 27) (Figure 4).
**Discussion**

Individualizing antineoplastic treatment in advanced NSCLC is becoming daily practice by the use of predictive biomarkers. Patients with non-squamous histology, prone to express low levels of thymidylate synthase, will often benefit from pemetrexed based regimen (15), while patients with EGFR-mutation positive tumors should be treated up-front with gefitinib (1). The backbone of chemotherapy are PBDs, often including vinorelbine, taxol or paclitaxel and a reliable biomarker for these compounds is thus greatly needed. Evidence regarding 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 of predictive value in our population. However, this feature was restricted to specific histopathological subgroups and reached significance only in patients with adenocarcinomas. Furthermore, the histopathological subtype proved 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 co-workers demonstrated 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 of 1.75, \( P = 0.012 \)) was found by Sèvé 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 cells 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 like ERCC1 (4-6, 20).

However, this method (H-score) was applied when the same group analysed the prognostic and predictive value of TUBB3 in an adjuvant NSCLC setting of 265 patients (11). 140 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 to the observation arm. However, Cox regression analysis was not significant for interaction between TUBB3 expression and chemotherapy. **No information on the histopathological 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 HR’s 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 et al. (22).

This last mentioned cross-validation study of 1149 patients could not prove TUBB3 of predictive value in the adjuvant chemotherapy group in accordance with Sèvé and coworkers (11). The observation could be due to the histopathological constitution of their population characterized by squamous cell carcinomas (47%) and accordingly male
predominance (80%) (22), compared to our population (28% and 61%, respectively).

Indeed, squamous cell carcinoma patients are prone to a poor prognosis in spite of TUBB3 status according to our results (discussed in detail later). Azuma and co-workers also failed to demonstrate 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 et al., increasing the risk of random observations and widening the CI’s. The available tissues are often small FFPE biopsy samples which always raise the unavoidable possibility of tumor heterogeneity and thus of variation in IHC coloration (Figure 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 the frequency of immunostainings. 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, while the taxol-containing regimen B was without major impact. This observation may suggest that TUBB3-expression is a less suited biomarker in prediction of taxane resistance. Indeed, the majority of studies exploring these compounds have reported varying results (12, 13, 16).

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 demonstrated that the survival-favourable TUBB3-neg adenocarcinoma patients suffered a larger deterioration in self-reported QOL perhaps due to more treatment induced toxicity, when compared to the TUBB3-pos adenocarcinoma patients. It has previously been shown that increased chemotherapy induced haematological toxicity and superior outcome are correlated until a certain threshold (23;24). If our findings are reproduced more focus should be addressed to prophylactic intervention in the subgroups at risk, i.e. adenocarcinoma patients having TUBB3-neg tumors. This could be achieved by randomizing biomarker panel stratified patients to intensive prophylactic regimen or standard of care. In addition, it could prove of great value to correlate TUBB3 status in normal and tumour 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. The 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 regimens. 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 a paclitaxel and vinorelbine, has proven superior in terms of survival compared to carboplatin especially in patients with non-squamous 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-pos adenocarcinomas (Table 2). However, 11 more patients in this subgroup were treated with the intensive regimen A, perhaps inducing an increased response, in comparison to the patients with TUBB3-neg tumors.

The occurrence of the many responders among patients with TUBB3 pos tumors is likely to have diminished the HR of 1.55 for TUBB3 pos expression but also raises a different issue. As mentioned, we have demonstrated increased PFS and OS in the TUBB3-neg 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 demonstrated that knockdown by RNA interference of TUBB3 decreases NSCLC cells’ anchorage-independent growth, a hallmark property of tumorigenic potential of the cancer cell and, in addition, leads to sensitivity to both 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 behaviour of the tumors.

As shown in Figure 3, there is a significant variation in survival among patients with TUBB3-negative tumors when divided up into the three main histopathological subtypes. The patients with TUBB3-neg adenocarcinomas appear to benefit the most from chemotherapy as compared to 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 bronchiolo-alveolar 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 two main pathways proposed for the development of the peripheral adenocarcinoma (26).

One is the EGFR mutated/amplificated pathway, often found in non-smokers, while the other is seen in smokers and has the following features. These tumors often harbour *K-ras*, *p53* mutations, and DNA-methylation of the tumor suppressor gene *p16(INK4/CDK2A)*, as compared to patients with squamous cell carcinomas, carrying a huge sequential accumulation of genetic and molecular abnormalities, such as 9p and 3p losses, VEGF overexpression, *p16* and *p53* inactivation as well as *D1* and *Bcl-2* overexpression among others (27). 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-neg 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, in spite of its otherwise survival favourable TUBB3 neg expression (*Figure 3*). A notion supported by similar results concerning ERCC1 (5). In contrast, no difference in outcome is observed when looking to the three TUBB3 pos histopathological subtypes in the same figure. This observation supports the above mentioned notion by Mccarroll and
co-workers (25) stating that TUBB3 pos tumors carry a dismal prognosis that possibly overwrites the potential predictive influence of histopathological differences.

In conclusion, we have demonstrated the predictive value of TUBB3 in advanced NSCLC patients having adenocarcinoma subtype when treated with MIAs, while an effect of TUBB3-neg expression is questionable in other histopathological 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.
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Legends to figures

Figure 1. Flow chart of the patients’ tissue samples through the TUBB3 tumor-marker study. Abbreviations: TS: tissue samples. QOL: quality of life.

Figure 2. A squamous cell carcinoma (magnification x 40, upper) showing an intense positive cytoplasmic staining for TUBB3 in contrast to an adenocarcinoma (magnification x 20, lower) with a more heterogeneous staining pattern.

Figure 3. Progression free survival (PFS) and overall survival (OS) in months for the entire patient population (upper) of patients with advanced non-small cell lung cancer (NSCLC) and patients with adenocarcinomas (middle) stratified according to the TUBB3 tumor-marker expression. OS in months for the 149 TUBB3-negative (lower left) and 112 TUBB3-positive (lower right) patients with advanced NSCLC according to histopathological subtype. Abbreviations: Neg: negative. Pos: positive. Adeno: adenocarcinomas. Other: Other carcinomas (NOS, large cell and adenosquamous carcinomas. Squamous: squamous cell carcinomas.

Figure 4. Alteration in self-reported quality of life (QOL) among 45 patients with advanced NSCLC (adenocarcinoma subtype) stratified according to the TUBB3 tumor-marker expression. Abbreviation: CI: Confidence Interval.
Figure 1. Flow chart of the patients’ tissue samples through the TUBB3 tumor-marker study. Abbreviations: TS: tissue samples. QOL: quality of life.
Table 1: Characteristics of the population in the TUBB3 tumor-marker study.

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Abbreviations: NO: Number, LDH: Lactate dehydrogenase, PS: Performance expression, NOS: Not otherwise specified. The histological subtype of NSCLC could not be classified on the basis of the available bioptic material.
Figure 2. A squamous cell carcinoma (magnification x 40, upper) showing an intense positive cytoplasmic staining for TUBB3 in contrast to an adenocarcinoma (magnification x 20, lower) with a more heterogeneous staining pattern.
Figure 3. Progression free survival (PFS) and overall survival (OS) in months for the entire patient population (upper) of patients with advanced non-small cell lung cancer (NSCLC) and patients with adenocarcinoma (middle) stratified according to the TUBB3 immunohistochemical expression. OS in months for the 149 TUBB3-negative (lower left) and 112 TUBB3-positive (lower right) patients with advanced NSCLC according to histopathological subtype. Abbreviations: Neg, negative; Pos, positive; Squamous, squamous cell carcinoma; Adeno, adenocarcinoma.
Table 2: Treatment outcome in the TUBB3 tumor-marker study with adenocarcinoma subtype.

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Abbreviations: PD: progressive disease, NC: no change, PR: partial response, CR: complete response
**Figure 4:** Alteration in self-reported quality of life (QOL) among 45 patients with advanced NSCLC (adenocarcinoma subtype) stratified according to the TUBB3 tumor-marker expression. Abbreviation: CI: Confidence Interval.
Class III $\beta$-tubulin in advanced NSCLC of adenocarcinoma subtype predicts superior outcome in a randomized trial

Adam Christian Vilmar, Eric Santoni-Rugiu and Jens Benn Sørensen

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