Increased Expression of Id Family Proteins in Small Cell Lung Cancer and its Prognostic Significance

Laleh Kamalian, John R. Gosney, Shiva S. Forootan, Christopher S. Foster, Zheng Z. Bao, Carol Beesley, and Youqiang Ke

**Abstract**

**Purpose:** To study the molecular pathology of human small cell lung cancer (SCLC), molecular biology approaches were used to identify genes involved in malignant progression of the cancer cells.

**Experimental Design:** Microquantity differential display was used initially to identify genes expressed differentially between normal and malignant cell lines. The differences were verified by Western blot. Immunohistochemical analysis was done on paired normal and malignant lung tissues and on tissues taken by biopsy to assess the expression status of candidate genes and their prognostic significance.

**Results:** *Inhibitor of DNA/differentiation (Id)* 1 gene was up-regulated in SCLC cells. Levels of Id1 in 8 of 10 cell lines were increased by 1.7- to 21.4-fold when compared with the benign cells. A similar increase was also found in levels of Id2 and Id3. On 26 pairs of lung tissues, all four Id proteins were significantly (Wilcoxon Signed Rank Test, *P < 0.001-0.005*) overexpressed in cytoplasm of the malignant cells. In nuclei of SCLC cells, Id1 expression was significantly reduced, whereas the levels of Id2, Id3, and Id4 were significantly (Wilcoxon Signed Rank Test, *P < 0.001*) increased. Immunohistochemical staining on biopsy specimens showed that the increased expression of Id2 in cytoplasm of cancer cells, not the other three proteins, was significantly associated with the increased survival of SCLC patients.

**Conclusion:** Changed expression profiles of Id proteins may play important roles in malignant progression of SCLC, and the increased Id2 in cytoplasm is a novel prognostic factor to predict the patient outcomes.

Neuroendocrine tumors of lung consist of a spectrum of lesions, from low-grade typical carcinoid, through immediate-grade atypical carcinoid and highly malignant large cell neuroendocrine carcinoma, to the most malignant small cell lung cancer (SCLC; refs. 1, 2). SCLC is one of the most aggressive, disseminative, and probably one of the least understood cancers (3). It is considered a distinct clinicopathologic entity because of the specific clinical manifestations, the unique pathologic features, and sensitivity to chemotherapy (4). It accounts for 20% of all bronchogenic carcinomas (4–6). At the time of diagnosis, 60% to 70% of the patients have already developed extensive disease (4, 5). Average survival is 10 to 16 months (4).

Recently, efforts have been made to study the origins of neuroendocrine tumor cells of the lung and to classify them by their gene expression profiles (7–9). Because most previous studies were done with a small number of tissue samples, the results from different investigators were not in total agreement. According to gene expression profiles, it was reported that SCLC was closely related to, and probably derived from, bronchial epithelial cells; whereas pulmonary carcinoids were related to neural cress-derived brain tumors (7). However, DNA microarray analysis of gene expression profiles identified two prognostically significant subtypes of high-grade lung neuroendocrine tumors independent of SCLC and large cell neuroendocrine carcinoma (9). A recent study showed that the comparative proteomics of pulmonary tumors with neuroendocrine differentiation matched the morphologic classification except for atypical carcinoid and large cell neuroendocrine carcinoma, but SCLC revealed differences in their proteomics according to tumor stage (8).

Despite the recent advances, our knowledge of the molecular pathology of neuroendocrine tumors of lung remains limited. To investigate the molecular mechanism underlying the malignant progression of these tumors, particularly SCLC, we have recently done studies to identify possible candidate genes involved in tumorigenicity and metastasis of SCLC cells. Using microquantity differential display (MDD) developed in our laboratory (10), we have compared the gene expression profiles between the normal bronchial epithelial cell line Beas-2b (11) and a malignant SCLC cell line Lu-165 (12). A cDNA fragment was identified to be up-regulated in Lu-165 cells. After this...
Id Proteins in SCLC

Yoshinobu Ohsaki (Asahikawa Medical College, Asahikawa, Japan), H417 and H345 were gifts from Carol Thomas (School of Clinical Science, University of Liverpool, Liverpool, United Kingdom), and H82 was a gift from Dr. Paul Edwards (Department of Pathology, University of Cambridge, Cambridge, United Kingdom). All cell lines (except Beas-2b and U2020) were grown and maintained as suspension cultures in RPMI 1640 (BioWittaker; BE12-167F) supplemented with 10% (v/v) FCS (Biosera), l-glutamine (2 mmol/L), penicillin (100 units/mL), and streptomycin (100 µg/mL). Beas-2b and U2020 were grown as adherent cultures with the same condition.

Detection of Id proteins in cultured cells. Expression of Id proteins in different cell lines was detected by Western blotting using enhanced chemiluminescence light-emitting nonradioactive kit (Amersham Pharmacia Biotech.) as described previously (19). Total protein in each sample (50 µg) was quantified with a Coomassie Protein Assay Reagent kit (Pierce). Protein extracts prepared from different cell lines were subjected to SDS-PAGE in 15% (v/w) polyacrylamide gels. Membranes were first incubated with preblocking reagents and then incubated with different antihuman Id protein polyclonal antibodies (Santa Cruz Biotechnology) at 1/100 dilution for 1.5 h at room temperature. Membranes were then incubated with horseradish peroxidase–conjugated swine anti-rabbit antibody (DAKO), and bound antibodies were revealed by chemiluminescence (Amersham BioTech) and recorded on Kodak XAR-5 film. Intensities of Id protein bands were determined by scanning densitometry using Alpha-Imager 2000 software (Alpha Innotech). The values of the bands were obtained by measuring the areas below the peaks. Relative levels of Id protein expression in different malignant cell lines were determined by comparing the intensities of the bands with that of the Beas-2b cells. Blots were incubated with a specific antihuman actin monoclonal antibody (Sigma) to correct the possible loading errors.

Human lung tissue samples. The human lung tissues used in this work included two sets of samples collected during the period of 1995 to 2001 and stored in Pathology Archive of the Royal Liverpool University Hospital. One set of tissues consisted of 26 pairs of formalin-fixed, paraffin-embedded blocks, each pair containing a section of SCLC tumor and a section of the normal bronchial epithelium obtained from the same patient. Another set of samples with follow-up data was 93 formalin-fixed, paraffin-embedded biopsy blocks that were diagnosed as SCLC cases. Histologic sections (4 µm) of formalin-fixed, paraffin-embedded tissues were cut and processed, as described previously (20). The tissue sections were examined independently by two qualified pathologists and classified as normal bronchial epithelium and SCLC. This study was approved by the Liverpool Science Ethics Committee in accordance with the Medical Research Council guidelines (project reference number, Ke-07/H1001/102).

Immunohistochemical staining of the human tissues. Expression of the four Id proteins in human tissues was detected by immunohistochemistry with the procedures similar to those described previously (21, 22). Antigens were retrieved with microwave pretreatment and incubated with different anti-Id protein polyclonal antibodies (Santa Cruz Biotechnology) at a dilution of 1/100 for 1 h at room temperature. Bound antibodies were detected with 200 µL of Chemmate Envision horseradish peroxidase from Envision System Horseradish Peroxidase kit (Dakocytomation) for 30 min, and the reaction was visualized with 3’-3’-diamino-benzidine for 10 min. Samples were counterstained with hematoxylin, and sections were mounted with dibutylphthalate xylene. To confirm its specificity, the antibody was incubated with 10 µg/mL of different recombinant Id proteins (Santa Cruz Biotechnology) at 4°C overnight, which completely blocked any immunolabelings. Intensity of staining was classified by both the percentages of the cells stained and the intensity of the staining. In this way, the final scores of 0 to 3 were obtained (0, negative; 1, weak; 2, moderate; and 3, strong). Samples of prostate cancer and benign prostatic hyperplasia were used as positive control, as described previously (18).

Statistical methods. Disregulation of Id protein expression in 26 pair of normal and malignant samples was assessed by Wilcoxon
Signed-Rank test using the Statistical Package for Social Sciences, Version 14.0 (SPSS Inc.). Correlation between different Id protein staining and the length of patient survival was evaluated using Kaplan-Meier survival curve analysis. Differences between patients groups were assessed by Log-Rank test. Statistical significance was defined as a $P$ value of <0.05.

**Results**

**Identification of Id1 as a differentially expressed gene.** To identify differentially expressed genes between the bronchial epithelial cell line Beas-2b and the malignant SCLC cell line Lu-165, mRNA extracted from both cell lines was transcribed into double-stranded cDNA and subjected to MDD analysis. A cDNA subset (Fig. 1A) was found to contain a differentially expressed cDNA fragment (arrow), which was recovered from the denaturing gel. Nucleic acids sequence analysis revealed that this fragment was a 661 bp cDNA molecule. Comparing with the DNA sequences of National Center for Biotechnology Information gene data bank confirmed that it was a segment of Id1 cDNA.

**Verification of the difference in levels of Id1 expression between normal and malignant cells.** To verify the difference in the level of Id1 between normal and malignant cells, Western blot analysis was done to measure the Id1 expression in normal Beas-2b cells and in 10 malignant SCLC cell lines (Fig. 1B). Expression of Id1 was detected in all 10 malignant cell lines. Further quantitative analysis (Fig. 1C) showed that in 8 of 10 SCLC cell lines, the levels of Id1 expression were higher than that detected in normal Beas-2b cells. When the level of Id1 in normal Beas-2b cells was set at 1, it increased from 1.7 (U2020) to 21.4 (Lu165). Although one cancer cell line H-345 did not express Id3, its levels of expression in 9/10 SCLC cell lines were higher than that detected in normal Beas-2b cells (Fig. 2A). When the level of Id3 in normal Beas-2b cells was set at 1, it increased from 1.7 (U2020) to 12.1 (GLC-19) among the 9 malignant cell lines (Fig. 2C). Western blot analysis showed that Id4 expression might be too low to be detected in either the normal Beas-2b cells or in any of the 10 malignant SCLC cell lines (data not shown).

**Expression status of Id family proteins in normal and malignant lung tissue.** To compare the expression status of Id family proteins expressed in normal and malignant tissues, immunohistochemical staining was done on 26 pairs of tissue samples: each pair consisted of sections taken from a SCLC tumor and those taken from adjacent normal bronchial epithelium obtained from the same patient. Some representative stains were shown in Fig. 3. The detailed results of immunohistochemical analysis were summarized in Table 1. Although both cytoplasmic and nuclear stains were observed for Id1, Id3, and
Immunohistochemical staining was also done on an archival set of a large number of SCLC biopsy samples to further examine the expression status of the four Id proteins in carcinomas. The detailed results of biopsy sample staining were shown in Fig. 4. Similar to that observed in the 26 pairs of lung tissues, Id1 expression in the SCLC biopsy samples was mostly cytoplasmic and Id2 expression in nuclei was negative. In the cytoplasm, 69 of 91 (76%) cases exhibited positive Id1 staining, and 82 of 91 (90%) cases exhibited positive Id2 staining. Staining was done on 89 and 93 cases with the antibodies against Id3 and Id4, respectively. In cytoplasm, 65 (73%) and 77 (83%) were positive, most of which (47 of 65 and 63 of 77, respectively) were weakly stained. In nuclei, 80 (90%) and 77 (83%) were positive, most of which (68 of 80 and 52 of 77, respectively) were moderately to strongly stained.

**Prognostic significance of the increased expression of Id proteins.** The relationship between increased expression of each Id protein and patient survival was assessed by Kaplan-Meier survival analysis. Details of Id protein expression status and average survival time of the patients are shown in Table 2. Among the 91 cases stained with antibody against Id2, the average survival of the 38 cases with negative/weak cytoplasmic stain was 3 months. The average survival of the 53 cases with moderate/strong cytoplasmic stain was significantly (Log Rank test, \( P = 0.001 \)) increased to 10 months (Fig. 5). For the other three Id proteins, their increased expression was not significantly associated with the patient outcome (Table 2).

**Discussion**

Recently, Id proteins have been widely investigated for their properties related to tumorigenesis. Id proteins promote the proliferation of normal cells (23) and some malignant cells (14, 24). Experiments in embryos, tumor xenografts, and cultured cells showed that Id proteins could function as promoters in several important steps during malignant progression, including inhibition of differentiation (16, 25), invasiveness (26), and angiogenesis (27–29). Although Id proteins lack definite characteristics of oncogenes, they exhibit oncogene mimicry functions (16), cooperate with some important oncogenes such as \( \text{myc} \) (30) and \( \text{Ras} \) (31, 32), and work against tumor suppressors such as the retinoblastoma protein (33). Id proteins have been shown to prolong cell survival by blocking the \( p16^{INK4A}/\text{retinoblastoma} \) pathway and \( p53 \); both are important in cell senescence (34). Furthermore, they have an antiapoptotic function through activation of nuclear factor-\( \kappa \)B signaling pathway (35). Dysregulation of Id proteins have been found in many types of cancers, including liver (36), prostate (37), ovary (38), breast (39), pancreas (40), squamous cells of head and neck (41), cervix (42), and thyroid (43). There are also reports of deregulation of mRNAs for Id proteins in carcinomas of colon, rectum, endometrium, melanoma, Ewing's sarcoma, seminoma, gastric adenocarcinoma, neural tumors, and leukemia (16, 44).

In contrast to the extensive study of Id proteins in other cancers, their possible involvement in the development and metastasis of SCLC has not been previously investigated. In this work, we initially identified that \( \text{Id1} \) was overexpressed in the malignant SCLC cell line Lu-165 when compared with the normal lung epithelial cell line Beas-2b by MDD analysis. Then we showed that 8 of 10 SCLC cell lines overexpressed 1.7- to 21.4-fold Id1 than Beas-2b cells at the protein level. Although the level of Id4 was barely detectable in these cell lines, Id2 and Id 3 were expressed in higher levels in 8 and 9 of 10 malignant cell lines, respectively, than the normal Beas-2b cells in which...
the expression of Id2 was not detected. These results showed that malignant cells either increased their level of Id1 and Id3 or started to produce Id2. Thus, the increased expression of these 3 Id proteins was associated with the malignant characteristics of the cells, indicating they may play some roles for the malignant transformation.

Surgery is not considered a standard treatment for SCLC after diagnosis (4, 5, 45), and thus, archival samples of these tumors are usually not abundant. We have collected limited numbers of tumor tissues from cases dated from 1995 to 2001 in the Royal Liverpool University Hospital archive. From these cases, 26 were confirmed to be typical SCLC; for each of which used in this study, a normal bronchial epithelium sample was taken from the same patient as control. Pairing of the samples provided an excellent resource to compare the expression of four Id proteins between the malignant SCLC cells and their normal counterparts. Using immunohistochemical staining, we found that expression of all four proteins was significantly increased in the cytoplasm of the malignant cells comparing to the marginal normal epithelium. Whereas Id3 and Id4 were expressed in higher levels in the nuclei of the malignant cells, nuclear expression of Id2 was not statistically different.

### Table 1. Immunohistochemical detection of the expression of Id proteins in SCLC and their normal counterparts

<table>
<thead>
<tr>
<th>Malignant to normal</th>
<th>Overexpression</th>
<th>Same expression</th>
<th>Underexpression</th>
<th>( P ) value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Id protein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Id1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasm no. (%)</td>
<td>14 (54%)</td>
<td>10 (38%)</td>
<td>2 (8%)</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Nuclei no. (%)</td>
<td>0 (0%)</td>
<td>3 (12%)</td>
<td>23 (88%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Id2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasm no. (%)</td>
<td>21 (81%)</td>
<td>4 (15%)</td>
<td>1 (4%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Nuclei no. (%)</td>
<td>3 (11%)</td>
<td>15 (58%)</td>
<td>8 (31%)</td>
<td>0.675</td>
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<tr>
<td>Id3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasm no. (%)</td>
<td>23 (88%)</td>
<td>2 (8%)</td>
<td>1 (4%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Nuclei no. (%)</td>
<td>3 (12%)</td>
<td>0 (0%)</td>
<td>23 (88%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Id4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasm no. (%)</td>
<td>18 (69%)</td>
<td>8 (31%)</td>
<td>0 (0%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Nuclei no. (%)</td>
<td>18 (69%)</td>
<td>6 (23%)</td>
<td>2 (8%)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

**NOTE:** Altogether, 26 pairs of tissue samples were stained. Each pair consisted of a SCLC tumor section and a sample of normal adjacent bronchus epithelium from the same patient.

*\( P \) values represent the significance of the difference of the protein expression levels between the paired benign and malignant samples using Wilcoxon Signed Rank Test.
Although Id1 exhibited a lower staining score in the nuclei of SCLC cells, the total amount of this protein is generally upregulated in the malignant cells because Western blot detected an increase of Id1 protein in the whole cell protein content.

Immunohistochemical staining showed that Id1 protein within SCLC cells was localized mainly in cytoplasm, as it does in some other cancer cells such as prostate (37). The increased expression of Id1 in cytoplasm significantly correlated with the malignant property of lung tissues. Conversely, it was observed that the expression of Id1 in the nuclei of the SCLC cells was reduced. Thus, not only the level of Id1 expression but also the pattern of its distribution was associated with the malignant changes of the lung epithelial cells. Further investigation is needed to understand the biological significance of both the increased level and the changed distribution pattern of Id1 in malignant changes of the SCLC cells. It has been reported that the localization of Id2 is different among different cancers. In prostate cancer, Id2 is expressed in both cytoplasm and nuclei (18). In pancreatic carcinoma, it expressed only in the cytoplasm (40). Whereas in squamous cell carcinoma (46) and cervical cancer (42), its expression was confined to nuclei only. In this work, we found that Id2 is expressed predominantly in the cytoplasm of the SCLC cells. It is not clear whether the different distribution patterns observed in different types of cancers relate to the possible different roles played by increased Id2. Similar differences in distribution patterns among different cancer types were also observed in Id3, which was cytoplasmic in prostate cancer (18) but nuclear in squamous cell carcinoma (46) and cervical cancer (42). The results in this work showed that Id3 in both cytoplasm and nuclei of the SCLC cells was significantly increased and the intensity of nuclear expression seemed to be greater than that in cytoplasm, indicating that large amount of Id3 protein was concentrated in nuclei where it probably functions as a cancer promoter.

The location of Id4 in our study was stronger in the nuclei, similar to that reported for high-grade prostate carcinoma (18). It was noticeable that the expression of Id4 in most cases was either weak or moderate. This observation plus the undetectable amount of this protein in Western blot analysis suggests that this protein might function with very small amounts within the malignant cells. Previous investigations have always separated Id4 from other Id proteins with regard to its pattern of distribution and function during development (47, 48). In prostate cancer, Id4 was suggested to have a role in distant metastasis rather than tumorigenesis (18). Understanding the precise role of Id4 in SCLC cells requires further investigation.

![Fig. 4. Immunohistochemical examination of the expression status of different Id proteins in SCLC biopsy samples. A, nuclear expression of Id proteins. B, cytoplasmic expression of Id proteins.](image)

<table>
<thead>
<tr>
<th>Table 2. Correlation of the expression of different Id proteins to patient survival</th>
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<tbody>
<tr>
<td><strong>Survival analysis</strong></td>
</tr>
<tr>
<td><strong>Id protein</strong></td>
</tr>
<tr>
<td>Id1</td>
</tr>
<tr>
<td>Nuclei</td>
</tr>
<tr>
<td>Id2</td>
</tr>
<tr>
<td>Nuclei</td>
</tr>
<tr>
<td>Id3</td>
</tr>
<tr>
<td>Nuclei</td>
</tr>
<tr>
<td>Id4</td>
</tr>
<tr>
<td>Nuclei</td>
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<tr>
<td>Cytoplasm</td>
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</table>

*Group 1, cases with negative/weak staining; Group 2, moderate/strong staining.
†Id1 and Id2 did not have any moderate/strong nuclear expression. Therefore the comparison was not possible.
To investigate the correlation of Id protein dysregulation and the SCLC patient survival after diagnosis, we used a large number (n = 89–93) of biopsy samples of confirmed cases of SCLC. The original 26 pairs of tissues were not used in survival analysis because these tissues had been surgically removed, which is not the standard treatment of SCLC. Assessment of the relationship between the Id protein status and patient survival showed that although Id1, Id3, and Id4 are all significantly overexpressed in SCLC, the increased expression levels are not significantly associated with the length of the patient survival (Table 2). However, cytoplasmic expression of Id2 does show a significantly (Table 2) positive correlation with patient survival (P = 0.001). When Id2 was negatively or weakly expressed in the cytoplasm of SCLC cells, the average patient survival was 3 months, whereas when moderately or strongly expressed, average survival time of the patients was increased to 10 months. This result suggested that the increased cytoplasmic Id2 is a positive factor for patient survival. Immunostaining also showed that no Id2 was detected in nuclei of SCLC cells in the biopsy samples, whereas in marginal bronchial epithelial cells, Id2 protein does present in nuclei (Table 1). It is possible that in SCLC cells, part of the increased cytoplasmic Id2 protein is gained from that lost in the nucleus. High level expression of Id2 can inhibit the activity of the tumor suppressor retinoblastoma protein and, thus, to increase cell proliferation and cell cycle progression (15, 16). These tumorigenicity-promoting functions were done in the nucleus, not the cytoplasm (49). In those SCLC cases expressing high level of cytoplasmic Id2, it may be possible that the diminishment of the biologically active Id2 occurred through shifting the protein from nucleus to cytoplasm. Thus, the increment of cytoplasmic Id2 may have greatly reduced its tumorigenicity-promoting effect and, hence, has contributed to the longer patient survival.

In summary, Id1, Id2, and Id3 are overexpressed in SCLC tissues and cell lines, and Id4 is overexpressed in SCLC tissues. Association of the increased expression of these proteins with malignant characteristics of the cells indicated possible important roles played by these proteins in the malignant progression. Although the increased Id1, Id3, and Id4 in SCLC cells is not significantly correlated patient outcomes, the increased expression of Id2 in cytoplasm of SCLC may be a highly relevant prognostic factor to predict patient survival.

Acknowledgments

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