An Apoptosis Methylation Prognostic Signature for Early Lung Cancer in the IFCT-0002 Trial

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Abstract

Purpose: To evaluate prognostic and predictive molecular biomarkers in early-stage non–small cell lung carcinoma (NSCLC) receiving neoadjuvant chemotherapy.

Experimental Design: The IFCT-0002 trial compared two neoadjuvant regimens in 528 stages I to II NSCLC patients. DNA extraction of snap-frozen surgical samples taken from 208 patients receiving gemcitabine-cisplatin or paclitaxel-carboplatin regimens allowed for the identification of 3p allelic imbalance, Ras association domain family 1A (RASSF1A) and death-associated protein kinase 1 (DAPK1) promoter methylation, and epidermal growth factor receptor, K-ras, and TP53 mutations. Multivariate analysis identified prognostic and predictive effects of molecular alterations. A Bootstrapping approach was used to assess stability of the prognostic models generating optimism corrected indexes.

Results: RASSF1A methylation correlated significantly with shorter disease-free survival (DFS; adjusted HR = 1.88, 95% CI: 1.25–2.82, P = 0.0048) and shorter median overall survival (OS; adjusted HR = 2.01, 95% CI: 1.26–3.20, P = 0.020). A computed bootstrap resampling strategy led to a prognostic model, including RASSF1A, DAPK1, and tumor stage, dividing patients into three prognostic groups, with median OS ranging from 34 months for high-risk patients (HR for death = 3.85, 95% CI: 1.79–6.40) to more than 84 months for moderate (HR = 1.85, 95% CI: 0.97–3.52) and low-risk patients (reference group; P = 0.0044). In addition, RASSF1A methylation predicted longer DFS in patients treated with paclitaxel-carboplatin compared with gemcitabine-cisplatin (adjusted HR = 0.47, 95% CI: 0.23–0.97, Pinteraction = 0.042).

Conclusions: Following neoadjuvant chemotherapy, RASSF1A methylation negatively impacted prognosis of early-stage NSCLC. Along with DAPK1 methylation and tumor stage, RASSF1A methylation allowed definition of three subgroups with strikingly different prognosis. Conversely, significantly longer DFS following paclitaxel-based neoadjuvant chemotherapy for patients whose tumors showed RASSF1A methylation suggested its predictive interest in stages I and II NSCLC. Clin Cancer Res; 18(10): 2976–86.

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Translational Relevance

Ras association domain family 1A (RASSF1A) and death-associated protein kinase 1 (DAPK1) are apoptosis-regulating genes, frequently and precociously inactivated in lung cancer by the promoter methylation. However, to date, their contribution to prognosis in early lung cancer is solely based on retrospective studies.

In a prospective neoadjuvant chemotherapy phase III trial, our bootstrap-validated prognostic score, including two apoptosis gene methylation patterns and tumor stage, identified a subset of early-stage non–small cell lung carcinoma (NSCLC) patients with RASSF1A methylation but wild-type DAPK1, who may not benefit from perioperative platinum-based chemotherapy doublets. In contrast, RASSF1A methylation may predict longer survival in patients receiving paclitaxel-based doublet therapy possibly by interfering with tubulin cytoskeleton network as shown here by RASSF1A siRNA-mediated knockout in bronchial cells. Although this molecular-based apoptotic predictor still needs to be prospectively validated, it highlights the need for alternative, molecular-driven treatments in early-stage NSCLC patients for whom survival is unsatisfactory.

Materials and Methods

Patients and the Bio-IFCT-0002 trial

The IFCT-0002 randomized trial design (ClinicalTrials.gov id. NCT00198354) was reported previously (8), with its details provided in Supplementary Data. In short, during a 4-year period, 528 eligible patients provided written informed consent to participate in the clinical trial and optionally supply tumor material for biomarker analyses. Study approval was obtained from the sponsor’s Institutional Ethic Committee (Besançon University Hospital, France). The trial was conducted in accordance with the Declaration of Helsinki. The ancillary study Bio-IFCT-0002 was designed by a steering committee and conducted according to a detailed protocol available on request.

Banking and pathology process

Snap-frozen surgical specimens were collected in centers with facilities to store frozen tissues at −80°C. The submitted histologic diagnosis was used in the development of prognostic scores. In 140 patients, 7 mL blood samples were also collected for allelic imbalance (AI) analyses at the time of inclusion, with processed plasma being stored at −80°C. Tissue blocks were cut ensuring that the frozen sections contained at least 50% tumor cells. A total of 221 samples were made available for molecular analyses. The snap-frozen slides were processed for RNA and DNA extraction using a MagNA Pure LC Robotic workstation (Roche). The DNA yield from 208 samples was sufficient to allow for multiple independent PCR amplifications, including DNA methylation-specific PCR (MS-PCR) of DAPK1, RASSF1A, and p16, as well as mutation analysis of epidermal growth factor receptor (EGFR), K-Ras, and TP53 genes. CDH13, RASSF1A pyrosequencing, and AI determination on chromosomes 3p and 9p were possible in 167, 137, and 115 samples, respectively, with enough tumor DNA or corresponding plasma DNA samples remaining (Supplementary Fig. S1). Specimen sampling time points were the dates of surgical resection, either after 2 chemotherapy cycles (perioperative arm: n = 109; nonresponder patients in the preoperative arm: n = 10) or after 4 chemotherapy cycles (nonprogressive patients in the preoperative arm: n = 89; Supplementary Fig. S2).
MS-PCR assay and pyrosequencing

Bisulfite DNA conversion used a CpGenome DNA modification kit (MP Biomedicals) with MspI amplification serving as DNA quantity and quality control following bisulfite treatment. The method for MS-PCR was previously reported (15).

For pyrosequencing, 2 μL bisulfite-treated DNA was amplified in a total volume of 50 μL that contained 20 pmol of each primer (Supplementary Table S1), 1.5 mmol/L MgCl₂, and 1.25 U of FastStart Taq DNA polymerase (Roche). PCR product (40 μL) was bound to Streptavidin Sepharose High Performance (GE Healthcare), then purified, washed, denatured with a 0.2 mol/L NaOH solution and washed again. Thereafter, 0.3 μmol/L of pyrosequencing primer (AGGAAAATATGTTTAGTGA for CDH13 and GGTTAGTTTGTTTTT for RASSF1A) was annealed to the purified single-stranded PCR product. The pyrosequencing was carried out on a PyroMark ID system (Qiagen) following the manufacturer’s instructions and the percent of methylation determined the methylation index (MF; ref. 16).

Western blotting RASSF1A analysis

Paraffin-embedded blocks with sufficient tumor content (>50%) were randomly selected from 5 patients with methylated RASSF1A promoters and 5 wild-type RASSF1A promoters. Following the macrodissection of the tumor cell component, proteins were extracted with a Qproteome FFPE Tissue Kit (Qiagen) according to the manufacturer’s instructions. Protein samples (25 μg) were resolved on 10% (w/v) SDS-PAGE and electrotransferred onto nitrocellulose membranes. RASSF1A immunodetection used an affinity-purified mouse monoclonal RASSF1A (3F3, 1 of 300) antibody (Santa Cruz Biotechnology). Hela cervical cancer cells and renal HEK93 cells served as positive and negative controls of the RASSF1A expression, respectively. Only 3 protein extracts of each status were shown in the upper part of the figure. Differences in RASSF1A protein content from tumors with a methylated- or unmethylated-RASSF1A promoter were tested by one-way ANOVA followed by the Fisher exact test.

EGFR, K-Ras, and TP53 mutations and 3p AI

A multiplex allele-specific oligonucleotide PCR (MASO-PCR) assay for EGFR mutations was previously reported, identifying the 14 most frequent molecular events, which accounted for 90% of EGFR exon 19 and 21 gain of function mutations (Supplementary Table S2) and were checked by direct genomic sequencing (17).

K-Ras mutations were determined by peptide nucleic acid (PNA)–mediated PCR (18) and sequencing the positive PNA–PCR products.

TP53 mutation analysis employed denaturing high-performance liquid chromatography (DHPLC) to screen for mutations in exons 5 to 9 while DNA specimens with variant DHPLC profiles were sequenced (19).

For 3p and 9p microsatellite analysis, “normal” DNA from circulating lymphocytes as well as paired tumor DNA were amplified by fluorescent PCR using 4 microsatellite markers distributed along each of the 3p and 9p chromosomes (20). Cutoff values for significant AIs were determined based on previous studies (20).

RASSF1A siRNA-mediated downregulation in human bronchial epithelial cells

Isogenic human bronchial epithelial cells (HBEC)3 and HBEC3-RasG12V bronchial cells, immortalized by constitutive expression of cyclin-dependent kinase 4 (CDK4) and human telomerase reverse transcriptase, with either wild-type or K-Ras G12V (16), were kindly provided by Dr. Michael White (UT Southwestern Medical Center, Dallas) and grown in keratinocyte serum–free medium supplemented with EGF and bovine pituitary extracts at 37°C in 5% CO₂.

A RASSF1 siRNA was previously described (21) and shown to downregulate RASSF1 A, D, E, F, and G isoforms, all of them being downregulated by RASSF1A 1α promoter methylation in human tumors. Lipofectamine-mediated HBEC cell transfection (Lipofectamine RNAiMax; Invitrogen) was carried out with 20 nmol of such an RASSF1 siRNA, along with a negative scrambled control siRNA. Immunofluorescence studies were carried out according to standard protocols with RASSF1A (mAb clone eb114-10H1, 1:50; ebiosciences), or α-tubulin (1:100) antibodies, and Alexa-fluor, red-labeled anti-mouse secondary antibody (1:300), the nuclei being colored by DAPI. α-Tubulin and RASSF1A were visualized with a confocal laser scanning fluorescence microscope (FluoView FV1000; Olympus). Images were captured with FV1000 software and processed with a FV10-ASW-1.7 viewer.

Statistical analysis

The characteristics of patients with and without molecular analyses, as well as the associations between molecular and clinical characteristics were compared using χ² or Fisher exact tests for qualitative variables and Student t tests for quantitative variables. Disease-free survival (DFS) was calculated as the time from surgery to the date of recurrence or the date of death for patients who died without recurrence. In patients alive without recurrence, DFS was calculated from the date of surgery to the date of last news or to the end point date. Overall survival (OS) was calculated as the time from surgery to death from any cause or the date of last news or the end point date. The prognostic analyses initially treated 18 gene alterations classified into 3 different categories (Supplementary Table S3). Univariate Cox models were applied to select the most promising markers (threshold P = 0.20). For each of the selected genes, a multivariate Cox model was then applied to adjust for potential confounders (clinical characteristics associated with DFS or OS at P < 0.20). Hochberg method for multiple comparisons was used to correct the multivariable P values in each of the 3 categories (22). As a complementary analysis, the prognostic value of RASSF1A MI, was also investigated in a subset of patients with enough remaining DNA for pyrosequencing. The fractional polynomial method was used.
to account for a potential nonlinear effects (23). Adjusted HRs with 95% CIs were calculated. Survival curves were generated with the Kaplan–Meier method. The same strategy was used for the predictive value analyses, which were based on the interaction tests in the Cox models analyzing the relation between gene alterations and chemotherapy regimen.

All the biomarkers with a P value below 0.20 in the univariate analyses, and with less than 10% of values missing, were included in a backward multivariate Cox model along with the baseline clinical characteristics to identify multigene prognostic DFS and OS models. Internal validation of the models was achieved by bootstrap resampling (1,000 samples), with all the steps of the model development process being replicated on each bootstrapped sample (24). Optimism corrected concordance indexes (c-indexes) were also calculated (24).

Statistical significance was set at P < 0.05. Data were analyzed with SPSS software, version 15.0 and the surVcomp package of R software.

Results

Patients' characteristics

Demographic factors were similarly distributed in patients with (n = 208) and without (n = 320) molecular analyses with the exception of histology and response to chemotherapy, as adenocarcinoma was more frequent in patients with molecular analyses (46.2% vs. 32.8%, P = 0.0051), while response to chemotherapy was less frequent (55.4% vs. 41.8%, P = 0.0026; Supplementary Table S4). In contrast, the “molecular” group included more patients with stage I tumors (70.7% vs. 61.9%, P = 0.038). Median DFS was 40.9 months in patients with molecular analyses and 39.8 months in the other patients (P = 0.85). As for all patients, median follow-up was 59 months, and median OS was more than 71 months (P = 0.55).

RASSF1A and DAPK promoter gene methylation

MS-PCR for RASSF1A was successful in 202 out of 208 samples. RASSF1A promoter gene methylation was detected in 44 of 202 (21.8%) specimens. Results were confirmed by 2 laboratories (Grenoble and Caen) using independent bisulfite-modified DNA aliquots and PCR amplifications. Pyrosequencing was carried out in 137 samples, including all positive specimens by MS-PCR, which revealed an MI ranging from 10% to 67%.

RASSF1A methylation and K-Ras mutations (n = 41 of 206, 19.7%) or EGFR mutations (n = 16 of 201, 7.9%) were not mutually exclusive (data not shown). RASSF1A methylation was more frequent in specimens with 3p AI (30.3% vs. 11.7%, RR = 2.39, 95% CI: 1.20–5.63, P < 0.01) as a bona fide tumor suppressor gene. This association remained significant after adjusting for clinical factors such as sex, age, and smoking status relating to RASSF1A methylation at P < 0.20 (adjusted HR = 2.56, 95% CI: 1.17–5.61, P = 0.018). In addition, there was a 2-fold higher frequency of RASSF1A methylation in specimens with TP53 mutations (36.5% vs. 16.8%; RR = 2.18, 95% CI: 1.30–3.65, P = 0.0035), another apoptosis gene indirectly regulated by RASSF1A. Again, the association remained significant after adjusting for potential confounding factors (adjusted HR = 2.07, 95% CI: 1.21–3.52, P < 0.01).

Immunoblot analysis of protein extracts with anti-RASSF1A antibodies confirmed that RASSF1A promoter gene methylation assessed by our MS-PCR assay induced a significant decrease in RASSF1A protein expression (P < 0.001, ANOVA; Supplementary Fig. S3).

MS-PCR for DAPK1 was successful in 198 out of 208 samples, with 99 (50%) samples displaying DAPK1 promoter gene methylation. No significant differences in demographic or tumor characteristics were observed between patients with or without RASSF1A or DAPK1 gene methylation (Table 1).

Survival and promoter genes methylation

Univariate analysis of DFS according to DAPK1 or RASSF1A methylation status showed a nonsignificant improvement in DFS for patients with DAPK1 promoter gene methylation (adjusted HR = 0.73, 95% CI: 0.50–1.07, P = 0.11). In contrast, patients with methylated RASSF1A had a median DFS of 16.7 months, which was significantly lower than that of patients with unmethylated RASSF1A (DFS = 61.2 months; HR = 2.02, 95% CI: 1.35–3.03; P = 0.0068; Fig. 1).

In the multivariate analysis adjusted for confounding variables (histology, number of CT cycles, tumor size, and stage), only RASSF1A methylation predicted a shorter DFS (HR = 1.88, 95% CI: 1.25–2.82; corrected P = 0.0048), whereas no survival disadvantage was found in patients with methylated DAPK1, p16, or CDH13 (data not shown). Quantitative methylation of RASSF1A promoter was evaluated by pyrosequencing in 137 patients with enough DNA, giving an MI expressed as a continuous variable. In this subset of patients, there was no significant difference in RASSF1A MI according to the different treatment arms (data not shown). The linearity of RASSF1A MI effect on DFS was not rejected, and indeed, RASSF1A MI correlated with DFS in univariate as well as in multivariate analysis giving an adjusted HR = 1.18, (95% CI: 1.10–1.27), corrected P = 0.0000064 for a MI increase of 5%.

The OS prognostic role for DAPK1 promoter gene methylation was significant in univariate analysis (HR = 0.56, 95% CI: 0.36–0.88; P = 0.011), but not significant in multivariate analysis with Hochberg correction (adjusted HR = 0.61, 95% CI: 0.39–0.96; corrected P = 0.12, Fig. 2B).

RASSF1A methylation status had a significant effect on OS. Median OS for patients with RASSF1A methylation was 32.9 months compared with more than 84 months for those without methylated RASSF1A (HR = 2.13, 95% CI: 1.34–3.37; P = 0.0013, Fig. 2A). In the Cox model, the adjusted HR for death was 2.01 (95% CI: 1.26–3.20, corrected P = 0.020) in patients with RASSF1A methylation. Again, analysis of prognostic value of quantitative RASSF1A MI, in a subset of 137 patients, showed an association with OS, in
univariate as well as multivariate analyses, giving a very stable adjusted HR = 1.18 (95% CI: 1.09–1.27), corrected
P = 0.000084 for an MI increase of 5%.

Finally, the prognostic value of RASSF1A methylation was further validated with a resampling bootstrap procedure (1,000 replications), in which all steps of the statistical analysis (i.e., univariable selection of genes, selection of and adjustment for clinical confounders, and Hochberg correction) were replicated on each bootstrapped sample. RASSF1A methylation was associated with significantly shorter DFS and OS in respectively 70% and 69% of the samples, with respectively 0.63 (95% CI: 0.58–0.68) and 0.64 (95% CI: 0.58–0.70) corrected c-indexes, confirming the predictive accuracy of RASSF1A methylation for worse prognosis.

### Survival, promoter gene methylation, and chemotherapy

In univariate analysis, the interaction between RASSF1A gene methylation and the chemotherapy regimen was

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**Table 1. Characteristics of Bio-IFCT-0002 patients with RASSF1A and DAPK1 promoter gene methylation analysis**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All patients (n = 202)a</th>
<th>RASSF1A methylated (n = 44)</th>
<th>RASSF1A unmethylated (n = 158)</th>
<th>p(^2)</th>
<th>All patients (n = 198)b</th>
<th>DAPK1 methylated (n = 99)</th>
<th>DAPK1 unmethylated (n = 99)</th>
<th>p(^2)</th>
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<td>Sex</td>
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<tr>
<td>Male</td>
<td>158 (78.2%)</td>
<td>38 (86.4%)</td>
<td>120 (75.9%)</td>
<td>0.14</td>
<td>154 (77.8%)</td>
<td>80 (80.8%)</td>
<td>74 (74.7%)</td>
<td>0.31</td>
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<td>Female</td>
<td>44 (21.8%)</td>
<td>6 (13.6%)</td>
<td>38 (24.1%)</td>
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<td>44 (22.2%)</td>
<td>19 (19.2%)</td>
<td>25 (25.3%)</td>
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<td>Age at inclusion</td>
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<tr>
<td>&lt;60 y</td>
<td>98 (48.5%)</td>
<td>17 (38.6%)</td>
<td>81 (51.3%)</td>
<td>0.14</td>
<td>95 (48%)</td>
<td>42 (42.4%)</td>
<td>53 (53.5%)</td>
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<tr>
<td>&gt;60 y</td>
<td>104 (51.5%)</td>
<td>27 (61.4%)</td>
<td>77 (48.7%)</td>
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<td>103 (52%)</td>
<td>57 (57.6%)</td>
<td>46 (46.5%)</td>
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<td>≤10</td>
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<td>2 (4.5%)</td>
<td>22 (13.9%)</td>
<td>0.089</td>
<td>24 (12.1%)</td>
<td>12 (12.1%)</td>
<td>12 (12.1%)</td>
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<td>&gt;10</td>
<td>178 (88.1%)</td>
<td>42 (95.5%)</td>
<td>136 (86.1%)</td>
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<td>174 (87.9%)</td>
<td>87 (87.9%)</td>
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<td>WHO PS(^a)</td>
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<td>166 (82.2%)</td>
<td>38 (86.4%)</td>
<td>0.41</td>
<td>162 (81.8%)</td>
<td>79 (79.8%)</td>
<td>83 (83.8%)</td>
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<td>1 or 2</td>
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<td>6 (13.6%)</td>
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<td>36 (18.2%)</td>
<td>20 (20.2%)</td>
<td>16 (16.2%)</td>
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<td>Histology</td>
<td>SCC</td>
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<td>14 (31.8%)</td>
<td>0.50</td>
<td>71 (35.9%)</td>
<td>40 (40.4%)</td>
<td>31 (31.3%)</td>
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<td>Non-SCC</td>
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<td>127 (64.1%)</td>
<td>59 (59.6%)</td>
<td>68 (68.7%)</td>
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<td>Arm (ITT)</td>
<td>Gemcitabine 4 cycles PRE</td>
<td>53 (26.2%)</td>
<td>14 (31.8%)</td>
<td>0.39</td>
<td>53 (26.8%)</td>
<td>30 (30.3%)</td>
<td>23 (23.2%)</td>
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<tr>
<td></td>
<td>Gemcitabine 2 cycles PERI</td>
<td>52 (25.7%)</td>
<td>11 (25%)</td>
<td></td>
<td>51 (25.8%)</td>
<td>26 (26.3%)</td>
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<tr>
<td></td>
<td>Paclitaxel 4 cycles PRE</td>
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<td>9 (20.5%)</td>
<td>0.80</td>
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<td>21 (21.2%)</td>
<td>22 (22.2%)</td>
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<td>Paclitaxel 2 cycles PERI</td>
<td>53 (26.2%)</td>
<td>10 (22.7%)</td>
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<td>22 (22.2%)</td>
<td>29 (29.3%)</td>
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<td>Gemcitabine</td>
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<td>56 (56.6%)</td>
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<td>Paclitaxel</td>
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<td>94 (47.5%)</td>
<td>43 (43.4%)</td>
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<td>No. of cycles</td>
<td>3–4 cycles</td>
<td>87 (43.1%)</td>
<td>18 (40.9%)</td>
<td>0.74</td>
<td>84 (42.4%)</td>
<td>43 (43.4%)</td>
<td>41 (41.4%)</td>
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<td>0-1-2 cycles</td>
<td>115 (56.9%)</td>
<td>26 (59.1%)</td>
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<td>114 (56.7%)</td>
<td>56 (56.6%)</td>
<td>58 (58.6%)</td>
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<td>pStage(^d)</td>
<td>0, I</td>
<td>122 (60.4%)</td>
<td>23 (52.3%)</td>
<td>0.24</td>
<td>121 (61.1%)</td>
<td>60 (60.6%)</td>
<td>61 (61.6%)</td>
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<td>II, III, IV</td>
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<td>21 (47.7%)</td>
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<td>77 (38.9%)</td>
<td>39 (39.4%)</td>
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<td>cT</td>
<td>1</td>
<td>51 (25.2%)</td>
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<td>0.41</td>
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<td>2 + 3</td>
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<td>Response(^f)</td>
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<td>86 (44.3%)</td>
<td>46 (47.9%)</td>
<td>40 (40.8%)</td>
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</tr>
</tbody>
</table>

\(^a\)Two hundred and two successful RASSF1A methylation results, 6 nondetermined.

\(^b\)One hundred and ninety eight successful DAPK methylation results, 10 nondetermined.

\(^c\)All c-stages, but 2 noneligible patients with clinical stage IIIA, were I to II (inclusion criteria in the trial).

Pathologic analysis led to reclassification of 7 patients into p-stage IV because of a distant parenchymal metastasis nodule in another lobe (1997 Revised International System for Staging Lung Cancer, used at time of initiation of this trial), but would be classified as p-T4 and p-stage IIIA according to the 2007 7th edition of the staging system.

\(^d\)Four patients were not evaluable for response.

\(^e\)World Health Organization scores for performance status range from 0 to 2, with a score of 0 indicating no symptoms, 1 mild symptoms, and 2 moderate symptoms.

\(^f\)P values were calculated by the \(\chi^2\) test or Fisher exact test when appropriate.
statistically significant for DFS (interaction ratio = 2.40, 95% CI: 1.04–5.52; P = 0.040; HR carboplatin-paclitaxel versus cisplatin-gemcitabine = 1.32; 95% CI: 0.85–2.06 for patients with unmethylated RASSF1A gene compared with HR = 0.55, 95% CI: 0.27–1.12 for patients with methylated RASSF1A; Fig. 3). In the methylated group, treatment with paclitaxel plus carboplatin extended DFS by 24 months compared with treatment with gemcitabine plus cisplatin. After adjusting for histology, number of cycles, tumor size, and stage, the interaction remained significant (adjusted interaction ratio = 3.22, 95% CI: 1.35–7.66; corrected P = 0.033; adjusted HR carboplatin-paclitaxel vs. cisplatin-gemcitabine = 1.52, 95% CI: 0.96–2.41; P = 0.076 in the unmethylated group compared with adjusted HR = 0.47, 95% CI: 0.23–0.97; P = 0.042 in the other group). The interaction did not reach significance for OS, despite a nearly 2-fold increase in HR (carboplatin-paclitaxel vs. cisplatin-gemcitabine) in patients with methylated RASSF1A versus those with unmethylated RASSF1A (interaction ratio = 1.81, 95% CI: 0.70–4.70; P = 0.22). A poor median OS was observed in patients with methylated RASSF1A treated with gemcitabine plus cisplatin (30.3 months) versus those treated with paclitaxel plus carboplatin (70 months). In OS multivariate analysis, the interaction ratio was 2.42 in methylated patients, but no statistical significance was achieved (95% CI: 0.90–6.53 with adjusted HR carboplatin-paclitaxel versus cisplatin-gemcitabine = 0.44, 95% CI: 0.19–0.99; corrected P = 0.24).

Unlike the results for RASSF1A methylation, no significant interaction was noted between DAPK1 promoter methylation and chemotherapy regimen for DFS and OS (data not shown).

RASSF1 downregulation drastically impacts tubulin cytoskeleton in bronchial cells

We aimed to check that RASSF1A extinction could actually impact tubulin cytoskeleton of bronchial epithelial cells, thus giving a rationale for the predictive value of RASSF1A gene methylation in patients receiving tubulin-interacting paclitaxel. The efficacy of RASSF1 siRNA was tested by quantitative RT-PCR and actually downregulated RASSF1 expression by more than 80% in lipofectamine-mediated cell transfection, from H24 to H96 after 20 nmol siRNA transfection, as compared with lipofectamine alone or with a commercial scrambled negative control siRNA (data not shown).

As shown in Fig. 4, RASSF1 siRNA induced a round-up cell phenotype with a drastic tubulin redistribution along cytosol in the perinuclear region. Such a phenotype was observed in both isogenic HBEC3, Ras wild-type cell line and its counterpart expressing a mutant activated Ras protein (data not shown).

We carried out a web search with String 8.2 (Search Tool for the Retrieval of Interacting Genes/Proteins) software (http://string82.embl.de). Only published data coming...
from experiments were explored, generating a protein interaction network scheme around RASSF1A protein (Supplementary Fig. S4). Along with apoptosis and cell-cycle regulating proteins, several tubulin network regulating proteins were found to actually belong to RASSF1 interactome, such as microtubule-interacting proteins MAP1S and MAP1B, and integral components of microtubules such as tubulin β-1 chain (TUBB1); tubulin α-1 chain (TUBA4A, α-tubulin 1), tubulin gamma-1 chain (TUBG1), tubulin β-chain-5 chain (TUBB5), or tubulin β-2A chain (TUBB2A).

We also found DAXX protein, a proapoptotic Fas-interacting partner, in the RASSF1A interaction network with a high probability score, a finding of interest because elevated cellular levels of DAXX protein were shown to increase paclitaxel sensitivity of breast cancer cell lines (25).

**Prognostic score including apoptotic gene methylation status**

A backward Cox regression analysis applied to all the biomarkers selected in the univariate analyses (with <10%
missing values) as well as baseline clinical characteristics resulted in a DFS model that included histology (non-squamous vs. squamous), stage, and RASSF1A methylation, with adjusted HRs for disease progression being 1.69 (95% CI: 1.12–2.56, \(P = 0.013\)), 1.96 (95% CI: 1.31–2.93, \(P = 0.0010\)), and 1.99 (95% CI: 1.33–2.99, \(P = 0.0009\)), respectively (Supplementary Table S3). Internal validation, whereby all steps of the model development process were replicated on each bootstrapped sample, showed that the RASSF1A variable was present in 76% of the resulting models. Corrected c-index was 0.60 (95% CI: 0.54–0.66) when RASSF1A methylation status was added. Three groups of patients with different prognosis were defined with the prognostic score derived from the Cox regression model, namely low-risk (lower quartile), moderate-risk (second and third quartile), and high-risk patients (upper quartile). Data analysis revealed a median DFS more than 84 months for low-risk patients (reference category), 52.0 months for moderate risk (HR = 1.71, 95% CI: 0.97–3.05), and 12.0 months for high-risk patients (HR = 3.25, 95% CI: 1.81–5.84; \(P < 10^{-5}\), Fig. 5A).

The same procedure was applied to OS, resulting in a different final model that included tumor stage, RASSF1A methylation, and DAPK methylation, with HRs for death being 1.86 (95% CI: 1.17–2.95, \(P = 0.0084\)), 2.18 (95% CI: 1.36–3.51, \(P = 0.0013\)), and 0.53 (95% CI: 0.34–0.83, \(P = 0.0058\)), respectively (Supplementary Table S5). Internal validation showed that RASSF1A and DAPK were present in 71% and 70% of models, respectively. Corrected c-index was 0.62 (95% CI: 0.55–0.69) for the final model. Three groups of patients were similarly classified as low-risk (lower quartile), moderate-risk (second and third quartile), and high-risk patients (upper quartile). These 3 groups were mainly defined by the apoptosis 2-gene methylation signature, with median OS more than 84 months in low-risk (reference category) and moderate-risk patients (HR = 1.85, 95% CI: 0.97–3.52), but only 34 months for high-risk (HR = 3.38, 95% CI: 1.79–6.40; \(P = 0.00044\); Fig. 5B).

**Discussion**

Our study indicated that RASSF1A promoter methylation was a strong independent prognostic factor for the stages I and II lung cancer patients who were included in our phase III trial on perioperative chemotherapy. As compared with patients with methylated RASSF1A, patients with unmethylated RASSF1A exhibited a DFS and OS four and 3 times longer, respectively. Internal validation of this prognostic value was shown by a bootstrap resampling methodology. Furthermore, we used an alternative molecular assay, pyrosequencing, giving a quantitative evaluation of RASSF1A promoter methylation (MI). RASSF1A MI correlated with a worse DFS and a worse OS with highly significant \(P\) values (\(10^{-5}\) to \(10^{-3}\)) in multivariate analyses with correction for multiple comparisons. We therefore postulate that the loss of power induced by this subset analysis in only 137 patients was compensated by the analysis of promoter methylation intensity as a continuous variable without any pre-specified cut point. There was no advantage of one chemotherapy arm over the other in the whole group (8). However, patients with methylated RASSF1A experienced a significant increase in DFS when treated with paclitaxel-carcaboplitin as opposed to gemcitabine-cisplatin, with an adjusted HR of 0.47 favoring the paclitaxel arm and a positive interaction test (corrected \(P = 0.033\)).

RASSF1A methylation analysis was conducted in 208 patients, which represented almost half of patients with incomplete histologic response. Baseline factors did not significantly differ between patients with or without molecular analysis, with both patient subgroups displaying similar DFS and OS.

The tissue samples used to develop our prognostic model were taken after patients received chemotherapy, and thus, we cannot exclude the interference of treatment on this prognostic model. However, a recent paper examined the gene methylation chemotherapy-induced variations in breast and ovarian cancer cell lines using high-definition DNA methylation profiling. Among the 14 genes and 800 CpG islands analyzed, half of the tested genes did not show any change in DNA methylation level (26). Furthermore, RASSF1A promoter gene methylation was not increased in anthracycline-selected drug-resistant
breast or ovarian cancer cells (26). Conversely, in some resistant breast cancer cells but not ovarian cancer cells, methylation of CpG islands in RASSF1A promoter was reduced without inducing mRNA reexpression, suggesting that RASSF1A downregulation was maintained to escape from chemotherapy-induced cell death. In fact, in the proximal promoter and exons 1 regions, the specific role of 14 transcriptionally active CpG sites of the 33 CpG proximal promoter and exon 1 regions, the specific role of transcriptionally active, while our pyrosequencing primer hybridizes with the +74 to +97 sequence, exploring a downstream longer region of 136bp containing 14 CpG islands. Differences in selected primer pairs may account for both functional and frequency differences in previous studies dealing with RASSF1A methylation in early lung cancer (27). Hence, the transcriptional consequences of RASSF1A methylation may not be directly inferred from the overall intensity or frequency of RASSF1A promoter methylation, which vary according to the length of the amplified promoter fragment containing the CpG islands. The functional silencing of RASSF1A would only derive from the methylation of a combination of specific transcriptionally important CpG sites. Similarly, several distinct tumor subpopulations may exhibit different levels of RASSF1A methylation and silencing, resulting in apparently discordant levels of methylation and RASSF1A expression. Moreover, only biallelic promoter methylation or, consistent with Knudson 2-hit hypothesis, monoallelic methylation and 3p21.3 loss of heterozygosity or changes in chromatin structure on the other allele, result in complete RASSF1A mRNA downregulation. Given these uncertainties, we sought to explore actual RASSF1A protein expression in 10 tumor samples that were selected on the basis of their tumor cell content being more than 50% (Supplementary Fig. S2). We found a significant downregulation of RASSF1A protein expression in the samples detected as RASSF1A-methylated using the MS-PCR assay, highlighting the functional relevance of RASSF1A promoter methylated sequences in this assay. For the other genes analyzed in our study with previously published methylation-specific primers (Supplementary Table S1), the frequency of promoter gene methylation did not essentially differ from that previously reported for DAPK1, CDH1, p16, TIMP3, and CDH13 in chemotherapynaive patients, suggesting a low interference of neoadjuvant treatment on gene methylation frequency (6, 30).

In other retrospective studies on early lung cancer, the RASSF1A gene methylation was identified as a putative prognostic factor (13, 30–33). The lack of prognostic influence of p16 methylation found in our study was previously reported (30, 34), with the IALT group confirming that p16 immunohistochemistry did not influence prognosis in early-stage NSCLC (35). The p16 promoter is not the sole mechanism in the inactivation of the CDKN2A locus, which also includes chromosome 9p21 deletion (36). In a previous work involving the same series of patients, we reported that 9p21 AI tended to be a predictor of worse prognosis in a multivariate model with Hochberg correction (HR = 1.61, 95% CI: 0.90–2.90, P = 0.13; ref. 37).

In addition to our study being a prospective controlled trial, our findings are strengthened by the use of Hochberg correction for multiple testing. Other studies, all of them retrospective in nature, identified RASSF1A gene methylation as a prognostic marker in the whole study population or in subgroups of patients (13, 32, 33, 38, 39). The study of Brock and colleagues included 51 stage I NSCLC patients presenting tumor recurrence within 40 months of complete surgical resection and 116 stage I patients without any recurrence. The authors found that RASSF1A methylation only tended to predict recurrence (HR = 1.86, P = 0.07), while p16 and CDH13 methylation were independently associated with tumor recurrence (HR = 3.55 and 2.33, respectively), whereas DAPK1 methylation had no prognostic impact (7). Aside from tumor stage (stages I and II for our series vs. stage I for Brock), another major difference with the Brock study was our use of Hochberg correction for multiple hypothesis testing. In fact, when Riaz applied the Bonferroni correction to the Brock data, only 2 of the initial 11 positive correlations were still significant (40).

Our results showing RASSF1A gene methylation to be a predictor for longer DFS in patients receiving taxane-based doublet therapy are in accordance with previous in vitro findings. Our data also align with the identified role of RASSF1A in regulating the tubulin cytoskeleton by directly interfering with the tubulin network during mitosis (41, 42; Supplementary Fig. S4) and with the phenotypic effect of siRNA RASSF1A depletion in our bronchial cell model. Alternatively, depletion of RASSF1A protein by promoter gene methylation (or siRNA) could act by increasing the pool of free DAXX protein, one of its multiple cell partners, previously shown to be a trigger of paclitaxel response (25).

However, our analysis of the predictive value of RASSF1A methylation has some obvious methodologic limitations. Although the interaction did not reach significance for OS, given the CI width for the interaction ratio, we cannot rule out that a predictive effect would have been found with a greater sample size. Indeed, the difference in OS for patients with methylated RASSF1A receiving paclitaxel (70 months) versus gemcitabine treatment (30 months) was clinically meaningful, thus supporting the need for confirmatory studies.

In contrast, DAPK1 methylation had a favorable effect on DFS and OS, which, however, was not significant when applying Hochberg correction. Although some published studies reported a similar positive influence (43, 44), others showed a negative impact of DAPK1 methylation (45–47). These discrepancies may be explained by the low levels (up to 8.9%) of DAPK1 gene methylation in normal lymphocytes (48, 49), whereas RASSF1A methylation was shown to be virtually absent in blood lymphocytes taken from 164 healthy blood donors.
using pyrosequencing-based high-throughput analyses (50). As our patients received neoadjuvant chemotheraphy, we cannot exclude that the positive influence of DAPK1 methylation may simply reflect a lymphocyte inflammatory infiltration of the tumor as secondary to overall chemotherapy efficacy.

In our study, we developed 2 different prognostic models for DFS and OS, allowing us to identify 3 subgroups of patients with strikingly different DFS and OS in a somewhat homogeneous cohort of patients. Internal validation, based on a bootstrapping procedure that included all steps of the model development process, showed the stability of RASSF1A methylation for DFS and OS and of DAPK1 methylation for OS in the selected prognostic models. However, an obvious limitation of our results is derived from the negativity of the overall clinical study; consequently, our results derived from a subgroup analysis must be interpreted with caution. Therefore, we suggest our OS composite classifier, including 2 apotopic gene methylation statuses, be evaluated in further independent prospective trials involving both adjuvant and neoadjuvant treatments, just as cDNA microarrays studies are currently carried out to customize adjuvant therapy in confirmatory trials.

Disclosure of Potential Conflicts of Interest

E. Bergot has honoraria from Speakers Bureau from Actelion and Lilly. V. Westeel has honoraria from Speakers Bureau from Lilly and is a consultant on the advisory board of Lilly. D. Moro-Sibilot has honoraria from Speakers Bureau from Eli Lilly, Astra Zeneca, Roche, Boehringer, and Pﬁzer. G. Zalcman has honoraria from Speakers Bureau from Lilly and BMS and is a consultant on one advisory board of BMS. No potential conﬂicts of interest were disclosed by the other authors.

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An Apoptosis Methylation Prognostic Signature for Early Lung Cancer in the IFCT-0002 Trial

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