Mitogen-Activated Protein Kinase Activation in Lung Adenocarcinoma: A Comparative Study between Ever Smokers and Never Smokers

Giannis Mountzios,1,2 David Planchard,1,2 Benjamin Besse,1 Pierre Validire,4 Philippe Girard,5 Christine Devisme,3 Meletios-Athanasios Dimopoulos,6 Jean-Charles Soria,1,7 and Pierre Fouret2,8

Abstract

**Purpose:** There are major differences affecting genes in adenocarcinomas in ever and never smokers. However, data on whether mitogen-activated protein kinase (MAPK) activation state differs according to smoking status are limited.

**Experimental Design:** Expression of activated extracellular signal–regulated kinases, c-Jun NH2-terminal kinases, and P38 enzymes (pP38) were evaluated by means of immunohistochemistry in 188 chemonaive patients with surgically resected lung adenocarcinoma. Cell viability of the lung adenocarcinoma cell line HCC827 was studied after treatment with cisplatin or the P38 MAPK inhibitor SB 203580.

**Results:** Thirty-seven of 44 never smokers [84%; 95% confidence intervals (95% CI), 70-92%] expressed high pP38 levels compared with 45 of 104 ever smokers (43%; 95% CI, 34-53%; \( P < 0.0001 \)). The proportion of never smokers expressing high c-Jun NH2-terminal kinase levels (72%; 95% CI, 57-83%) was greater than that of ever smokers (53%; 95% CI, 44-62%; \( P = 0.03 \)). The proportion of ever smokers expressing high extracellular signal–regulated kinase levels (51%; 95% CI, 42-59%) was similar to that of never smokers (57%; 95% CI, 42-71%; \( P = 0.47 \)). Never smokers were 10.5 times (95% CI, 3.5-31.5) more likely to express high pP38 levels after adjustment for variables linked to smoking status, including age, sex, and histologic subtype. None of the activated MAPKs predicted for overall survival. Cell viability of HCC827 was significantly reduced after exposure to SB203580 alone or when combined with cisplatin.

**Conclusions:** Life-long nonsmoking is associated with high activated P38 levels in patients with lung adenocarcinoma. Activated P38 can contribute to the viability of adenocarcinoma cells in never smokers, but is not predictive for overall survival.

There is overwhelming evidence that tobacco smoking is the major cause of lung cancer. However, even in people who have never smoked, lung cancer accounts for ~15,000 deaths annually in the United States (1). Among histologic types of non–small cell lung cancer, adenocarcinoma is the less strongly associated with smoking and has become the most common type in many Western countries (2).

There are striking differences affecting several genes in tumors occurring in ever smokers and never smokers (2, 3). Tobacco carcinogens are responsible for G/T transversions in target genes including both the \( p53 \) tumor suppressor gene and the \( Ras \) oncogenes (4, 5). K-Ras mutations are almost entirely limited to ever smokers, predominantly with adenocarcinoma histology. Conversely, the incidence of epidermal growth factor receptor mutations is much higher in never smokers and women (6). The methylation profiles of lung cancers in ever smokers and never smokers are also very different (7). Among chromosomal aberrations, a gain at 16p seems more frequent in never smokers (8).

Many oncogenic proteins are members of or interact with cytoplasmic signaling cascades, and transformation is often a direct result of the deregulation of a cytoplasmic signal transduction pathway (9). Mitogen-activated protein kinases (MAPK) constitute an evolutionarily preserved family of protein kinases that act as cytoplasmic mediators of signal transduction pathways critical for cellular proliferation and survival. In multicellular organisms, there are three well-characterized subfamilies of MAPKs, including the extracellular signal–regulated kinases (ERK), ERK1 and ERK2; the c-Jun NH2-terminal kinases (JNK);
and the four P38 enzymes α, β, γ, and δ. Activated ERK and JNK can lead to increased proliferation and survival, whereas the P38 MAPK pathway is implicated in the suppression of tumorigenesis (10). Contrary to expectations, Greenberg and colleagues (11) have reported selective P38 activation in non–small cell lung cancer. Vicent and colleagues (12, 13) have reported that ERK, JNK, and P38 are activated in non–small cell lung cancer, but the degree of their activation is variable.

Current evidence indicates that lung cancer in never smokers is a distinct entity with unique molecular and biological characteristics (2, 14). However, data on whether MAPK activation state differs according to smoking status are limited.

**Patients and Methods**

**Clinical data.** Using a single institution clinical database (Institut Mutualiste Montsouris, Paris, France), 217 patients with lung adenocarcinoma who had undergone curative surgical treatment between January 1995 and December 2003 were retrospectively identified. Complete clinical data including follow-up information and detailed smoking history were obtained either by exhaustive electronic or manual query of clinical databases or by postal or personal communication with the patients, their families, and at least two treating physicians. This review led to the exclusion of 29 cases due to incomplete clinical records (19 cases), prior treatment by neoadjuvant chemotherapy (2 cases), or because the metastatic origin of the thoracic tumor could not be ruled out (8 cases). Hence, a total of 188 chemonaive patients with primary lung adenocarcinoma and available formol-fixed, paraffin-embedded tumor samples were studied. According to the WHO definition (15), a patient was considered as an ever smoker if he or she admitted having smoked at least 100 cigarettes in his or her life, whereas patients who denied any active tobacco exposure or had smoked less than 100 cigarettes in their life were defined as never smokers. The study was carried out according to national legal regulations.

**Tissue microarrays and immunohistochemistry.** The procedure of tissue microarray fabrication has been previously described (16).

**Table 1. Patient characteristics**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Ever smokers (n = 143)</th>
<th>Never smokers (n = 45)</th>
<th>Total (n = 188)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (y)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>59 (34-83)</td>
<td>71 (38-86)</td>
<td>63 (34-86)</td>
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</tr>
<tr>
<td>&lt;60 y</td>
<td>73 51%</td>
<td>10 22%</td>
<td>83 44%</td>
<td></td>
</tr>
<tr>
<td>≥60 y or more</td>
<td>70 49%</td>
<td>35 78%</td>
<td>105 56%</td>
<td></td>
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<tr>
<td>Gender (n)</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Male</td>
<td>94 66%</td>
<td>3 7%</td>
<td>97 52%</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>49 34%</td>
<td>42 93%</td>
<td>91 48%</td>
<td></td>
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<tr>
<td>T (n)</td>
<td></td>
<td></td>
<td></td>
<td>0.19</td>
</tr>
<tr>
<td>1</td>
<td>52 36%</td>
<td>21 47%</td>
<td>73 39%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>84 59%</td>
<td>24 53%</td>
<td>108 57%</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>7 5%</td>
<td>0 0%</td>
<td>7 4%</td>
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</tr>
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<td>N (n)</td>
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</tr>
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<td>94 66%</td>
<td>37 82%</td>
<td>131 70%</td>
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</tr>
<tr>
<td>1</td>
<td>23 16%</td>
<td>3 7%</td>
<td>26 14%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>23 16%</td>
<td>5 11%</td>
<td>28 15%</td>
<td></td>
</tr>
<tr>
<td>Not classified (n)</td>
<td>3 2%</td>
<td>0 0%</td>
<td>3 2%</td>
<td></td>
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<tr>
<td>Tumor-node-metastasis (n)</td>
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<td></td>
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<tr>
<td>IA</td>
<td>42 29%</td>
<td>19 42%</td>
<td>61 32%</td>
<td></td>
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<tr>
<td>IB</td>
<td>47 33%</td>
<td>18 40%</td>
<td>65 35%</td>
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<td>II</td>
<td>27 19%</td>
<td>3 7%</td>
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<td>IIIA</td>
<td>24 17%</td>
<td>5 11%</td>
<td>29 15%</td>
<td></td>
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<tr>
<td>Not classified (n)</td>
<td>3 2%</td>
<td>0 0%</td>
<td>3 2%</td>
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<td>1 2%</td>
<td>5 2.5%</td>
<td></td>
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<tr>
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<td>1 2%</td>
<td>5 2.5%</td>
<td></td>
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<tr>
<td>Complete</td>
<td>135 94%</td>
<td>43 96%</td>
<td>180 95%</td>
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<tr>
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<td>26 58%</td>
<td>87 46%</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>67 47%</td>
<td>17 38%</td>
<td>84 45%</td>
<td></td>
</tr>
<tr>
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<td>2 4%</td>
<td>17 9%</td>
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<td>43 96%</td>
<td>164 87%</td>
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</tr>
<tr>
<td>Yes</td>
<td>22 15%</td>
<td>2 4%</td>
<td>24 13%</td>
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<td>38 84%</td>
<td>149 79%</td>
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<tr>
<td>Yes</td>
<td>32 22%</td>
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<td>Second cancer (n)</td>
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<td>0.97</td>
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<td>137 96%</td>
<td>43 96%</td>
<td>180 96%</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>6 4%</td>
<td>2 4%</td>
<td>8 4%</td>
<td></td>
</tr>
</tbody>
</table>

*Positive surgical margins.
† No lymphadenectomy performed.
Immunostaining of tumor samples was done with Vectastain Elite ABC kit using the instructions of the manufacturer. To enhance epitope exposure, deparaffinized slides were heated at 98°C in 10 mmol/L citrate buffer (pH 6) for 30 min. Incubation with the primary antibody [phospho-ERK Thr202/Tyr204 antibody, dilution 1:400, and phospho-P38 Thr180/Tyr182 antibody, dilution 1:800 (both antibodies from Cell Signaling Technology); phospho-JNK Thr183/Tyr185 antibody, dilution 1:1,600 (Santa Cruz Technology)] was done at 4°C overnight. The chromogen used was NovaRED (Vector), whereas the counterstain used was Mayer's hematoxylin. As negative controls, the primary antibodies were omitted.

Immunohistochemical evaluation. The slides were scanned at high resolution (VM3 virtual scanner, Ziemmens, Germany), thus enabling the study of an identical high-quality image for each spot by two independent readers (G. Mountzios and P. Fouret) in various magnifications. The samples of ever smokers and never smokers were arrayed on separate slides, but they were labeled and analyzed in batches including irrelevant samples without knowledge by the observers. A map of the tissue microarray blocks was used to record the results and attribute them to the correct patient. An independent quality control verified the agreement between coordinates of spots on tissue microarray slides, patient numbers, and paraffin-embedded samples.

For each case, three cores were studied. If all three cores were not interpretable because of poor tissue quality, or few cancer cells, the corresponding case was excluded from the analysis. Importantly, all

Fig. 1. Representative cases of strong positive staining (H score = 3; left) and of true negative (H score = 0; right) for pERK, pJNK, and pP38. Internal positive controls (white arrows). Original magnification, ×20.
cases without positive internal control (macrophages, lymphocytes, endothelial cells, and fibroblasts) were considered invalid and were excluded from the analysis.

Because the activation of each MAPK induces its translocation into the nucleus, only the nuclear staining (noted as pERK, pJNK, and pP38) was recorded. In order to semi-quantitatively evaluate the immunohistochemical staining for each marker in a homogenous and comparable manner, the H score method was used (17). Briefly, the percentage of the stained tumor cells (assigning 0, 0.1, 0.5, or 1 for 0%, 1-9%, 10-49%, or ≥50% of stained cancer cells, respectively) and the intensity of staining (on a scale from 0 for absence of staining to 3 for strong staining) were recorded. The H score was calculated as the product of the percentage and intensity scores. All discordant cases were resolved within consensus meetings.

**Experiments with the HCC827 cell line.** The HCC827 cell line (American Type Culture Collection) was maintained in RPMI supplemented with 10% fetal bovine serum, 2 mmol/L of l-glutamine at 37°C, 5% CO₂. Cells were seeded at 3 × 10⁵ cells per well in 96-well microplates in a final volume of 100 μL/well culture medium. Twenty-four hours later, the cells were treated with cisplatin (Merck) at 50 or 100 μmol/L for 1 h or with SB 203580 (Calbiochem) at 10 μmol/L alone for 24 h or 30 min prior to cisplatin. Control treatments contained 0.1% DMSO. After treatments, cells were washed with PBS and supplemented with fresh medium. Measurements of viable cell mass were done 5 days later using a colorimetric-based reaction in accordance with the protocol of the manufacturer (Roche). The absorbance was measured at 450 nm with an ELISA plate reader. Cell viability was expressed as viable cell mass following a given treatment normalized to that of parallel cultures of untreated cells (viable cell mass, %). Each experiment was done in triplicate with three measurements for each condition. The results shown are the mean values of three such experiments.

**Statistical analysis.** For each activated MAPK, the H scores were compared according to smoking status using the Mann-Whitney U test. The H score median values were a priori selected as the cutoff point for dichotomization of activated MAPK expression in “high” and “low.” When several cases had a score equal to the median, the proportion of cases with H scores equal to or superior to the median was >50%. The proportions of high and low expressers were compared in never smokers and in ever smokers using the χ² test or Fisher’s exact test. Their 95% confidence intervals (95% CI) were calculated using the method of Agresti and Coull. The logistic regression models of each activated MAPK were done using smoking status and smoking-associated characteristics as exposure variables. Overall survival (OS) from the date of diagnosis up to January 2006 was used for the survival analysis. The OS curves were plotted using the Kaplan-Meier method, and compared with the log-rank test. Cox proportional hazards models were developed to examine whether activated MAPK expression was prognostic after adjustment for clinical variables. All tests were two-sided, and the chosen level of significance was P < 0.05.

**Results.**

**Patient characteristics.** Among the 188 validated cases, there were 143 ever smokers (76%) and 45 never smokers (24%). Median age at diagnosis was 63 years (range, 34-86 years). Two-thirds (67%) of the patients had stage I disease. Never smokers were mostly women (42 of 45 or 93%, P < 0.0001), and were significantly older than ever smokers (median age, 71 and 59 years for never smokers and ever smokers, respectively; P = 0.0007). The percentage of adenocarcinomas with bronchoalveolar (BAC) features was significantly higher in never smokers (15 of 45 or 33%) compared with ever smokers (20 of 143 or 14%, P = 0.004). There were no other differences between ever smokers and never smokers (Table 1).

**MAPK activation in ever smokers and never smokers.** At least one valid spot was available for 159, 155, and 148 cases regarding pERK, pJNK, and pP38, respectively. Staining was stronger for both pERK (median H score = 1.5) and pJNK (median H score = 2) compared with pP38 (median H score = 0.5). Representative examples of cases with strong positive and negative staining for all three MAPKs are provided in Fig. 1.

Using the Mann-Whitney U test, there was no difference in pERK expression between ever smokers and never smokers (P = 0.92; Fig. 2A; Table 2). The same analysis done for pJNK showed that this marker was expressed at higher levels in never smokers compared with ever smokers (P = 0.012; Fig. 2B; Table 2). Similarly, pP38 was expressed at significantly higher levels in never smokers compared with ever smokers (P < 0.0001; Fig. 2C; Table 2).

Using the median value as the cutoff point to identify high expression levels, the proportion of ever smokers expressing
high pERK levels (59 of 117 or 51%; 95% CI, 42-59%) was similar \((P = 0.47)\) to that of never smokers (24 of 42 or 57%; 95% CI, 42-71%). The proportion of ever smokers expressing high pJNK levels (59 of 112 or 53%; 95% CI, 44-62%) was lower \((P = 0.03)\) than that of never smokers (31 of 43 never smokers or 72%; 95% CI, 57-83%). Similarly, 45 of 104 ever smokers (43%; 95% CI, 34-53%) expressed high pP38 levels compared with \((P < 0.0001)\) 37 of 44 never smokers (84%; 95% CI, 70-92%).

**Multivariate logistic regression analysis of pP38 and pJNK.** As pP38 and pJNK were both associated with smoking status in univariate analysis, the logistic regression method was used to determine whether the expression of pP38 or pJNK at high levels were explained by smoking status after adjustment for clinical or pathologic variables linked to smoking status, including sex, age at diagnosis, and presence of BAC features (Table 1). Never smokers had a 10.5 odds of expressing high pP38 levels compared with ever smokers (95% CI, 3.5-31.5; \(P < 0.0001\)) after adjustment for sex, age at diagnosis, and presence of BAC features. On the other hand, never smokers were not more likely to express high pJNK levels than ever smokers after adjustment for the same variables (odds ratio, 3.5; 95% CI, 0.9-12.3; \(P = 0.07\)). In a forward variable selection procedure, none of the individual factors, but only the combination of sex and BAC features were responsible for the decrease in odds ratio for pJNK level.

As never smokers were mostly women, we also did a logistic regression analysis for pP38 expression restricted to the female population of our cohort: female never smokers had a 12.2 odds of expressing high pP38 levels compared with female ever smokers (95% CI, 3.7-40.2; \(P < 0.0001\)) after adjustment for age and BAC features. In the subgroup of ever smokers, there was no difference in pP38 expression according to whether patients had quit smoking for ≥1 year or not \((P = 0.42)\). The development of lung cancer in never smokers remains a partially understood phenomenon. Various etiologic, genetic, and molecular differences have been reported between these cancers and tobacco-related non–small cell lung cancer (2, 6–8, 16). The aim of this study was to establish whether the activated MAPK expression profile differs in ever smokers and never smokers with lung adenocarcinoma. Our results provide evidence that life-long nonsmoking is associated with a partially understood phenomenon. Various etiologic, genetic, and molecular differences have been reported between these cancers and tobacco-related non–small cell lung cancer (2, 6–8, 16). The aim of this study was to establish whether the activated MAPK expression profile differs in ever smokers and never smokers with lung adenocarcinoma. Our results provide evidence that life-long nonsmoking is associated with the activated p38 pathway, but the strength of the association should be considered with caution given the small number of never smokers. Whereas activated ERK levels were similar in ever smokers and never smokers, both activated JNK and activated P38 were expressed at higher levels in never smokers than in ever smokers (2, 6–8, 16).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio</th>
<th>95% CI (low)</th>
<th>95% CI (high)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor-node-metastasis stage (IA, IB, II, IIIA)</td>
<td>1.32</td>
<td>1.01</td>
<td>1.73</td>
</tr>
<tr>
<td>pERK (H score)</td>
<td>0.85</td>
<td>0.66</td>
<td>1.08</td>
</tr>
<tr>
<td>pJNK (H score)</td>
<td>0.83</td>
<td>0.57</td>
<td>1.21</td>
</tr>
<tr>
<td>pp38 (H score)</td>
<td>1.01</td>
<td>0.72</td>
<td>1.41</td>
</tr>
</tbody>
</table>

**Discussion**

The development of lung cancer in never smokers remains a partially understood phenomenon. Various etiologic, genetic, and molecular differences have been reported between these cancers and tobacco-related non–small cell lung cancer (2, 6–8, 16). The aim of this study was to establish whether the activated MAPK expression profile differs in ever smokers and never smokers with lung adenocarcinoma. Our results provide evidence that life-long nonsmoking is associated with the activated p38 pathway, but the strength of the association should be considered with caution given the small number of never smokers. Whereas activated ERK levels were similar in ever smokers and never smokers, both activated JNK and activated P38 were expressed at higher levels in never smokers.
compared with ever smokers. However, it is noted that never smokers in this cohort were older and much more often women, and their tumors more frequently comprised BAC features. Nevertheless, the P38 pathway was 10 times more likely activated in never smokers compared with ever smokers after adjustments for these potentially confounding covariates. Instead, after taking sex and BAC features together into account, activated JNK was not significantly associated with never smokers status. Therefore, smoking status is the patient characteristic that predicts for P38 pathway activation state within lung adenocarcinoma cells. Admittedly, the low number of men among never smokers limits the ability of multivariate modeling to properly adjust for a confounding effect of gender. However, the relationship between pP38 and smoking status was preserved even when the analysis was restricted to women. Some of the negative results reported here may have been generated by low statistical power of some of our analyses, which is indicated by the wide confidence intervals for the observed proportions. Thus, we cannot rule out that pERK expression is actually higher in never smokers compared with ever smokers, although the 95% CI overlapped to a large extent (42–71% for never smokers and 42–62% for ever smokers). However, it is noted that never smokers are related to the distinct molecular changes that characterize those entities.

Among MAPKs, P38 can act as a tumor suppressor—a possibility that is puzzling when considering the high P38 levels seen in tumors such as lung adenocarcinomas, mainly in the subgroup of never smokers. A possible reason could be that P38 action is different in the context of adenocarcinoma cells in never smokers, as those tumors have unique molecular and biological characteristics. In an effort to support this hypothesis, we studied the effects of P38 pharmacologic inhibition on cell growth in the epidermal growth factor receptor mutant (delE746_A750) adenocarcinoma cell line HCC827, which is derived from a nonsmoking patient and does not harbor K-Ras mutations. We showed that P38 activity does not inhibit, but rather, contributes to cell growth in the HCC827 model. On the other hand, it seems well-established that P38 suppresses cell growth by inducing apoptosis or senescence in several models characterized by Ras-induced proliferation (21–23). In light of the selectivity of P38 tumor suppression for Ras-transformed cells, we speculate that the high activated P38 levels seen in never smokers may be explained by the lack of K-Ras mutations, although the specific aberrations in MAPK or interacting pathways that are responsible for P38 signaling in lung adenocarcinoma remain to be determined.

In conclusion, among patients with lung adenocarcinoma, the P38 pathway is much more frequently activated in never smokers compared with ever smokers. The P38 pathway can contribute to cell growth in adenocarcinoma in never smokers. Further studies in cells in never smokers may lead to the discovery of important, yet unknown, alternative pathways of lung carcinogenesis, to which P38 pathway activation may contribute.

### Disclosure of Potential Conflicts of Interest

The authors have declared no conflict of interest.

### Acknowledgments

Estelle Taranchon, Theofil Dutu, and Mustapha Erman participated in the TMA construction. The TMA slides were scanned at the European Organization for Research and Treatment of Cancer by D. Jarnin. The P38 inhibitor was a generous gift from V. Camara-Clayette. We thank Jean Tréndian and Isabelle Monnet for their help in collecting clinical data.

### References


Tobacco smoke can rapidly and reversibly alter signal transduction in lung cells (18). The lack of difference in pP38 expression between current and former smokers does not necessarily imply that pP38 is not involved in tobacco-related lung carcinogenesis on a pathway linked to an early-stage effect of tobacco. However, it is noted that the higher pP38 level seen in adenocarcinomas in never smokers compared with ever smokers is related to the distinct molecular changes that characterize those entities.

Fig. 3. Viability of HC C827 cells following treatment with CDDP or P38 MAPK inhibitor SB203580. Cells were seeded at a density of 2 × 10^5 cells/well in 96-well tissue culture plates, and on the following day, cells were treated with the designated agents. Viable cell mass was assessed 5 d later using WST-1 assay and was expressed as the percentage of the value obtained in a parallel untreated culture. All measurements were carried out in triplicate and experiments repeated thrice. Columns, mean; bars, SE.

### References


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Mitogen-Activated Protein Kinase Activation in Lung Adenocarcinoma: A Comparative Study between Ever Smokers and Never Smokers

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