Inhibitor of Differentiation-1 as a Novel Prognostic Factor in NSCLC Patients with Adenocarcinoma Histology and Its Potential Contribution to Therapy Resistance

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Abstract

Purpose: High inhibitor of differentiation-1 (Id1) levels have been found in some tumor types. We aimed to study Id1 levels and their prognostic impact in a large series of stages I to IV non-small cell lung cancer (NSCLC) patients. Experiments in cell lines and cells derived from malignant pleural effusions (MPE) were also carried out.

Experimental Design: A total of 346 NSCLC samples (three different cohorts), including 65 matched nonmalignant tissues, were evaluated for Id1 expression by using immunohistochemistry. Additional data from a fourth cohort including 111 patients were obtained for Id1 mRNA expression analysis by using publicly available microarrays. In vitro proliferation assays were conducted to characterize the impact of Id1 on growth and treatment sensitivity.

Results: Significantly higher Id1 protein levels were found in tumors compared with normal tissues ($P < 0.001$) and in squamous carcinomas compared with adenocarcinomas ($P < 0.001$). In radically treated stages I to III patients and stage IV patients treated with chemotherapy, higher Id1 levels were associated with a shorter disease-free survival and overall survival in adenocarcinoma patients in a log-rank test. A Cox model confirmed the independent prognostic value of Id1 levels for both stages I to III and stage IV patients. In silico analysis confirmed a correlation between higher Id1 mRNA levels and poor prognosis for adenocarcinoma subjects. In vitro Id1 silencing in radio/chemotherapy-resistant adenocarcinoma cells from MPEs restored sensitivity to both therapies.

Conclusions: In our series, Id1 levels showed an independent prognostic value in patients with adenocarcinoma, regardless of the stage. Id1 silencing may sensitize adenocarcinoma cells to radiotherapy and chemotherapy. Clin Cancer Res; 17(12); 4155–66. ©2011 AACR.

Introduction

Lung cancer is the leading cause of cancer deaths among men and women in the United States and Europe. It has been estimated that 1.3 million new cases of lung cancer occur yearly worldwide, resulting in more than 180,000 annual fatalities in Europe (1) and 160,000 in the United States (2).

Although early-stage non-small cell lung cancer (NSCLC) can initially be radically treated, approximately 30% of stage I patients (3), 50% of those with stage II (4), and more than 70% of stage III individuals (5) will relapse and die from the disease. In addition, the vast majority of patients diagnosed with metastatic disease will die within the first 18 months following diagnosis (6).

Thus, in all stages of NSCLC, it is essential to identify specific biomarkers (7) that are able to predict survival. These biomarkers would help to identify patients who would benefit from treatment and those at risk of relapse to determine the best therapeutic option for each scenario. Furthermore, this identification would add more useful biomarkers to the so-called molecular circuits for solid tumors (8).

In the present manuscript, we have studied the biomarker inhibitor of differentiation-1 (Id1), a member of the helix-loop-helix protein family (9), which has been shown to be expressed in a variety of tumor types (10). For the last several years, the impact of Id1 expression on endothelial
and tumor cells from different types of cancer by using animal models and human cancer samples has been investigated. Some studies using diverse preclinical models have shown that Id1 plays a key role in tumor cell differentiation (11), proliferation (12), angiogenesis (13–15), and metastasis (16, 17).

In addition, immunohistochemical (IHC) analysis of Id1 expression in human cancer specimens has markedly improved because of the recent validation (18) of a highly specific monoclonal antibody (Biocheq 195–14) against Id1, which produces consistent and reliable results in a variety of tumor types (17, 19, 20).

Regarding lung cancer, preliminary data from 3 recent reports based on a small series of patients have shown Id1 protein expression in human samples of small cell lung cancer (SCLC; ref. 21) and NSCLC (20, 22). Id1 expression has also been shown in NSCLC (22) at the mRNA level. However, no clear evidence (or even contradictory results) has been published on Id1 expression in tumor tissue compared with normal tissue and the correlation between Id1 levels and patient’s clinical outcome has never been addressed.

On the basis of tumor samples from more than 400 patients with all stages of NSCLC, the objective of our study is to investigate Id1 protein levels and their relationship with prognosis in NSCLC. We present data suggesting that Id1 is a prognostic factor in NSCLC and a role for this protein in chemotherapy and radiotherapy (RT) resistance using in vitro studies in primary cell lines.

Materials and Methods

Patients and tissue samples

This study included 457 NSCLC patients who were divided into the following 4 different cohorts: A (n = 101), B (n = 211), C (n = 34), and D (n = 111). Detailed clinical and pathologic characteristics of cohorts A, B, and C are summarized in the Supplementary Table S1. The patients included in cohort D have previously been described by Bild and colleagues (23). The biological material used was formalin-fixed paraffin-embedded tissue sections of tumor and nonmalignant lung. The nonmalignant lung tissue was obtained from the same resection piece where the tumor was located. The pathologist selected nonmalignant tissue as far as possible from the primary tumor. Samples were stored in their respective biobanks. A nonsmoker was defined as someone who had smoked less than 100 cigarettes in his or her lifetime.

Cohort A. A series of 101 patients diagnosed with NSCLC who underwent surgical resection at our institution (Clinica Universidad de Navarra, Pamplona, Spain) from 2000 to 2009 were included in this study. A total of 65 tumor samples from this cohort were obtained with their paired nonmalignant lung tissues. The inclusion criteria were NSCLC histology, absence of cancer within the 5 years prior to lung cancer surgery, and no neoadjuvant chemotherapy. A total of 57 patients were treated with surgery and 44 with surgery followed by adjuvant treatment (chemotherapy alone or RT and concomitant chemotherapy), according to pathologic findings within the tumor sample resected. The median follow-up for this cohort was 43 months. The median disease-free survival (DFS) was 53 months. Overall survival (OS) was not reached (estimated percentage of patients alive at 5 years was 78.4%).

Cohort B. A second cohort of 211 NSCLC patients who underwent surgical resection at the MD Anderson Cancer Center (Houston, TX) from 2000 to 2003 was used. The inclusion criteria were the same as described above. A total of 186 patients were treated with surgery and 25 were surgically resected followed by adjuvant treatment (chemotherapy alone or RT and concomitant chemotherapy) according to pathologic findings within the tumor sample. Patients in this cohort were followed for a median time of 66 months and the median DFS and OS were 68 and 99 months, respectively with an estimated percentage of patients alive at 5 years of 63.5%.

Cohort C. This cohort was composed exclusively of stage IV NSCLC patients. A total of 34 patient samples were obtained prior to chemotherapy at the Clínica Universidad de Navarra (Pamplona, Spain) from our tissue biobank (collection period time 2000–2006). Eligible patients were 18 years of age or older at the time of treatment, had a histologically confirmed diagnosis of stage IV NSCLC and were eligible to be treated with a platinum–taxane combination regimen in a first-line setting (case selection is explained in the Supplementary Data). A total of 32 patients were followed until death and 2 were still alive in our last follow-up (November, 2010). Median follow-up was 8 months and median progression-free survival (PFS) and OS were 4 and 8 months, respectively. Response Evaluation Criteria in Solid Tumors were applied for response assessment as previously published (24).
Histologic diagnosis was carried out according to the 2004 WHO classification (25), and reporting recommendations for tumor marker prognostic studies criteria were followed throughout the study (26). Stages were defined according to the American Joint Committee on Cancer guidelines, version 6th. The study protocol was approved by the ethical committee of each institution.

IHC and Western blot analyses

Id1 expression was evaluated with indirect immunoperoxidase staining of formalin-fixed and paraffin-embedded tissue sections. Endogenous peroxidase activity was quenched by using 3% hydrogen peroxidase in methanol for 30 minutes. Antigen retrieval was carried out in EDTA (1 mmol/L, pH 8) for 20 minutes in a microwave, and sections were then incubated with the anti-Id1 monoclonal antibody (1:100; Biocheck) overnight at 4°C. Previous validation of the specificity of this antibody has been carried out (18). Detection was conducted with the Advance HRP system (Dako). Immunostaining was developed with 3-3′-diaminobenzidine, and sections were counterstained with hematoxylin.

The primary antibody was omitted in negative controls. Staining scores were established (H-score) as previously described. Staining scores were established by semiquantitative analysis. The extension and intensity of the staining was assessed compared with a known external positive control (1 weak, 2 moderate, and 3 strong staining). A final score, called H-score, was calculated by adding the products of the percentage cells stained at a given staining intensity (0–100) and the staining intensity (0–3; ref. 27). The median of the score for Id1 expression silencing was carried out using RNA interference by using siRNA hairpins (sc-29356-sh; Santa Cruz Biotechnology). The transfection of siRNA was conducted with Lipofectamine 2000 with a molar ratio of DNA/lipid of approximately 1:3 (Invitrogen). At 24 hours after transfection, puromycin (2 μg/mL) was added to select transfected cells. These cells were collected and used for functional assays.

Expression dataset from microarray analysis

Gene expression and clinical data from 58 primary lung adenocarcinomas (AC) and 53 squamous cell carcinomas (SCC; cohort D, n = 111) were obtained from Bild and colleagues (23), and the dataset was retrieved from the Gene Expression Omnibus (GSE3141; www.ncbi.nlm.nih.gov/geo). Id1 mRNA levels were determined by analysis of the signal intensity of the probe set 208937_s_at. Details of the patient selection criteria and methods for data normalization have been described by Bild and colleagues (23).

Characterization of NSCLC cells obtained from pleural effusions

Malignant pleural effusions (MPE) from 8 patients were prospectively obtained according to the protocol. After the patients signed an informed consent, an aliquot of each MPE was sent to the Department of Pathology to confirm the presence of tumor cells. Then, primary cells were isolated from MPEs. Each sample was centrifuged, and the pellet was resuspended in complete ACL-4 medium for primary cells (28).

Finally, MPE samples from 2 different patients (designated as Gon8 and Mai9), which were resistant to RT and carboplatin, respectively, were used in the in vitro experiments (for characteristics of these patients, see the Supplementary Data).

RNA interference-mediated silencing of Id1 expression

Id1 expression silencing was carried out using RNA interference by using siRNA hairpins (sc-29356-sh; Santa Cruz Biotechnology). The transfection of siRNA was conducted with Lipofectamine 2000 with a molar ratio of DNA/lipid of approximately 1:3 (Invitrogen). At 24 hours after transfection, puromycin (2 μg/mL) was added to select transfected cells. These cells were collected and used for functional assays.

Cell proliferation and clonogenic assay

Cells were plated in 96-well plates (2 × 10⁶ cells/mL) in triplicate. Then, Gon8 cells were exposed to radiation (0, 4, and 8 Gy), and Mai9 cells were treated with 0, 25, and 200 μg/mL carboplatin. A549 cells were used as positive controls because of their sensitivity to radio- and chemotherapy. After 3 days, cell proliferation was measured by using MTT (Roche) assays according to the manufacturer’s protocol. The plate absorbance was read at 490 nm.

Colon cancer formation assays were conducted by plating 500 cells/well into 100-mm culture dishes in triplicate. After a 12-day incubation at 37°C and 5% CO₂, cells were fixed with formaldehyde and stained with 2% crystal violet. The number of colonies was then counted, and the surviving fraction of treated cells was normalized to the surviving fraction of the corresponding controls.

Radiation exposure

Gon8 cells and A549 cells (as positive radiosensitive cells) were irradiated by using 15 MeV electrons (Primus Linear Accelerator, Siemens) at 4 and 8 Gy. Nonirradiated controls were handled identically to the irradiated cells. After irradiation, cultures were maintained under normal cell culture conditions.
Statistical analysis

The association between Id1 expression and clinicopathologic factors was analyzed by Pearson’s χ² test. Fisher’s exact test was used when appropriate. Unpaired 2-tailed Student’s t-tests were used to analyze comparisons between the 2 groups. An ANOVA test was used when appropriate to determine significant differences between groups. For survival analysis, PFS and DFS were calculated from the date of surgery or treatment initiation to the date of recurrence or death. OS was calculated from the date of surgery or treatment initiation to the date of death. Follow-up was calculated from the date of surgery or treatment initiation to the date of death, lost to follow-up or last contact with the patient. Kaplan–Meier curves and the log-rank test were used to analyze differences in survival time (the median of the H-score was selected as a cutoff point) and to evaluate the significance of Id1 mRNA expression in lung cancer survival. Univariate and multivariate Cox proportional hazards analysis were used to assess the prognostic role of Id1. Only those variables with P < 0.1 in univariate analysis were included in the multivariable analysis.

Statistical analyses were conducted with the SPSS 15.0 software. A P < 0.05 was considered statistically significant.

Results

Patient characteristics and clinical features

Three different cohorts (A–C) of NSCLC patients were evaluated by using immunohistochemistry analysis in this study. The main clinical and pathologic characteristics of these cohorts are summarized in Supplementary Table S1. The distribution of histologies was 41% AC and 53% SCC in cohort A and 61% AC and 38% SCC in cohort B.

Cohort C (n = 34) exclusively comprised stage IV patients (70% AC and 18% SCC) who had received a median of 4 cycles (range, 1–8) of platinum–taxane combination chemotherapy. After palliative chemotherapy, 35.3% of patients experienced a radiological response, 29.4% showed stable disease, and 35.3% showed disease progression.

Id1 protein expression in normal and NSCLC specimens

In nontumor lung tissues, the vast majority of the tissue showed no staining (Fig. 1A). Id1 was specifically expressed in the nuclei of tumor cells and endothelial cells.

The first immunohistochemistry analysis was carried out in cohort A with their matched nonmalignant lung tissue. Our analysis showed a significant increase in Id1 protein expression in tumors compared with their nontumor counterparts (median H-score 114.0 ± 7.10 vs. 31.6 ± 3.46, P < 0.001; Supplementary Fig. S1A).

In Supplementary Table S3, we have described the relationship between Id1 levels and different clinicopathologic factors by χ² test. A significant correlation between Id1 levels and histology was found in cohorts A and B and a trend toward statistically significance was found in cohort C. Correlation between Id1 and sex was also found in cohort B. No correlation between the patients’ smoking history, treatment received or stage, and the expression of Id1 in tumors was found in any of the cohorts.

Differential expression of Id1 in lung AC and SCC

We next compared Id1 expression in the 2 main histologies of NSCLC patients, AC and SCC. In cohort A, we observed that AC tissues expressed significantly less Id1 levels than SCC tissues (P < 0.001; Supplementary Fig. S1B). This result was then validated in cohort B (P < 0.001; data not shown). The third analysis, conducted in cohort C, confirmed that AC tissues expressed significantly less Id1 protein (P = 0.019; data not shown) than SCC tissues. Therefore, from stages I to IV NSCLC, Id1 protein was differentially expressed in lung AC (low levels) and SCC (high levels) specimens. Representative IHC staining images of SCC and AC samples are shown in Figure 1A.

High Id1 protein levels predict a poor outcome in stages I to III

Next, we studied the relationship between Id1 expression and NSCLC prognosis in cohort A (n = 101). No association between Id1 levels and OS was found (P = 0.25). However, a significant association between higher Id1 expression levels and shorter DFS, was found (P = 0.043; Supplementary Fig. S1C). No correlation was found when adjusting by treatment received. Moreover, in the subset of AC, a similar trend in both OS and DFS unfavorable to high Id1 levels, was also found (P = 0.23 and P = 0.11, respectively) whereas no correlation was found in SCC tumors for any of the outcomes. This finding suggested that in AC, despite lower Id1 protein levels than SCC tumors, Id1 levels could be associated with patient’s outcome. With these preliminary results found in a cohort with a limited number of patients, we carried out a similar study in cohort B, which was composed of more than twice the number of patients in cohort A.

In cohort B, we observed a significantly shorter median DFS (P = 0.025) and OS (P = 0.001; Fig. 1B) among patients with higher Id1 levels. When we stratified by histologies, the same association between high Id1 levels and poor prognosis in AC histology was found for both DFS and OS (P = 0.008 and P = 0.003; Fig. 1B). No such significant association was observed for SCC patients.

Similar results were observed when we divided patients according to adjuvant treatment administration. In patients who did not receive adjuvant treatment and showed high Id1 levels in tumors, DFS and OS times were decreased, with a trend toward statistical significance for DFS (P = 0.078; Supplementary Fig. S2A) and a significant decrease for OS (P = 0.007; Supplementary Fig. S2A). In the group that received adjuvant treatment, a trend toward an association between a shorter DFS and
OS and higher Id1 levels was found ($P = 0.10$ and $P = 0.076$, respectively). We then conducted similar analyses after stratifying by histology. In patients who did not receive adjuvant therapy and had AC histology, poor median DFS, and OS times were found for those patients with high Id1 levels ($P = 0.047$ for DFS and $P = 0.017$ for OS; Supplementary Fig. S2A). Among patients treated with adjuvant therapy, in the AC subgroup, similar results, unfavorable to high Id1 levels, were found in DFS and OS ($P = 0.038$ for both; Supplementary Fig. S2B). In contrast, the levels of Id1 in SCC specimens showed no statistical differences in DFS or OS in any of the groups (data not shown).

With these results suggesting that Id1 might be a prognostic factor in AC histology, a multivariate analysis in AC patients of cohort B was done. In the multivariate analysis, we observed that Id1 was an independent prognostic factor for both, DFS [HR = 2.070 (95% CI: 1.258–3.406); $P = 0.004$] and OS [HR = 2.712 (95% CI: 1.554–4.736); $P < 0.001$], in AC patients (Tables 1 and S2).
High Id1 protein expression correlates with a poor outcome in stage IV patients

To study the potential role of Id1 as a useful prognostic factor in stage IV NSCLC patients, we studied cohort C (Supplementary Tables S1 and S3). Interestingly, the median PFS and OS times of high and low Id1-expressing tumor groups were significantly different, and an unfavorable association was found with high Id1 levels (Fig. 2A).

When this cohort was stratified by histology, Id1 also exhibited a prognostic value in AC. Median PFS and OS times were significantly decreased in patients with high levels of Id1 in AC. A median PFS of 1 month was observed among high Id1-expressing patients compared with 7 months in low Id1-expressing patients ($P = 0.008$). When histologic types were analyzed separately, high Id1 mRNA levels were associated with poor survival in patients with AC ($P = 0.045$; Fig. 2B) but not in patients with SCC ($P = 0.801$). These results were in accordance with our observations on protein expression in the previous cohorts, suggesting that Id1 levels could predict prognosis in AC NSCLC patients but not in SCC patients.

Expression of Id1 in both AC and SCC cell lines

We next investigated the role of Id1 in NSCLC in vitro. Id1 expression was detected in all 8 NSCLC cell lines analyzed by using Western blotting. All 4 AC (SKLU-1, LXF289, H322, and H23) and 4 SCC (H520, HCC15, H157, and H58) cell lines showed Id1 protein expression (Fig. 3A). However, SCC cell lines showed higher Id1 levels compared with AC cells (Fig. 3A). This pattern was similar to the findings observed in tumor samples from patients.

Influence of Id1 inhibition on the proliferation of NSCLC cells

To determine the effect of Id1 inhibition on the proliferation of NSCLC cell lines, we selected 2 AC (LXF-289 and H23) and 2 SCC (H157 and H520) lung cancer cell lines. We then used Id1 shRNA plasmids to silence Id1 expression (Fig. 3B). Cells were also transfected with a scrambled

| Table 1. Multivariate Cox proportional hazards model by using OS and DFS/PFS in Cohort B and C. |
|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
|            | COHORT B    |             | COHORT C    |             |             |
| DFS        | OS          | DFS         | OS          | DFS         | OS          |
| Id H-score | P           | HR (95% CI) | P           | HR (95% CI) | P           |
| Low        | High        | 2.070 (1.258–3.406) | 0.004 | 2.712 (1.554–4.736) | 0.001 | 6.895 (1.685–28.211) | 0.007 | 3.452 (1.106–10.778) | 0.033 |
| Age, y     | ≥70         | 2.279 (1.372–3.786) | 0.001 | 3.124 (1.747–5.588) | 0.001 | 3.800 (1.060–13.624) | 0.040 | – | – |
| Sex        | Male        | – | – | – | – | – | – | 0.251 (0.068–0.921) | 0.037 |
|            | Female      | – | – | – | – | – | – | – | – |
| Stage      | I–II        | – | – | – | – | – | – | – | – |
|            | III         | 2.989 (1.639–5.452) | 0.001 | 1.862 (0.925–3.748) | 0.082 | – | – | – | – |

Abbreviation: NA, not applicable.

analyses by using previously published microarray data (23). Data for DFS could not be analyzed because of a lack of information in the public database. Higher levels of Id1 mRNA expression were associated with a poorer OS ($P = 0.038$; Fig. 2B). When histologic types were analyzed separately, high Id1 mRNA levels were associated with poor survival in patients with AC ($P = 0.045$; Fig. 2B) but not in patients with SCC ($P = 0.801$). These results were in accordance with our observations on protein expression in the previous cohorts, suggesting that Id1 levels could predict prognosis in AC NSCLC patients but not in SCC patients.
shRNA sequence control (Mock). In preliminary experiments, we observed that both Id1 protein levels (Fig. 3B) and proliferation (not shown) of Mock-transfected cells and their corresponding wild-type cells were similar. Different degrees of Id1 inhibition were found in cells after gene silencing. The Id1 protein inhibition rates were 48.72% (H520), 47.25% (H23), 36.17% (LXF-289), and 21.54% (H157) compared with their respective Mock-transfected controls (Supplementary Fig. S3A). Clonogenic assays were performed to assess the influence of Id1 inhibition on cells. In H157 and H520 cells, the proportion of cell colonies was significantly reduced in comparison with controls [34.85% ± 3.80 \( P = 0.01 \) and 68.92% ± 3.82 \( P = 0.007 \), respectively; Fig. 3C and Supplementary Fig. S5A]. The values for LXF-289 and H23 cells compared with their controls were 72.66% ± 0.14 \( P = 0.0081 \) and 34.65% ± 1.28 \( P = 0.0007 \), respectively (Fig. 3C). Thus, we concluded that Id1 inhibition decreased the clonogenic capacity of NSCLC cells not only in AC cells but also in SCC cells.

Figure 2. A, Kaplan–Meier survival curves showing OS and PFS according to the Id1 histoscore for cohort C. B, Kaplan–Meier survival curves showing the association between Id1 mRNA expression levels and OS.
viously described. A decrease in Id1 levels was observed in
ferative effect, an Id1 shRNA plasmid was used, as pre-
primary AC cells
Id1 downregulation impairs the proliferation of their sensitivity to therapy.
data prompted us to examine whether Id1 downregulation
a much higher expression than Mai9 cells (Fig. 4B). These
expressed Id1 protein (Fig. 4B), although Gon8 cells had
Western blot analysis revealed that these primary cells
from whom they were obtained (Supplementary Data).
found to be calretinin-negative, mesotelin-negative and
cell lines were immunophenotypically characterized to
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Figure 3. A, detection of Id1 expression in both AC and SCC cell lines by
using Western blot. The amount of protein was normalized to GAPDH. B, Id1 inhibition in NSCLC cells. Differential Id1 levels between wild-type (wt), Mock and shId1 analyzed by using Western blot in H157, LXF-289, H520 and H23 cells. C, influence of Id1 downregulation on NSCLC cell proliferation. Id1 inhibition dramatically reduced the number of colonies. (‘, P < 0.05; “, P < 0.01; ‘’, P < 0.001).

Therapy-resistant primary AC cells express Id1
Two primary NSCLC cell lines obtained from MPEs, Gon8, and Mai9, which were selected because of their resistance to RT and carboplatin, respectively, were used for in vitro analysis (Supplementary Data). Both primary cell lines were immunophenotypically characterized to determine their subtype. IHC features of lung AC were found to be calretinin-negative, mesotelin-negative, and CK20-negative and CK7-positive (data not shown) and TTF1-positive (Fig. 4A), which was similar to the patients from whom they were obtained (Supplementary Data). Western blot analysis revealed that these primary cells expressed Id1 protein (Fig. 4B), although Gon8 cells had a much higher expression than Mai9 cells (Fig. 4B). These data prompted us to examine whether Id1 downregulation would impair the proliferation of these cells and restore their sensitivity to therapy.

Id1 downregulation impairs the proliferation of primary AC cells
To ascertain whether Id1 knockdown had an antiproliferative effect, an Id1 shRNA plasmid was used, as previously described. A decrease in Id1 levels was observed in both primary cell lines after transfection with the Id1 shRNA plasmid (Fig. 4C). Densitometric quantification indicated that Id1 levels were reduced to 72.80% (shId1-Gon8) and 29.77% (shId1-Mai9) compared with Mock controls (Supplementary Fig. S3B).

Clonogenic assays were conducted to study the influence of Id1 inhibition on cell growth. Id1 knockdown in these cells resulted in a dramatic decrease (>60%; P < 0.05) in the cell clonogenic capacity compared with Mock for both cell lines (Fig. 4D and Supplementary Fig. S5B). These results confirmed that Id1 downregulation impaired tumor cell growth.

Id1 downregulation sensitizes primary AC cell lines to RT and chemotherapy treatment
Taking into account the aggressive clinical characteristics of the tumors in the patients (Supplementary Data) from which primary AC cells were isolated and considering their resistance to radio- or chemotherapy, we hypothesized that knocking down Id1 would reverse this resistance. We conducted MTT assays with Mock-transfected Gon8 (RT-resistant), shId1-Gon8 cells, Mock-transfected Mai9 (carboplatin-resistant) cells, and shId1-Mai9 cells. The lung cancer cell line A549 was used as a positive control (radio- and chemosensitive).

Gon8 control cells, shId1-Gon8 cells, and A549 cells were treated with different RT doses (0, 4, and 8 Gy). In agreement with our hypothesis, radiation (4 and 8 Gy) did not significantly affect cell proliferation compared with that observed in nonradiated Gon8 cells (P > 0.05; Fig. 5A). In contrast, the cell growth of shId1-Gon8 cells was significantly reduced after 8 Gy (P < 0.001) compared with untreated shId1-Gon8 cells and also significantly reduced (P < 0.05) compared with untreated Gon8 control cells (Fig. 5A). These results showed that Id1 downregulation sensitized RT-resistant cells to undergo irradiation-mediated cytotoxicity.

Mai9 Mock and shId1-Mai9 cells were exposed to 0, 25, and 200 μg/mL carboplatin. As expected, only high doses of carboplatin (200 μg/mL) induced cytotoxicity in Mai9 control cells. Treatment with 25 μg/mL carboplatin did not induce significant changes in Mai9 cell proliferation, but shId1-Mai9 cells treated with the same dose exhibited a significant reduction in cell growth (P < 0.05; Fig. 5B). Moreover, a significant difference (P < 0.05) was observed between Mai9 and shId1-Mai9 cells when both were treated with 200 μg/mL carboplatin (Fig. 5B). These results show that inhibition of Id1 potentiates the cytotoxic effect of carboplatin and confirm that Id1 inhibition sensitizes resistant cells to chemotherapy.

Discussion
In this study, for the first time, the correlation between Id1 (protein and mRNA) expression levels and prognosis in NSCLC is investigated by analyzing samples from more than 400 patients. In addition, the in vitro role of Id1 in commercially available NSCLC tumor cell lines and cells
derived from pleural effusions from our own patients was also addressed.

In the first part of the study, we compared the levels of Id1 in 65 normal human lung tissue samples with matched NSCLC tumor samples by using immunohistochemistry. Our analyses showed a significantly higher expression of Id1 protein in tumor tissue compared with normal lung tissue. Recently, Bhattacharya and colleagues (22) compared Id1 mRNA expression levels in NSCLC tumors and matched normal samples in a short series of specimens. Surprisingly, the majority of the 30 tumor samples studied (with AC and SCC histology) showed significantly lower Id1 expression compared with matched normal tissues but no immunohistochemistry analysis was conducted to confirm the presence of the protein. Although a potential posttranslational regulation of Id1 cannot be ruled out, no other studies on Id1 mRNA levels in NSCLC samples are available and several reports in other tumors have shown Id1 overexpression in neoplastic tissue at the mRNA level (11, 29, 30).

Interestingly, SCC showed significantly stronger staining than AC. This result was also observed in vitro, although Id1 silencing affected growth in both AC and SCC cell lines. One potential explanation for this finding could be that the level of Id1 expression is tumor histology dependent in epithelial tumors, including lung cancer. According to this hypothesis, high constitutive expression would characterize most of the nonadenocarcinoma histology tumors, such as SCC or transitional cell carcinoma, independent of their prognosis. Previous work by Perk and colleagues (18) has reported that a high proportion of patients with transitional cell carcinoma (or even in situ carcinomas) of the urinary bladder show high Id1 tumor cell expression. However, only advanced and highly dedifferentiated prostate and breast AC showed Id1 expression (18).

Although no correlation between the patients’ smoking history and Id1 expression was found in our study, it is tempting to hypothesize that Id1 expression could be related to tobacco exposure of the tumor cell. Thus, some tobacco-linked cancer histologies, such as SCC of the lung...
or transitional cell carcinoma of the bladder, would express a higher level of Id1 independently of the prognosis. According to our results, it seems clear that Id1 confers particular malignant properties to lung cancer AC, despite AC expressing lower Id1 levels than SCC. Different molecular alterations have been associated with specific NSCLC histologies (31), and these biological differences might be reflected in the differential response seen in clinical trials between AC and SCC in NSCLC. Future studies should determine whether Id1 activates specific molecular pathways related to malignant behavior in lung AC, which could aid in treatment selection.

Because of the discordant data between Id1 protein and Id1 mRNA by Bhattacharya and colleagues (22), we next investigated whether the expression of Id1 mRNA levels was also associated with survival in NSCLC patients. The results from the in silico analysis of 111 NSCLC patients showed agreement with the observation made at the protein level in our study. In accordance with the results of Id1 protein expression, when the OS was compared between the different histologies (AC vs. SCC), high levels of Id1 mRNA were associated with poor survival only in patients with AC.

Our results from 3 independent clinical series with stages I to IV NSCLC show for the first time that tumor Id1 levels correlate with survival in patients with AC histology, regardless the disease stage. High levels of Id1, at both protein and mRNA levels, were associated with poor survival in AC patients. Interestingly, this suggests that Id1 may not only be a reflection of tumor burden or disease progression but a common feature of a more aggressive and poorer prognosis NSCLC subtype from early stages. No previous prognostic significance of the Id1 levels in tumor cells in lung cancer has been proven in NSCLC (20) or SCLC (21) patients. These discrepant results may be because of the fact that the clinical series evaluated by Rothschild and colleagues (20) was too small to show significant differences, whereas the anti-Id1 antibody used for the IHC analysis in the study by Kamalian and colleagues (21) was very likely a nonspecific polyclonal antibody (18).

Finally, we carried out in vitro validation experiments of our findings in patients by using not only commercially available human NSCLC cell lines but also tumor cells derived from MPEs from metastatic patients. Our results showed significant but variable Id1 protein expression between the different cell lines. According to the in vitro findings, SCC cell lines expressed higher Id1 levels compared with the AC cell lines. Interestingly, in both cases, Id1 expression was associated with tumor proliferation. When its expression was significantly inhibited, sensitivity to radio- and chemotherapy was restored. Previous in vitro studies have found that increasing the expression levels of Id1 in NSCLC cell lines produces an enhancement in invasion (32) and migration (22). Reports in other tumor types have also shown that Id1 plays an important role in proliferation and therapy resistance (12, 33, 34).

Our study has some potential limitations. Mainly, it is a retrospective analysis of cases and comprises a limited and heterogeneous series of patients. Thus, a validation analysis in a large-scale prospective homogeneous series of patients is warranted.

**Conclusions**

Id1 is widely expressed in NSCLC tumors and it may represent an independent novel prognostic factor among patients with AC histology. This result warrants further investigation of Id1 expression in prospectively designed clinical trials. Moreover, Id1 contributes to NSCLC cell proliferation and may be crucial for radio- and chemotherapy resistance. Thus, Id1 represents a new potential drug target candidate to be explored in the development of new antineoplastic agents.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
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