High TUBB3 Expression, an Independent Prognostic Marker in Patients with Early Non–Small Cell Lung Cancer Treated by Preoperative Chemotherapy, Is Regulated by K-Ras Signaling Pathway

Guénâelle Levallet1,2, Emmanuel Bergot1,3, Martine Antoine6, Christian Creveuil4, Adriana O. Santos9, Michelle Beau-Faller10, Florence de Fraipont12, Elisabeth Brambilla13, Jérôme Levallet5, Franck Morin5, Virginie Westeel15, Marie Wislez11, Didier Debieuvre16, Fatémé Dubois1, Isabelle Rouquette17, Jean-Louis Pujol18, Denis Moro-Sibilot14, Jacques Camonis9, Gérard Zalcman1,3, on behalf of the Intergroupe Francophone de Cancérologie Thoracique (IFCT)

Abstract

We assessed the prognostic and predictive value of β-tubulin III (TUBB3) expression, as determined by immunohistochemistry, in 412 non–small cell lung cancer (NSCLC) specimens from early-stage patients who received neoadjuvant chemotherapy (paclitaxel- or gemcitabine-based) in a phase III trial (IFCT-0002). We also correlated TUBB3 expression with K-Ras and EGF receptor (EGFR) mutations in a subset of 208 cryopreserved specimens. High TUBB3 protein expression was associated with nonsquamous cell carcinomas (P < 0.001) and K-Ras mutation (P < 0.001). The 127 (30.8%) TUBB3-negative patients derived more than 1 year of overall survival advantage, with more than 84 months median overall survival versus 71.7 months for TUBB3-positive patients [HR, 1.58; 95% confidence interval (CI), 1.11–2.25]. This prognostic value was confirmed in multivariate analysis (adjusted HR for death, 1.51; 95% CI, 1.04–2.21; P = 0.031) with a bootstrapping validation procedure. TUBB3 expression was associated with nonresponse to chemotherapy (adjusted HR, 1.31; 95% CI, 1.01–1.70; P = 0.044) but had no predictive value (taxane vs. gemcitabine). Taking account of these clinical findings, we further investigated TUBB3 expression in isogenic human bronchial cell lines only differing by K-Ras gene status and assessed the effect of K-Ras short interfering RNA (siRNA) mediated depletion, cell hypoxia, or pharmacologic inhibitors of K-Ras downstream effectors, on TUBB3 protein cell content. siRNA K-Ras knockdown, inhibition of RAF/MEK (MAP–ERK kinase) and phosphoinositide 3-kinase (PI3K)/AKT signaling, and hypoxia were shown to downregulate TUBB3 expression in bronchial cells. This study is the first one to identify K-Ras mutations as determinant of TUBB3 expression, a chemoresistance marker. Our in vitro data deserve studies combining standard chemotherapy with anti-MEK or anti-PI3K drugs in patients with TUBB3-overexpressing tumors. Mol Cancer Ther; 11(5); 1203–13. ©2012 AACR

Introduction

Non–small cell lung cancers (NSCLC) are highly metastatic and often drug-resistant, so even after early diagnosis and resection with curative intent, patient prognosis remains poor (1). Efforts have been made to improve overall survival (OS) of patients with early NSCLC by lowering the distant metastasis rate with adjuvant or neoadjuvant chemotherapy (2–5). However, lung adenocarcinoma (LAC) is the most common NSCLC subtype, and a recent cisplatin evaluation (LACE) meta-analysis of adjuvant chemotherapy trials demonstrated that cisplatin does not improve OS or disease-free survival (DFS) (6). The overall benefit of adjuvant therapy is thus modest yet improves OS and DFS significantly in patients with node-positive or those at high-risk of relapse (1,7,8). Moreover, several recent randomized phase III trials showed that addition of targeted therapies improved the survival of patients with advanced stage NSCLC (9–12). Although promising, these advances have not translated into survival improvements in patients with resected NSCLC (6,13,14). In patients with resected NSCLC, neither the correlative molecular status nor classical genetic mutations, such as EML4-ALK fusions and K-RAS, EGFR mutations have shown significant impact on survival (15). Therefore, molecular strategies other than targeted therapies are needed to improve outcomes in this population. In this context, TUBB3 gene was recently found to be correlated with K-Ras oncoprotein expression and EGF receptor gene amplification in our previous works (16,17) and was suggested as a promising biomarker in NSCLC (18,19). An important clue is that TUBB3 is not a classic chemoresistance marker. In vitro studies using human bronchial cell lines only differing by K-Ras gene status revealed that K-Ras knockdown, inhibition of RAF/MEK (MAP–ERK kinase) and phosphoinositide 3-kinase (PI3K)/AKT signaling, and hypoxia were shown to downregulate TUBB3 expression in bronchial cells. This study is the first one to identify K-Ras mutations as determinant of TUBB3 expression, a chemoresistance marker. In our in vitro data, we further investigated TUBB3 expression in isogenic human bronchial cell lines only differing by K-Ras gene status and assessed the effect of K-Ras short interfering RNA (siRNA) mediated depletion, cell hypoxia, or pharmacologic inhibitors of K-Ras downstream effectors, on TUBB3 protein cell content. siRNA K-Ras knockdown, inhibition of RAF/MEK (MAP–ERK kinase) and phosphoinositide 3-kinase (PI3K)/AKT signaling, and hypoxia were shown to downregulate TUBB3 expression in bronchial cells. This study is the first one to identify K-Ras mutations as determinant of TUBB3 expression, a chemoresistance marker. Our in vitro data deserve studies combining standard chemotherapy with anti-MEK or anti-PI3K drugs in patients with TUBB3-overexpressing tumors.

www.aacrjournals.org

1203

Published OnlineFirst March 12, 2012; DOI: 10.1158/1535-7163.MCT-11-0899

Molecular Medicine in Practice

High TUBB3 Expression, an Independent Prognostic Marker in Patients with Early Non–Small Cell Lung Cancer Treated by Preoperative Chemotherapy, Is Regulated by K-Ras Signaling Pathway

Guénâelle Levallet1,2, Emmanuel Bergot1,3, Martine Antoine6, Christian Creveuil4, Adriana O. Santos9, Michelle Beau-Faller10, Florence de Fraipont12, Elisabeth Brambilla13, Jérôme Levallet5, Franck Morin5, Virginie Westeel15, Marie Wislez11, Didier Debieuvre16, Fatémé Dubois1, Isabelle Rouquette17, Jean-Louis Pujol18, Denis Moro-Sibilot14, Jacques Camonis9, Gérard Zalcman1,3, on behalf of the Intergroupe Francophone de Cancérologie Thoracique (IFCT)
cisplatin-based chemotherapy only found a 5-year absolute benefit of 5.4% (HR, 0.89; P = 0.004). This benefit was restricted to stage II and III patients, and OS rate at 5 years remained below 60% (6). In 2001, the French Intergroupe Francophone de Cancérologie Thoracique (IFCT) initiated a large phase III trial to evaluate the tolerance and efficacy of gemcitabine-cisplatin versus paclitaxel–carboplatin neoadjuvant chemotherapies (7). The trial also assessed patient survival and rates of recurrence after preoperative or perioperative chemotherapy and investigated potential markers of cancer cell resistance, which could help to identify subgroups of patients who are unlikely to benefit from perioperative chemotherapy (8). Among those putative markers is the structural protein class III β-tubulin (TUBB3; refs. 9–11). The TUBB3 gene was initially thought to encode a neuron-specific protein (12–14) and to be a marker of taxane resistance or cancer aggressiveness (15). High expression of TUBB3 correlates with low response rates in patients treated with taxane- or vinca-alkaloid–containing regimens and with reduced survival in patients with NSCLC (16, 17), breast (18), or ovarian cancer (19). However, the literature is conflicting, probably as a result of variation in tumor stage at analysis and trial design (11), and recent work also reported TUBB3 as a marker of resistance to DNA-targeting drugs (20). TUBB3 expression is highly regulated by hypoxia-inducible factor-1α (HIF-1α), which achieves transcriptional control of TUBB3 by binding at the 3’ flanking region in paclitaxel-resistant ovarian cancer cells (15). Epigenetic methylation of this 3’ enhancer region could alter HIF-1α–regulated TUBB3 expression (21). TUBB3 carboxyl-terminal region also undergoes numerous posttranslational modifications, such as polyglycylation, polyglutamylation, glycosylation, and finally phosphorylation, on serine residues (22–24). Such posttranslational changes modulate function of microtubules by affecting their dynamic properties during interphase and mitosis or by affecting their subcellular compartmentalization (24).

The aim of the translational ancillary study called “Bio-IFCT-0002” was to assess the predictive and prognostic value of TUBB3 expression in patients with stage I and II NSCLCs, treated with taxane- or antimetabolite-based preoperative chemotherapy in IFCT-0002 phase III trial. We also aimed to correlate TUBB3 expression with clinical, pathologic, and molecular (EGFR and K-Ras mutations) characteristics. Because we found an unexpected link between TUBB3 expression and K-Ras mutations, we investigated TUBB3 content in immortalized isogenic human bronchial epithelial cells, only differing by K-Ras downstream signaling effectors.

Materials and Methods

Patients and Bio-IFCT-0002 trial

Between 2001 and 2005, 528 patients were recruited to the IFCT-0002 phase III trial, approved by the Ethics Committee of Besançon University Hospital (Besançon, France). A specific informed consent was obtained for biologic studies (Bio-IFCT-0002), designed by a steering committee, conducted according to a detailed protocol, and granted by a National Clinical Research Grant (PHRC). Two platin-based perioperative chemotherapy regimens, gemcitabine plus cisplatin or paclitaxel plus carboplatin, and 2 chemotherapy schedules were compared in patients with resectable stage I or II NSCLCs. In the preoperative arm, patients received 2 courses of either chemotherapy regimens. Nonresponder patients underwent surgical resection, but patients with a partial response received 2 more cycles before surgical resection. In the perioperative arm, patients received 2 courses of either chemotherapy regimens and underwent surgical resection. Only responder patients received 2 additional adjuvant cycles. Results of this phase III trial have been presented elsewhere (7). Of the 467 patients who were finally operated and had no or only partial histologic response, 412 had available histologic material for reliable TUBB3 immunostaining study of their paraffin-embedded tumor specimen. Two hundred and eight patients could have cryopreserved histologic tumor specimens and therefore molecular analyses. K-Ras mutation analysis was possible in 206 patients of which 181 had both TUBB3 and K-Ras analyses (see flowchart, Supplementary Fig. S1)

Immunohistochemistry

After pretreatment with 0.01 mol/L citrate buffer (pH 6; Dako) for 20 minutes at 100°C, molecular marker was stained with automated immunohistochemical stainer (Dako). Slides were successively incubated at room temperature in 3% H2O2 for 5 minutes, then with TUBB3 monoclonal antibody (TUJ1 clone, generous gift from C. Dumontet, Inserm, U590, Université de Lyon, Lyon, France), diluted at 1:400 for 60 minutes at room temperature. Finally, antibody fixation was revealed by the EnVision+ Dual Link System (Dako). Internal positive controls were systematically evaluated (immunopositive endothelial cells and nerves). Slides were examined without knowledge of the individual patient data. The staining intensity of each tumor cell cluster, scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong), at ×40 magnification, was recorded. An overall immunohistochemical (IHC) composite score used 4 intensity immunostaining levels (0, 1, 2, 3) as previously reported in lung or breast cancer (17, 25, 26) and was calculated from the sum of the staining intensity multiplied by the distribution (0%–100%) from all parts of the slide, giving an H-score between 0 and 300, to take into account specimens with completely negative TUBB3 tumor staining but with TUBB3-positive internal controls.

TUBB3 IHC scores were first modeled as a continuous variable in the statistical analyses, without any predetermined arbitrary cutoff values. Fractional polynomial analysis was used to check the relationship was linear and to suggest an appropriate transformation in case of nonlinearity (27). Nonlinear associations were detected for each of the outcomes [nonresponse, progression-free survival...
(PFS), and OS. The relationship between TUBB3 score and the probability of nonresponse to neoadjuvant chemotherapy is depicted in Supplementary Fig. S2. Because the probability of nonresponse rapidly increases with increasing intensity of TUBB3 staining, until reaching a plateau, and as similar data were observed for survival analyses, a simple dichotomy of the TUBB3 score, that is, absence of TUBB3 staining (score = 0, “negative”) versus TUBB3 expression (score > 0, “positive”) was used as the appropriate form of coding.

Paraffin-embedded blocks were to be collected from all 492 patients with an incomplete histologic response. TUBB3 immunostaining was inconclusive in 31 patients because 2 different slides from the same pathologic block were negative for TUBB3 staining, including the internal positive control, or did not contain enough tumor cells. Blocks from 49 patients could not be collected. In total, paraffin-embedded tissues from 412 patients were the subject of this study (see flowchart, Supplementary Fig. S1).

K-Ras and EGFR mutations

Two hundred and eight snap-frozen specimens were collected in centers with facilities to bank frozen tissues at −80°C, and DNA extraction was centralized by the molecular biology platform of Grenoble University Hospital (Grenoble, France). The yield of DNA from 206 samples was sufficient to allow independent PCR amplifications for K-Ras mutations analysis with a sensitive peptidic nucleic acid (PNA)-mediated PCR (28). EGF receptor (EGFR) mutations were assessed by a multiplex allele-specific oligonucleotide (MASO) PCR assay in 201 specimens and verified by genomic sequencing as previously reported (ref. 29; see flowchart, Supplementary Fig. S1). This MASO-PCR assay for EGFR mutations identifies the 14 most frequent molecular events, which accounted for 90% of EGFR exon 19 and 21 alterations (29).

Cell culture and treatment

Isogenic HBEC3-wt and HBEC3-RasG12V bronchial cells, immortalized by constitutive expression of cyclin-dependent kinase 4 (CDK4) and human telomerase reverse transcriptase (hTERT), with either wild-type or K-RasG12V were kindly provided by Dr. Michael White (UT Southwestern Medical Center, Dallas, TX) and authenticated just before use with standard karyotyping techniques, checking for the presence of chromosomes 5 and 20 partial duplications, and XX chromosome pair (30). A549 cells (ATCC, # CCL-185) were obtained from the American Type Culture Collection (ATCC), cultured according to the suppliers’ recommendations and used for siRNA transfection in the 2 months following reception from ATCC.

K-Ras-specific siRNAs, K-Ras-1 (ACUUGUGGUA-GU GU GGAGCU) and K-Ras-2 (GGACUCUG AAGAU-GUACCU), were delivered with HiPerFect Transfection Reagent (Qiagen), according to manufacturer’s instructions.

For Raf, mitogen-activated protein (MAP)–ERK kinase (MEK), or phosphoinositide 3-kinase (PI3K) inhibition studies, when subconfluent at 30%, cells were starved for 24 hours in KSFM devoid of EGF and BPE, then cultured further for 48 hours in presence of PI3K (PIK90 25 μmol/L and LY294002 50 μmol/L), Raf (BAY43-9006 2 μmol/L and GW5074 1 μmol/L), or MEK (PD0325901 50 μmol/L and PD98059 50 μmol/L) inhibitors. For evaluation of TUBB3 expression in hypoxic cells, cells were first cultured at 37°C in 5% CO2. Confluent cells were maintained in the supplemented KSFM culture medium for 24, 48, or 72 hours at 37°C, either in normoxia, in a fully humidified atmosphere of 5% CO2, or in hypoxia in a chamber (IN VIVO2 1000; 3M) programmed at 0.1% O2 and 5% CO2 with an appropriate pressure of N2.

Immunofluorescent studies were conducted according to the standard protocols with TUBB3 (TUJ1 clone, 1:75), HIF-1α (1:50), or α-tubulin (1:100) antibodies, and Texas Red–labeled anti-mouse secondary antibody (1:300). Stainings were visualized with a confocal laser scanning fluorescence microscope (FluoView FV1000; Olympus). Images were captured by FV1000 software and processed with an FV10-ASW-1.7 viewer.

TUBB3 Western blotting

HBEC3-wt and HBEC3-RasV12 heat denatured cell proteins were resolved by 10% SDS-PAGE, electrophoresed onto nitrocellulose sheet, and probed with TUBB3 (TUJ1 clone) antibody at 1:3,000 dilution. A donkey anti-mouse IgG-horseradish peroxidase (HRP) complex and the enhanced chemiluminescence Advance Western Blotting Detection Kit (Amersham Biosciences) were used for detection of immunoreactive bands. For β-actin detection, the blots were stripped and reprobed with a monoclonal mouse anti-actin antibody (1:2,000 dilution) and goat anti-mouse IgG-HRP (Calbiochem). The immunoblots were scanned on the ProXPRESS Proteomic Imaging System (Perkin Elmer Life Science) and analyzed with the Total-Image Analysis Software (Nonlinear Dynamics Ltd.).

TUBB3 and K-Ras quantitative real-time PCR

Total RNA was extracted from A549, HBEC3-wt, or HBEC3-RasV12 cells and starved for 24 hours with TRI Reagent (Sigma-Aldrich) following the manufacturer’s instructions.

Total RNA (250 ng) was reverse-transcribed with random primers and 100 IU M-MLV reverse transcriptase at 37°C for 90 minutes, followed by 5 minutes of dissociation at 70°C. The resulting cDNAs were diluted (1:10) and used...
immunohistochemistry were compared using Fisher exact tests for qualitative variables, and Student t tests for quantitative variables. Associations between TUBB3 expression and clinical characteristics were evaluated by Fisher exact tests, Fisher exact tests, or Student t tests.

The prediction of nonresponse based on TUBB3 IHC scores or K-Ras mutations was assessed by log binomial regression to get a direct estimate of the risk ratio (RR). Prognosis values for PFS and OS, based on TUBB3 IHC scores, were assessed by Cox models. Interaction tests were used to evaluate predictive values. Median follow-up was estimated by the reverse Kaplan–Meier method. Kaplan–Meier survival curves were calculated. Multivariate log binomial regressions and Cox models were used to adjust for patients’ characteristics associated with the corresponding outcome (response, PFS, or OS) at \( P < 0.20 \) in univariate analysis. Discrimination of the proposed Cox models was estimated by the c-index (33). A first bootstrap resampling procedure (1,000 resamples) was used to evaluate the number of times TUBB3 expression reached significance in univariate and multivariate analyses. A second procedure (200 resamples) was used to correct the c-indexes for overoptimism. Data were analyzed with SPSS software, version 15.0 (SPSS for Windows, SPSS, Inc. 2000), the multivariable fractional polynomials (mfp) package (R package version 1.4.0, 2007) and performance assessment and comparison for survival analysis (survcomp) package (2011) of the R software.

Results

Patients and TUBB3 immunostaining characteristics

The study population included 328 males and 84 females. The mean age was 59.7 years (SD, 9.1; range 34–75 years), and the majority had an Eastern Cooperative Oncology Group (ECOG) performance status of 0 (\( n = 324 \); Table 1). Only 8% of patients were light smokers (<10 packs/y), and 62.6% of patients had nonsquamous histology. K-Ras and EGFR mutation status were available in 181 and 176 patients with concurrent TUBB3 analyses, respectively (see flowchart, Supplementary Fig. S1). No significant differences in patient characteristics were observed between the 412 patients with and the 80 patients without TUBB3 immunohistochemistry, but more patients with TUBB3 analysis had nonsquamous cell carcinoma (Supplementary Table S1).

The TUBB3 (TUJ1 clone) monoclonal antibody stained tumor cell cytoplasm but not stromal cells (Fig. 1). The staining intensity varied markedly among lung cancer samples and within the same slide, with strongly stained clusters of tumor cells sometimes adjacent to weakly stained tumor cells. Reliable immunostaining results were available from 412 slides; TUBB3 staining was positive in 285 pathologic specimens (69.2%) at any intensity and negative for 127 (30.8%).

TUBB3 staining was more frequently positive in women (\( P = 0.0016 \)), younger (\( P < 0.001 \)), nonsquamous cell carcinomas (\( P < 0.001 \)), and in patients who received fewer cycles of chemotherapy (\( P < 0.001 \)). TUBB3 status was not significantly affected by treatment arm, smoking, ECOG performance status, pathologic stage, or clinical T. K-Ras and EGFR mutations analysis could be conducted in, respectively, 181 and 176 specimens with TUBB3 immunostaining. K-Ras mutations were more frequent in patients with positive TUBB3 staining (\( P < 0.001 \)) than in TUBB3-negative samples, whereas TUBB3 immunostaining distribution did not correlate with EGFR status (\( P = 0.55 \)).

TUBB3 immunostaining associates with nonresponse to chemotherapy and poor PFS

TUBB3 expression was associated with nonresponse to chemotherapy in univariate (\( P < 0.001 \)) as well as multivariate analysis, adjusted for histology, clinical T, and smoking [adjusted HR, 1.31; 95% confidence interval (CI), 1.01–1.70; \( P = 0.044 \); Table 2]. Figure 2A shows PFS curves according to TUBB3 immunostaining, median follow-up of 55 months. TUBB3 immunostaining was associated with poor PFS in univariate (\( P = 0.015 \)) and multivariate analyses, adjusted for histology, number of cycles, clinical T, and stage (adjusted HR, 1.38; 95% CI, 1.00–1.90; \( P = 0.048 \); Table 2). Median PFS was 33.5 months for patients with TUBB3-positive
immunostaining versus 61.2 months for TUBB3-negative patients. Neither EGFR nor K-Ras mutations predicted PFS (data not shown). Validation by bootstrap resampling showed that TUBB3 positivity significantly predicted poor PFS in 70% of the samples in univariate analysis and 59% in multivariate analysis (corrected c-index, 0.640; 95% CI, 0.602–0.678).

TUBB3 immunostaining correlates with poor OS

OS curves are presented in Fig. 2B with median follow-up of 55 months. TUBB3 immunostaining correlated with poor OS in univariate (adjusted HR, 1.51; 95% CI, 1.04–2.21; P = 0.031; Table 2). Median OS was more than 84 months for TUBB3-negative patients than 71.7 months for TUBB3-positive patients. Again, neither K-Ras nor EGFR mutations had any significant impact on prognosis in the subset of patients in whom those analyses were conducted (data not shown). The bootstrap procedure showed that TUBB3 positivity was significantly associated with poor OS in 71% of the samples in univariate analysis and 61% in multivariate analysis (corrected c-index, 0.636; 95% CI, 0.590–0.682).

Table 1. TUBB3 immunostaining characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>TUBB3 = 0 (n = 127)</th>
<th>TUBB3 &gt; 0 (n = 285)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male</td>
<td>113 (89.0%)</td>
<td>215 (75.4%)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>14 (11.0%)</td>
<td>70 (24.6%)</td>
</tr>
<tr>
<td>Age at inclusion, mo (SD)</td>
<td>62.3 (8.2)</td>
<td>58.6 (9.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pack-year, m (SD)a</td>
<td>40.7 (21.6)</td>
<td>37.1 (20.8)</td>
<td>0.11</td>
</tr>
<tr>
<td>Performance</td>
<td>0</td>
<td>96 (75.6%)</td>
<td>228 (80.0%)</td>
</tr>
<tr>
<td></td>
<td>1 or 2</td>
<td>31 (24.4%)</td>
<td>57 (20.0%)</td>
</tr>
<tr>
<td>Histology</td>
<td>SCC</td>
<td>84 (66.1%)</td>
<td>70 (24.6%)</td>
</tr>
<tr>
<td></td>
<td>Non-SCC</td>
<td>43 (33.9%)</td>
<td>215 (75.4%)</td>
</tr>
<tr>
<td>Arm (ITT)</td>
<td>Gemcitabine 4 cycles PRE</td>
<td>35 (27.6%)</td>
<td>72 (25.3%)</td>
</tr>
<tr>
<td></td>
<td>Gemcitabine 2 cycles PERI</td>
<td>24 (18.9%)</td>
<td>78 (27.4%)</td>
</tr>
<tr>
<td></td>
<td>Paclitaxel 4 cycles PRE</td>
<td>31 (24.4%)</td>
<td>66 (23.2%)</td>
</tr>
<tr>
<td></td>
<td>Paclitaxel 2 cycles PERI</td>
<td>37 (29.1%)</td>
<td>69 (24.2%)</td>
</tr>
<tr>
<td>Number of cycles received</td>
<td>≤2</td>
<td>55 (43.3%)</td>
<td>178 (62.5%)</td>
</tr>
<tr>
<td></td>
<td>&gt;2</td>
<td>72 (56.7%)</td>
<td>107 (37.5%)</td>
</tr>
<tr>
<td>pStage</td>
<td>0, I</td>
<td>78 (61.4%)</td>
<td>195 (68.4%)</td>
</tr>
<tr>
<td></td>
<td>II, III, IV</td>
<td>49 (38.6%)</td>
<td>90 (31.6%)</td>
</tr>
<tr>
<td>cT</td>
<td>T1</td>
<td>21 (16.5%)</td>
<td>69 (24.2%)</td>
</tr>
<tr>
<td></td>
<td>T2 or T3</td>
<td>106 (83.5%)</td>
<td>216 (75.8%)</td>
</tr>
<tr>
<td>K-Rasb</td>
<td>WT</td>
<td>49 (94.2%)</td>
<td>92 (71.3%)</td>
</tr>
<tr>
<td></td>
<td>Mut</td>
<td>3 (5.8%)</td>
<td>37 (28.7%)</td>
</tr>
<tr>
<td>EGFRc</td>
<td>WT</td>
<td>46 (90.2%)</td>
<td>116 (92.8%)</td>
</tr>
<tr>
<td></td>
<td>Mut</td>
<td>5 (9.8%)</td>
<td>9 (7.2%)</td>
</tr>
</tbody>
</table>

NOTE: The mutation rate for K-Ras was 22%, and for EGFR it was 8%.
Abbreviations: cT, clinical tumor; ITT, intention to treat; Mut, mutation; pStage, pathologic stage; SCC, squamous cell carcinoma; WT, wild-type.
aInformation missing for 4 patients.
bInformation missing for 231 patients.
cInformation missing for 236 patients.
(paclitaxel vs. gemcitabine), 0.97 for TUBB3 score = 0; HR, 1.08 for TUBB3 score > 0; interaction ratio, 1.1; 95% CI, 0.61–2.0; \( P = 0.73 \); or OS (HR, 1.07 for TUBB3 score = 0; HR, 1.02 for TUBB3 score > 0, interaction ratio, 0.95; 95% CI, 0.47–1.9; \( P = 0.89 \)).

**TUBB3 and K-Ras mutation status**

With a sensitive PNA technique, K-Ras mutations were found in 40 of 181 specimens (22.1%). Relative risk of TUBB3 positivity in patients with K-Ras mutations compared with K-Ras wild-type patients was 1.42 (95% CI, 1.22–1.65; \( P < 0.001 \)). Contrasting with their lack of influence on survival, K-Ras mutations were significantly associated with nonresponse to chemotherapy, either in univariate analysis (RR, 1.44; 95% CI, 1.13–1.83; \( P = 0.011 \)) or in multivariate analysis adjusted on tobacco smoking (adjusted RR, 1.46; 95% CI, 1.15–1.86; \( P = 0.0022 \)). The association between TUBB3 expression and K-Ras mutations remained significant after adjusting for the factors related to TUBB3 staining (gender, age, histology, and number of cycles received; adjusted RR, 1.18; 95% CI, 1.03–1.36; \( P = 0.021 \)). We sought to explore further in vitro biologic mechanisms that could underlie the relationship between Ras signaling pathway and TUBB3 expression, we used a human bronchial cell line, HBEC, immortalized by CDK4 and hTERT but nontumorigenic, expressing (HBEC3-RasV12) or not (HBEC3-wt) the oncogene K-RasV12.

As shown by TUJ1 immunofluorescence (Fig. 3A) and immunoblotting (Fig. 3B) in HBEC3-wt and HBEC3-RasV12 cells, expression of a K-Ras mutant protein was associated with higher expression of TUBB3 protein. TUBB3 protein content in HBEC3-RasV12 was 1.5-fold significantly higher than in HEBC3-wt cells (\( P = 0.02 \), \( P < 0.001 \)).

**Table 2. Relationship between expression of TUBB3, nonresponse, PFS, and OS in patients from the bio-IFCT-0002 trial**

<table>
<thead>
<tr>
<th>Outcome</th>
<th>TUBB3 = 0 (n = 127)</th>
<th>TUBB3 &gt; 0 (n = 285)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonresponse*&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( n ) (%</td>
<td>47 (37.6)</td>
<td>171 (61.5)</td>
<td></td>
</tr>
<tr>
<td>RR (95% CI)</td>
<td>1</td>
<td>1.64 (1.28–2.09)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adj. RR (95% CI)</td>
<td>1</td>
<td>1.31 (1.01–1.70)</td>
<td>0.044</td>
</tr>
<tr>
<td>PFS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of events</td>
<td>59</td>
<td>166</td>
<td></td>
</tr>
<tr>
<td>Median, mo</td>
<td>61.2</td>
<td>33.5</td>
<td></td>
</tr>
<tr>
<td>HR (95% CI)</td>
<td>1</td>
<td>1.44 (1.07–1.95)</td>
<td>0.015</td>
</tr>
<tr>
<td>Adj. HR (95% CI)</td>
<td>1</td>
<td>1.38 (1.00–1.90)</td>
<td>0.048</td>
</tr>
<tr>
<td>OS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of events</td>
<td>36</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td>Median, mo</td>
<td>&gt;84</td>
<td>71.7</td>
<td></td>
</tr>
<tr>
<td>HR (95% CI)</td>
<td>1</td>
<td>1.58 (1.11–2.25)</td>
<td>0.011</td>
</tr>
<tr>
<td>Adj. HR (95% CI)</td>
<td>1</td>
<td>1.51 (1.04–2.21)</td>
<td>0.031</td>
</tr>
</tbody>
</table>

*Information missing for 9 patients.

*RR adjusted for histology, T, and smoking.

*HR adjusted for histology, number of cycles, T, and stage.
n = 7; ANOVA followed by Fisher’s (post hoc least significant difference) PLSD test], whereas the α-tubulin expression, used as an internal control, was stable. In addition, as shown in Fig. 3D, activation of EGFR, which turns K-Ras into an active form, also enhanced expression of TUBB3 either in K-Ras mutant or K-Ras wild-type bronchial cell lines. Surprisingly, TUBB3 mRNA content of the HBEC3 cell lines was not significantly different (Fig. 3C). Such data suggested that TUBB3 protein levels are dependent on posttranscriptional mechanisms regulating protein stability. TUBB3 overexpression was however a specific consequence of K-Ras activation, as 2 independent K-Ras siRNA reducing K-Ras expression by 70% to 80% (Supplementary Fig. S3) produced a consistent and significant decrease in TUBB3 content, compared with baseline (Supplementary Fig. S4). Thus, to precise the signaling modules downstream of K-Ras, which could regulate TUBB3 expression in HBEC3 cells, we used inhibitors with different degrees of specificity for PI3K (PIK90 25 μmol/L, BAY43-9006 2 μmol/L, and LY294002 50 μmol/L), Raf (BAV43-9006 2 μmol/L and GW5074 1 μmol/L) or MEK (PD0329901 50 μmol/L and PD98059 50 μmol/L) and observed a repeatedly (n = 4) significant TUBB3 expression decrease in HBEC3-wt cells (except with LY294002, which failed to properly inhibit p-AKT at the used concentrations and induced excessive toxicity at higher concentrations; Fig. 4).

Finally, while hypoxia has been previously reported to increase TUBB3 protein content in some ovarian cancer cells (15), we showed in HBEC3 cells that hypoxia induced TUBB3 decrease whatever was K-Ras status, wild-type or mutated. In fact, after 72-hour incubation period in 0.1% O₂, both HBEC3-wt and HBEC3-RasV12 cells showed only altered morphology (cell rounding) with rare features of apoptosis. At baseline, we repeatedly found differential expression of TUBB3 protein as described above, and after 24 hours of hypoxia, TUBB3 expression significantly decreased in both cell lines (Supplementary Fig. S5) whereas HIF-1α increased in the first 12 hours (Supplementary Fig. S5). However, the differential expression between HBEC3-wt and HBEC3-RasV12 remained constant (Supplementary Fig. S5). Again, no variation of TUBB3 mRNA was detected after 72 hours of hypoxia in either cell line (data not shown).

Discussion

Bio-IFCT-0002 showed a negative impact on prognosis for TUBB3-positive IHC staining, with differences in median PFS and OS of 30 months and more than 13 months, and HRs of 1.44 and 1.58, respectively (Table 2). This study also showed a significant association between TUBB3 expression and nonresponse to paclitaxel- or gemcitabine-based chemotherapy. Finally, our work identified K-Ras mutations as a determinant of TUBB3 expression.

We did not find a predictive effect of TUBB3 immunostaining in early-stage patients treated with tubulin-interacting agents (i.e., paclitaxel) compared with patients treated with other agents (i.e., gemcitabine in this study). On the basis of the 2 CIs of the interaction ratios (95% CI, 0.61–2.0 for PFS and 95% CI, 0.47–1.9 for OS), it can actually be inferred that a true ratio of 2 or more, suggestive of an important predictive effect, could be ruled out. However, a weaker effect, which would not have been detected because of the moderate sample size, remains possible.

Yet, our results confirm results from the pooled analysis conducted in 4 lung cancer adjuvant trials (LACE-bio study; ref. 34), which also failed to show a statistically significant interaction between TUBB3 and treatment assignment in predicting survival, in as much as 1,189 patients with early-stage NSCLCs. The prognostic impact of high TUBB3 content in patients treated with DNA-damaging agents, without tubulin-interacting agents, might be related to microtubule reorganization as a part of cellular response to DNA damage (20). Indeed, in HeLa cervical cancer cells, TUBB3 was recently shown to affect
the action of DNA-damaging agents, suggesting that altered expression of TUBB3 may also provide a cellular defense against diverse classes of DNA-targeting drugs (35). The association between TUBB3 overexpression and poor prognosis has also been reported for locally advanced or metastatic breast and lung tumors (18, 36–39), but again it was difficult to discriminate retrospectively between the prognostic effect of TUBB3 expression related to tumor aggressiveness and its ability to predict the response to and efficacy of chemotherapy.

The association between TUBB3 and chemoresistance may derive from its role during development, as TUBB3 is expressed during mesenchymal dedifferentiation. It is also associated with histologically high-grade malignancies (40), cell dedifferentiation, anaplastic transformation, and acquisition of progenitor- or stem cell–like phenotypic properties (12, 40, 41), a hallmark of cancer stem cells (42), which are often chemotherapy-resistant (43). Finally, it might also involve the invasive properties associated with cancer cells, as recently suggested by specific TUBB3 immunolocalization to invasive edges of human colorectal cancer (44).

We evaluated TUBB3 content in tumor tissues exposed to chemotherapy, and we cannot exclude that TUBB3 chemotherapy modulation could have led to a confounder effect in our series of patients. However, if in vitro modulation of chemosensitivity by TUBB3 overexpression has been previously documented (45, 46), on the other side, there is no evidence from literature that chemotherapy drugs (either taxane-based or not), could directly induce
Finally, we report a statistically significant unexpected association between K-Ras mutations and TUBB3 expression. Both alterations were associated with nonresponse to chemotherapy, but only TUBB3 predicted worse survival. EGFR mutations (that switch on K-Ras activity) had no significant effect on TUBB3 expression, survival, or nonresponse to chemotherapy (data not shown), but with a low prevalence (14 of 176 = 7.95%) in this series of Caucasian patients, and a possible lack of power for such a subgroup analysis. We therefore sought to explore K-Ras signaling and TUBB3 expression relationship with 2 isoergic HBE3 bronchial immortalized cell lines, only differing in respect of K-Ras mutant status. We found an increase of about 40% in TUBB3 protein content in cells expressing mutant K-Ras. These data were consistent with reports by Montgomery and colleagues (49), who used a pan-β-tubulin antibody to show higher levels of total β-tubulin protein in NIH3T3 cells overexpressing K-Ras, wt EGFR, HER2, or mutant EGFRvIII, compared with levels in the control NIH3T3 cells. We further showed that this differential protein expression could result from altered protein translation or turnover as both cell lines had similar TUBB3 mRNA content. Again, these findings are consistent with those by Montgomery and colleagues (49). These data also evoke the previous tubulin synthesis autoregulation, with selective tubulin mRNA degradation, induced by translation of β-tubulin amino-terminal sequence, leading to stable mRNA levels (50). Epigenetic mechanisms or posttranslational biochemical events such as phosphorylation could also account for TUBB3 expression regulation (42). Our data showing that EGF stimulation of bronchial cells also enhances TUBB3 expression, whatever is the K-Ras status (mutant or wild-type), suggest that, indeed, our clinical study was underpowered to detect a significant association between EGFR mutations and TUBB3 expression. We postulated that events regulating TUBB3 amounts could be controlled by K-Ras–induced signaling cascades. siRNA knockdown of K-Ras in HBE3 cells could indeed downregulate TUBB3 expression in EGF-treated HBE3 cells. The use of specific pharmacologic inhibitors of Raf, MEK, and PI3K, downstream effectors of K-Ras, actually influenced TUBB3 expression. Dissecting the precise mechanisms of tubulin cytoskeleton regulation and molecular actors interacting with these pathways could help to identify novel agents to treat patients with early NSCLC with TUBB3-expressing tumors, TUBB3 expression appearing to be a marker of poor prognosis and a general marker of chemoresistance. Our work raises the hypothesis that candidate therapeutic combinations could rely on MEK or PI3K inhibitors, which currently come to the clinics in early-phase trials, for patients with NSCLC.

**Disclosure of Potential Conflicts of Interest**

G. Zalcman received honoraria from speakers' bureaus of Lilly France and BMS. E. Brambilla is a consultant/on advisory board of Roche Diagnosis. J. Camonis is an employee of the Translational Research Department of the Institut Curie as head of Biophenics, a phenotyping facility of the Institut Curie as head of Biophenics, a phenotyping...
and imaging platform. D. Moro-Sibilot received honoraria from speakers bureaus of Eli-Lilly and Roche, and is a consultant on advisory board of Eli Lilly and Roche. V. Westeel received a commercial research grant from Roche for a distinct clinical trial, received honoraria from speakers’ bureaus of Roche, Eli Lilly, and Boehringer Ingelheim, is a consultant on advisory board of Roche and Eli Lilly, and does other work for Roche, Eli Lilly, and Asta Zeneca. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: G. Levallet, C. Creveuil, M. Beau-Faller, D. Moro-Sibilot, G. Zalcman
Development of methodology: G. Levallet, C. Creveuil, J. Levallet, F. Dubois, G. Zalcman, D. Moro-Sibilot
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. E. Levallet, E. Bergot, M. Antoine, A. O. Santos, F. de Fraipont, E. Brambilla, J. Levallet, F. Morin, V. Westeel, M. Wislez, E. Quoix, D. Debeuvre, F. Dubois, J. Rouquet, J.-L. Pujol, G. Zalcman
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Levallet, E. Bergot, C. Creveuil, A. O. Santos, F. Morin, F. Dubois, J.-L. Pujol, D. Moro-Sibilot, J. Camonis, G. Zalcman
Writing, review, and/or revision of the manuscript: G. Levallet, E. Bergot, C. Creveuil, M. Beau-Faller, F. de Fraipont, E. Brambilla, V. Westeel, M. Wislez, E. Quoix, J.-L. Pujol, D. Moro-Sibilot, J. Camonis, G. Zalcman

References

Grant Support
The study was supported by National Hospital Program for Clinical Research (PHRC) 2000; Association de Recherche contre le Cancer (ARC, 2001); Curi Institute grant for clinical research (CEIRC, 2001); National Program of Scientific Excellency from French National Cancer Institute (INCA, 2006); and Comité du Calvados de la Ligue contre le Cancer grant (2007) to G. Zalcman; French Society of Pulmonary Medicine (Société de Pneumologie de Langue Française, SPPF) 2008 grant to G. Levallet; and ARC grants #3131, #4845, Fondation de France grant #2008 05P019 to J. Camonis. A.O. Santos was supported by a postdoctoral fellowship from Fondation de France/Programme tumores solides.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 22, 2011; revised February 20, 2012; accepted February 27, 2012; published OnlineFirst March 12, 2012.
Molecular Cancer Therapeutics

High TUBB3 Expression, an Independent Prognostic Marker in Patients with Early Non–Small Cell Lung Cancer Treated by Preoperative Chemotherapy, Is Regulated by K-Ras Signaling Pathway

Guénaëlle Levallet, Emmanuel Bergot, Martine Antoine, et al.


Updated version  Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-11-0899

Supplementary Material  Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2012/05/07/1535-7163.MCT-11-0899.DC1

Cited articles  This article cites 48 articles, 19 of which you can access for free at: http://mct.aacrjournals.org/content/11/5/1203.full#ref-list-1

Citing articles  This article has been cited by 6 HighWire-hosted articles. Access the articles at: http://mct.aacrjournals.org/content/11/5/1203.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.