Loss of H-Cadherin Protein Expression in Human Non-Small Cell Lung Cancer Is Associated with Tumorigenicity

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ABSTRACT
Abnormalities in the H-cadherin gene have been described in several human cancers. However, their biological significance remains undetermined. To investigate the role of H-cadherin in non-small cell lung cancer (NSCLC), a chimera H-cadherin-green fluorescent protein (GFP) expressed in Cos-7 cells was used to identify an anti-H-cadherin antibody, HCD-1. Western blot analysis was performed in six NSCLC cell lines and 35 pairs of primary NSCLC tumors and nonmalignant lung tissue obtained from surgical resections using HCD-1. Loss of H-cadherin expression was seen in five (83%) of the six NSCLC cell lines, whereas loss of E-cadherin was seen in three (50%) of the six. H-cadherin expression was lost in 15 (43%) of 35 NSCLC surgical tumor specimens, whereas E-cadherin expression was lost in 6 (17%) of 35. H-cadherin was expressed in all of the nonmalignant lung tissue from all of the surgical specimens. Fourteen of 35 tumors were heterotransplanted s.c. in nude mice. Tumorigenicity in nude mice was associated with both loss of H-cadherin expression (P = 0.03) and loss of E-cadherin expression (P = 0.05). Loss of H-cadherin was also associated with a more advanced local tumor growth, although the difference was not significant. The results indicate that loss of H-cadherin is frequent in human NSCLC and suggest that it facilitates the implantation and local growth of human NSCLC tumors.

INTRODUCTION
Cadherins constitute a superfamily of Ca\(^{2+}\)-dependent intercellular adhesion molecules expressed on solid tissue cells. They connect cells in a homophilic and subclass-specific manner and are important in establishing and maintaining intercellular connections. In embryogenesis, the regulation of cadherin expression is involved in a variety of morphogenetic events (1). There is increasing evidence that cadherin-mediated adhesion plays a role in neoplastic cell behavior as well. Most recently, Cano et al. reported that expression of the Snail gene temporarily turns off the E-cadherin gene, resulting in induced tumorigenic growth and increased ability of cancer cells to invade and metastasize (2, 3). The H-cadherin gene, a new member of the cadherin superfamily, was recently isolated and has been mapped to 16q24 (4). H-cadherin, unlike other cadherin genes such as E-cadherin, N-cadherin, and P-cadherin, lacks the cytoplasmic domain (4). Abnormalities in the H-cadherin gene have been identified in human cancer cell lines and human primary tumors including lung (5), gastric (6), and ovarian (7) cancers. The biological significance of the alterations of the H-cadherin gene in human cancer remains undetermined. We have raised an anti-H-cadherin antibody HCD-1 and investigated the expression of H-cadherin protein in NSCLC (3), the leading cause of cancer-related death (8). Our studies indicate that loss of H-cadherin protein expression is frequent in NSCLC and is associated with tumorigenicity in nude mice.

MATERIALS AND METHODS
Construction of H-Cadherin-GFP-Recombinant cDNA and Production of Polyclonal Antibodies. A peptide spanning a predicted extracellular domain (158–171 amino acids) of the human H-cadherin was synthesized and conjugated to the carrier protein [keyhole limpet hemocyanin (KLH)] for polyclonal antibody production in rabbits. To identify specific anti-H-cadherin antibodies, a recombinant H-cadherin-GFP construct was prepared and expressed in Cos-7 cells. In brief, the cDNA encoding the full-length human H-cadherin was excised from pcDNA3 full-length human H-cadherin by BamHI and KpnI digestion and was subcloned into vector pEGFP-C2 (Clontech, Palo Alto, CA) after restriction by KpnI and BamHI digestion. The selected H-cadherin-GFP and pEGFP-C2 plasmid DNA were purified using Qiagen plasmid extraction kit (Qiagen, Inc., Valencia, CA) and were used to transfected Cos-7 cells by lipofectamine (Life Technologies, Inc., Gaithersburg, MD). The anti-H-cadherin rabbit sera were first screened by ELISA for reactivity against the same peptide. Positive anti-H-cadherin rabbit sera were then screened by immunoblot for reactivity against the H-cadherin-GFP protein expressed in Cos-7 cells. The selected rabbit anti-H-cadherin antibody, named HCD-1, was screened and affinity-purified with the same peptide by the SulfoLink column (Pierce, Rockford, IN).

Cell Lines and Patient Samples. Six human NSCLC cell lines (A549, H322, H460, H520, H596, and H661) were obtained from American Type Culture Collection (ATCC; Manassas, VA) and were cultured in RPMI medium supplemented...
with 10% FCS. Thirty-five surgical specimens of NSCLC tumors (26 adenocarcinomas, 7 squamous cell carcinomas, and 2 bronchoalveolar carcinoma) and nonmalignant lung tissue were obtained from the Lung Cancer Bank at the Kaplan Comprehensive Cancer Center (New York University School of Medicine). In all of the cases, nonmalignant lung tissue was also available for studying H-cadherin expression. Cos-7 cells were obtained from ATCC and were cultured in MEM supplemented with 10% FCS.

All of the tumor samples were from surgically removed specimens from patients with stage I-IIIA NSCLC. Three patients had received chemotherapy and/or radiotherapy prior to surgery. Distributions according to T and N status were as follows: 17 T1, 15 T2, 1 T3, 2 unknown, 22 N0, 4 N1, and 8 N2. Primary lung tumors and nonmalignant lung tissues were immediately snap-frozen after surgical resection and stored at −70°C. Tissue procurement was approved by the Joint Committee on Clinical Investigation and the Institutional Review Board.

Tumor Implantation in Nude Mice. The fresh tumor samples were cut into 1–2 mm3 pieces in sterile saline (9). Three or four fragments of nonnecrotic tissue were inoculated s.c. into the lower back and anterior chest of 6-to-8-week-old female Nu/Nu mice using a biomedical stainless steel needle (implant needle). Nude mice were maintained under standardized sterile conditions. Animals transplanted with NSCLC tumors were checked for tumor growth for a maximum of 36 weeks. Tumor formation measuring at least 5 mm in diameter was considered a positive take. Tumor formation was confirmed histologically in all cases. Temporary growth was defined as tumor formation followed by spontaneous regression before reaching a diameter of 5 mm. All of the other incidences were considered no growth.

SDS-PAGE and Western Blot. H-cadherin-GFP transfected Cos-7 cells, lung cancer cell lines and whole homogenates of nonmalignant or tumor tissues (0.1–0.5 g) were placed in SDS sample buffer containing protease inhibitors, 10 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 10 mg/ml aprotinin, and immediately boiled for 5 min. Volumes were adjusted to make all of the samples equal in protein concentration by the addition of SDS sample buffer, and proteins were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membrane and probed with anti-GFP antibody by Western blot analysis. Three rabbit sera were positive for reactivity against the synthetic peptide of H-cadherin by ELISA. One of those, only one, designated HCD-1, recognized the bands of recombinant H-cadherin protein detected with an anti-GFP antibody by Western blot analysis. HCD-1 was found to recognize chimera H-cadherin-GFP protein detected with an anti-GFP antibody by Western blot analysis (Fig. 1A). As a control, GFP protein was transiently expressed in Cos-7 cells as demonstrated by Western blot analysis. Three rabbit sera were positive for reactivity with the synthetic peptide of H-cadherin by ELISA. Of those, the anti-H-cadherin antibody recognizes the chimera H-cadherin-GFP protein of the predicted size with the same expression pattern as detected by an anti-GFP antibody by Western blot analysis. B, whole homogenates of human lung and heart tissue were probed with the anti-H-cadherin antibody.

RESULTS

Little is known about the biological role of H-cadherin protein in human cancers, although the H-cadherin gene has been reported to be inactivated in several human cancers including lung cancer (4–7, 10). We, therefore, developed an anti-H-cadherin antibody by raising polyclonal antibodies against a synthetic peptide spanning a predicted extracellular domain (151–172 amino acids) of human H-cadherin and constructing a recombinant cDNA composed of full-length human H-cadherin fused at the COOH terminus to GFP (recombinant H-cadherin-GFP). In the screening process, rabbit sera were screened first for reactivity against the synthetic peptide of H-cadherin by ELISA and then for reactivity to the chimera H-cadherin-GFP protein transiently expressed in Cos-7 cells as demonstrated by Western blot analysis. Three rabbit sera were positive for reactivity with the synthetic peptide of H-cadherin by ELISA. Of those, one, designated HCD-1, recognized the bands of chimera H-cadherin-GFP protein detected with an anti-GFP antibody by Western blot analysis (Fig. 1A). As a control, GFP protein was transiently expressed in Cos-7 cells and was detected as a single band at M1 = 27,000 with anti-GFP antibody (Fig. 1A). The immunizing peptide blocked the HCD-1 antibody interaction with M1 = 120,000 and 80,000 bands (data not shown). Although HCD-1 was found to recognize chimera H-cadherin-GFP protein, we needed to confirm that HCD-1 could detect the H-cadherin protein in normal human and animal tissues. As shown (Fig. 1B), two bands, approximately M1 = 120,000 and 80,000 were detected in the whole homogenates of human heart and lung. Also, HCD-1 was found to react with mouse heart and lung (data not shown).

To investigate the relationship of H-cadherin protein expression and tumor growth in human NSCLC, we first evaluated the expression of H-cadherin in six well-known NSCLC cell lines. Western blot analysis using HCD-1 revealed that H-cadherin protein was lost in 5 (83%) of 6 cell lines (Fig. 2B),
expression of human E-cadherin and A (data not shown). As shown in Fig. 2 identity of the H-cadherin PCR products by sequence analysis). We confirmed the gene in 4 (67%) of 6 cell lines (Fig. 2 A H-cadherin whereas RT-PCR analysis showed inactivation of E-cadherin in 3 (50%) of 6 cell lines (Fig. 2 B). Western blot analysis of E and H-cadherin expression in human NSCLC cell lines.

**Table 1** Loss of H- and E-cadherin in human NSCLC tumors

<table>
<thead>
<tr>
<th>Adenocarcinoma/bronchiolar-cell</th>
<th>Squamous cell</th>
<th>All n = 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-cadherin +</td>
<td>17 (61%)</td>
<td>3 (43%)</td>
</tr>
<tr>
<td>H-cadherin -</td>
<td>11 (39%)</td>
<td>4 (57%)</td>
</tr>
<tr>
<td>E-cadherin +</td>
<td>23 (82%)</td>
<td>6 (86%)</td>
</tr>
<tr>
<td>E-cadherin -</td>
<td>5 (18%)</td>
<td>1 (14%)</td>
</tr>
</tbody>
</table>

Fig. 2 Expression of H-cadherin by human NSCLC cell lines. A, expression of H-cadherin gene by RT-PCR. Top, RT-PCR of H-cadherin gene. Bottom, RT-PCR of β-actin. B, Western blot analysis of E and H-cadherin expression in human NSCLC cell lines.

whereas RT-PCR analysis showed inactivation of H-cadherin gene in 4 (67%) of 6 cell lines (Fig. 2A). We confirmed the identity of the H-cadherin PCR products by sequence analysis (data not shown). As shown in Fig. 2A, H-cadherin was detected as a single band of M_r 120,000 in H-460 cells. We examined expression of human E-cadherin and α-, β-, and γ-catenins in the same cell lines and found that E-cadherin was lost in 3 (50%) of 6 cell lines (Fig. 2B), whereas α-, β-, and γ-catenins were expressed in all of the cell lines (data not shown). Interestingly, the only cell line expressing H-cadherin (H460) did not express E-cadherin.

We then investigated the expression of H-cadherin and E-cadherin in 35 pairs of primary NSCLC tumor and nonmalignant lung tissue. Total loss of H-cadherin protein was observed in 15 (43%) of 35 NSCLC tumors and was not associated with any particular histological subtype (Table 1). Loss of E-cadherin was less frequently observed [6 (17%) of 35] and was mostly restricted to tumors that also had loss of H-cadherin (5 of 6 tumors with loss of E-cadherin also showed loss of H-cadherin; Table 1). Fig. 3 shows the expression of H-cadherin in five pairs of primary NSCLC and nonmalignant lung tissue. All of the nonmalignant lung tissues expressed H-cadherin. Interestingly, loss of H-cadherin was associated with a larger local tumor but the difference was not significant [5 (29%) of 17 T_1 were H-cadherin negative versus 9(60%) of 15 T_2; P = 0.15].

Fourteen tumors (10 adenocarcinomas and 4 squamous carcinomas) were heterotransplanted in nude mice. Six of the 14 primary tumors had loss of H-cadherin; 3 of the 14 had loss of E-cadherin; and 7 of 14 loss of either H-cadherin, E-cadherin, or both. Successful heterotransplantation was obtained in six cases (four adenocarcinomas and two squamous carcinomas) and was associated with loss of E-cadherin (100% in E-cadherin-negative tumors versus 27% in E-cadherin-positive tumors; P = 0.05), loss of H-cadherin (83% in H-cadherin-negative tumors versus 12% in H-cadherin-positive tumors; P = 0.03), and loss of either or both (86% in H- or E-cadherin-negative tumors versus 0% in H- and E-cadherin-positive tumors, P < 0.01; Fig. 4).

**DISCUSSION**

H-cadherin is a novel adhesion molecule that plays an important role not only in cell-cell adhesion but also in maintaining the normal cellular phenotype (5). Recent studies have revealed that reexpression of H-cadherin in breast cancer cells diminished the invasive potential of tumor cells in vitro and the formation of s.c. tumors in vivo (11). As a first step in studying the potential biological role of H-cadherin in human NSCLC, we performed an analysis of its expression in resected human NSCLC specimens and investigated its relationship with local growth and successful tumor heterotransplantation in nude mice.

To determine the expression of H-cadherin in NSCLC, we generated polyclonal antibodies against a synthetic peptide that is expressed in the extracellular domain of H-cadherin and identified a specific anti-H-cadherin antibody by using GFP as a probe. Lee (4) reported that the H-cadherin gene expresses two major mRNA transcripts of 3.7 kb and 4.1 kb in Northern analysis and encodes two products of M_r ~80,000 and 120,000 in vitro transcription and translation studies. We found that HCD-1 reacted with two distinctive molecular masses of M_r ~80,000 and 120,000 in human and mouse tissues. Such bands are identical to the size of H-cadherin protein. To our knowledge, HCD-1 is the first antibody developed for detecting H-cadherin protein by immunoblot. Interestingly, H-cadherin was detected as a single molecular mass of M_r ~120,000 by HCD-1 in the only human NSCLC cell line (H-460) that expresses H-cadherin. The significance of such variation of molecular mass is unclear. However, an important message from the present studies (12–15) is that cadherins may not function identically in different cell types. Thus, the fact that H-cadherin but not E-cadherin was expressed in H-460 cells suggests that H-cadherin may play an important biological role in H460 cells.
Abnormalities in H-cadherin gene have been reported in lung (5), breast (4, 10), gastric (6), and ovarian cancer (7). Our finding that the H-cadherin message is lost in four (67%) of six NSCLC cell lines is consistent with the results of Sato et al. (5), who reported that loss of expression and hypermethylation of the 5′ flanking region may be an important mechanism of inactivation of the H-cadherin gene (5). We also found that H-cadherin protein was lost in 5 (83%) of 6 NSCLC cell lines (Fig. 2). In contrast, E-cadherin, a tumor suppressor gene and a morphogenic factor in epithelial tumors (1, 16), was lost in three (50%) of six NSCLC cell lines.

Generally, E-cadherin expression has been found to inhibit not only the local invasiveness of cancer cells at primary sites but also the ability of circulating cancer cells to metastasize in lymph node and distant organs (16, 17–22). Recently, H-cadherin expression has been also found to reduce the invasive ability of breast cancer cells (MDAMB435) in vitro (11). However, our present results showed that neither the loss of H-cadherin nor E-cadherin expression in the primary lung tumor were associated with the presence of lymph node metastasis (data not shown); probably because we investigated expression of E- and H-cadherin only in the primary tumors.

The effect of H- and E-cadherin on tumor growth inhibition has been described previously using transfected tumor cell lines (4, 11, 16). However, the effect of the loss of H- and E-cadherin on the tumorigenicity of fresh human tumors implanted in nude mice has not been previously studied. In this study, we found that successful tumor implantation of human NSCLC tumors in nude mice was associated with the loss of E-cadherin (P = 0.05), loss of H-cadherin (P = 0.03), and loss of either or both (P < 0.01). We also found that the loss of H-cadherin tended to be associated with a larger local tumor; 5 of 17 T1 tumors were H-cadherin negative versus 9 of 15 T2 tumors (P = 0.15). However, the difference was not significant.

More recently, Cano et al. and Batlle et al. (2, 3) showed that a transcription factor Snail gene strongly represses the expression of E-cadherin and induces tumor growth. Our results are consistent with Cano’s results that down-regulation of H- and E-cadherin enhances human NSCLC growth. Although the mechanism by which cadherins inhibit tumor formation and growth is unknown, it is probably related to their ability to enhance cell-cell associations in the tissue microenvironment.

The determinants of successful implantation of fresh human tumor explants in nude mice have not been extensively studied and are probably multifactorial. A squamous histology and myc expression have been reported to be associated with a significantly higher take rate (23, 24), whereas the ability of the tumor-infiltrating lymphocytes to engraf and produce human immunoglobulin have been suggested to prevent tumor implantation (25). p53 status and VEGF expression by tumor cells were not found to be determinants of tumorigenicity (23, 26). Our study indicates that loss of cell adhesion molecules may be a major determinant of successful human tumor implantation in nude mice. These findings suggest that abrogation of the function of cell adhesion molecules by monoclonal antibodies or other methods could be a way to enhance the take rate of these tumors. This would widen the potential use of NSCLC hetero-transplants to perform individualized in vivo chemosensitivity testing, which we are actively exploring in our laboratory.

In conclusion, we have demonstrated that (a) H-cadherin is consistently expressed in nonmalignant human lung tissue; (b) H-cadherin is lost in most human NSCLC cell lines and about one-half of primary human NSCLC tumors; and (c) the loss of H-cadherin is associated with an enhanced tumorigenicity of human NSCLC tumors in nude mice. These results justify continuing exploring its biological role and potential exploitation as a therapeutic target.

REFERENCES


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