C-CAM1, a Candidate Tumor Suppressor Gene, Is Abnormally Expressed in Primary Lung Cancers

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ABSTRACT

Previous studies have shown that the expression of the cell-cell adhesion molecule (C-CAM1), located at chromosome 19, is down-regulated in several types of human cancers, including prostate and breast cancers. Two major isoforms of C-CAM1, the long or L-form C-CAM1 and the short or S-form C-CAM1, are derived from the C-CAM1 gene through alternative splicing. Tumor cells transfected with L-form C-CAM1, which contains a cytoplasmic domain, display significantly lower growth rates and less tumorigenicity in both in vitro and in vivo models compared with untransfected tumor cells, suggesting that L-form C-CAM1 may be a tumor suppressor. The transfection of the cytoplasmic domain of L-form C-CAM1 could also cause suppression of tumor growth, further supporting the role of L-form C-CAM1 in tumorigenesis. In contrast to reports of most of the tumor types tested, Ohwada et al. (Am. J. Respir. Cell Mol. Biol., 11: 214–220, 1994) reported that C-CAM1 was not down-regulated or even up-regulated in lung cancer. Because the cytoplasmic domain of L-form C-CAM1 is critical for the tumor suppressor function of C-CAM1, we hypothesized that switching of the isoform rather than down-regulation of C-CAM1 gene expression occurs during lung tumorigenesis. To test this hypothesis, we analyzed pairs of tumor tissue and corresponding normal-appearing lung tissue from 51 patients with non-small cell lung cancer (NSCLC) and 43 cell lines to determine expression profiles of L-form C-CAM1 and S-form C-CAM1 using reverse transcription-PCR. We found that L-form C-CAM1 was the predominant form (75%; 38 of 51) in normal-appearing lung tissue, whereas most (84%; 43 of 51) of the primary NSCLC tissue samples expressed predominantly S-form C-CAM1 (P < 0.0001). Similarly, 19 (79%) of the 24 NSCLC cell lines and 17 (85%) of the 20 small cell lung cancer cell lines expressed predominantly S-form C-CAM1. The frequent alteration of the C-CAM1 expression pattern suggests that C-CAM1 has an important role in lung tumorigenesis.

INTRODUCTION

C-CAM1 is an adhesion molecule that was originally identified and characterized in human bile (1). It is located at chromosome 19q13.2 within the cluster of CEA-related genes, and variable transcripts can be produced by an alternative splicing mechanism (2–4). Because C-CAM1 is structurally very similar to CEA, it was classified as a member of the CEA family (3).

C-CAM1 or CEACAM1 (also known as biliary glycoprotein, CD66a, CAM105, EctoATPase, HA4, or pp120) has two major isoforms identified by the inclusion or exclusion of exon 7 of the gene (5). The long form (L-form C-CAM1) includes exon 7, and its open reading frame stops within exon 9. The short form (S-form C-CAM1) excludes the 53-bp exon 7, possibly by an alternative-splicing mechanism, and its open reading frame stops within exon 8 because of a frame shift. Thus, S-form C-CAM1 lacks a 73-amino acid cytoplasmic domain that L-form C-CAM1 includes (Fig. 1).

Previous studies have shown that the expression of C-CAM1 is generally down-regulated in various types of tumors such as colorectal carcinomas, hepatomas, breast carcinomas, and prostate carcinomas (6–9). When murine L-form C-CAM1 was transfected into mouse colon carcinoma cells or human prostate, bladder, and breast carcinoma cells, cells transfected with L-form C-CAM1 displayed significantly lower growth rates and less tumorigenicity (10–14). Recent studies further indicate that the cytoplasmic domain of C-CAM1 is required to inhibit tumor growth (12, 15). In addition, Esterra et al. (16) reported that expression of the cytoplasmic domain alone of L-form C-CAM1 was sufficient to cause tumor suppression, suggesting that the cytoplasmic domain of L-form C-CAM1 is the tumor suppressor domain. These results suggest that C-CAM1 is a candidate tumor suppressor gene and thus warrants further investigation.

Paradoxically, however, C-CAM1 appears to be up-regulated in primary lung cancer samples when compared with adjacent normal-appearing lung tissues, according to results from one study in which Northern blot analysis and RNA in situ hybridization were

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3 The abbreviations used are: C-CAM, cell-cell adhesion molecule; CEA, carcinoembryonic antigen; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; RT-PCR, reverse transcription-PCR.
Because the cytoplasmic domain of L-form C-CAM1 is critical for the tumor suppressor function of C-CAM1, we hypothesized that isoform switching rather than down-regulation of C-CAM1 gene expression occurs during lung tumorigenesis. To test this hypothesis, we studied a panel of 51 primary NSCLCs and their corresponding normal-appearing lung tissues as well as 43 lung cancer cell lines including NSCLC and SCLC to determine relative expression levels of L-form C-CAM1 and S-form C-CAM1. We found that 75% of the normal-appearing lung tissues expressed predominantly L-form C-CAM1, whereas 84% of primary lung tumors and 84% of lung cancer cell lines expressed predominantly S-form C-CAM1. These findings support the hypothesis that the expression pattern of C-CAM1 is altered during lung tumorigenesis.

MATERIALS AND METHODS

Cell Lines and Tissue Specimens. cDNA from 19 NSCLC cell lines (H23, H157, H226, H441, H727, H1299, H1437, H1466, H1693, H1792, H1819, H1993, H2009, H2087, H2122, H2347, HCC44, HCC78, and HCC193) and 20 SCLC cell lines (H82, H128, H249, H289, H345, H378, H526, H735, H738, H841, H847, H889, H1092, H1105, H1184, H1304, H1450, H1618, H1672, and H1688) were kindly provided by Drs. Minna and Gazdar (The University of Texas Southwestern Medical Center, Dallas, TX). The lung cancer cell lines A427, A549, H460, H522, H1648, H1799, and H1944 were obtained from the American Type Culture Collection. These cell lines were grown in RPMI 1640 with 10% fetal bovine serum. Fifty-one sets of primary NSCLC specimens and corresponding normal-appearing lung tissues were obtained from surgically resected specimens collected in the Department of Pathology at the University of Texas M. D. Anderson Cancer Center and stored at −80°C until the experiment. There were 22 cases of squamous cell carcinoma, 24 cases of adenocarcinoma, 2 cases of large cell carcinoma, and 3 cases of carcinoid. All patients gave written consent for the use of their specimens, and the research protocol was approved by the institutional review board.

RNA Isolation and RT-PCR Analysis. The fresh tumor tissues and the corresponding normal-appearing lung tissues simultaneously obtained from patients with lung cancer were used for extraction of total RNA. Total RNA was isolated by using RNAzol B reagent (Biotecx Laboratories, Inc., Houston, Texas) according to the manufacturer’s protocol after electric tissue homogenization. Approximately 1–3 μg of total RNA from each sample was used to conduct reverse transcription reaction in a 20-μl volume using Superscript II RNase H− reverse transcriptase (Life Technologies, Inc., Grand Island, NY). The synthesized cDNA was either used immediately for PCR amplification or stored at −20°C for further analysis.

The PCR primers were designed to coamplify a cDNA fragment of 408-bp (L-form C-CAM1) or 355-bp (S-form C-CAM1) in a length that flanks three introns to avoid the amplification of possible contaminated genomic DNA. The forward primer and the reverse primer are located in exon 6 and the 3’ untranslated region, respectively.

Fig. 1  Schematic diagram of C-CAM1 and strategy for distinguishing the expression of L-form C-CAM1 and S-form C-CAM1 by RT-PCR. The exons of the human C-CAM1 gene are presented as boxes. Dashed lines represent the joining of exons to produce the alternative spliced mRNA isoforms. The corresponding amino acid sequences (in one-letter code) are shown for L-form C-CAM1 and S-form C-CAM1. The boldly underlined sequence represents the transmembrane domain. A1, B1, and A2, C2-set immunoglobulin domain. UT, untranslated region; ATG, the start codon; TAA or TAG, the stop codon. The primers for RT-PCR were designed to amplify a cDNA fragment of 408-bp (L-form C-CAM1) or 355-bp (S-form C-CAM1) in a length that flanks three introns to avoid the amplification of possible contaminated genomic DNA. The forward primer and the reverse primer are located in exon 6 and the 3’ untranslated region, respectively.
Taq DNA Polymerase (Qiagen, Inc., Chatsworth, CA). Amplification was carried out with an initial denaturing step at 95°C for 15 min, followed by 40 cycles of 95°C for 30 s, 60°C for 1 min, and 70°C for 1 min in a thermal cycler (Hybaid; PCR Express, Middlesex, United Kingdom). Omission of cDNA was used as a negative control. The PCR products were mixed with 6× loading buffer containing 0.5 mg/ml of ethidium bromide and separated by electrophoresis on a 2% agarose gel.

Sequencing of the L-Form C-CAM1 and S-Form C-CAM1.

After electrophoresis, the expected bands containing the 408-bp fragment or the 355-bp fragment (L-form C-CAM1 or S-form C-CAM1) were recovered using the QIAquick gel extraction kit (Qiagen, Chatsworth, CA). The sequences of L-form C-CAM1 and S-form C-CAM1 were determined using the AmpliCycle sequencing kit (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, New Jersey) with a γ-³²P-labeled primer according to the protocol from the manufacturer. Each amplified product (3 µl) was run on a 6% Long-Ranger gel (FMC BioProducts, Rockland, ME) and exposed to film.

Experimental Determination of Relative mRNA Copy Numbers.

RT-PCR products from H1944 and 17B, representing L-form C-CAM1 and S-form C-CAM1, respectively, were cloned directly into a PCR 2.1-TOPO vector (Invitrogen Co., Carlsbad, CA) according to the manufacturer’s protocol, and the clones were sequenced to confirm the presence of the respective forms of C-CAM1 cDNA fragments, respectively. DNA from the plasmids containing either L-form C-CAM1 or S-form C-CAM1 was quantified and mixed to create a panel with different concentrations of either plasmid DNA. PCR was performed as described above to determine the relative copy numbers and amplification efficiency.

Statistical Analysis.

The McNemar’s test was used to determine the true association between changes in the gene expression profile and primary NSCLC. The χ² test or Fisher’s exact test was used to determine differences in frequency of expression profiles and associations with clinical and histopathological parameters. All tests were two-sided, and Ps were considered significant when they were ≤0.05.

RESULTS

To test whether expression of L-form C-CAM1 is reduced in primary NSCLC, we analyzed 51 pairs of primary tumor and corresponding normal-appearing lung tissue specimens that were available in our tissue bank. All of the patients were treated by complete surgical resection of their primary tumors at M. D. Anderson Cancer Center between September 1996 and October 1998. No patients had undergone chemotherapy or radiation therapy prior to surgery and tissue acquisition. Pathological diagnosis and tumor differentiation status were reviewed and confirmed by our staff pathologist (B. L. K.). The tumors included 22 squamous cell carcinomas, 24 adenocarcinomas, 2 large cell carcinomas, and 3 carcinoids. Patients’ ages ranged from 33 years to 82 years, with a median age of 65.5 years. Thirty patients were men, and 21 were women. Among 47 patients with available smoking status, 38 were current smokers, 4 were former smokers, and 5 were nonsmokers. Smokers were defined as those who smoked cigarettes for ≥10 pack/years. Former smokers were smokers who had quit smoking for >1 year. Pathological staging of patients with NSCLC was determined according to the revised 1997 staging system for lung cancer from American Joint Commission on Cancer.
**C-CAM1** Expression Pattern in Normal Lung Tissues.

In normal-appearing lung tissues obtained from 51 patients with NSCLC, 38 (75%) showed L-form C-CAM1 as the predominantly expressed form. Fig. 2 shows examples of the expression patterns of normal-appearing lung tissues from patients with NSCLC. To exclude the possibility that PCR amplification might favor a smaller size of DNA fragment, we designed an in vitro experimental approach to determine the efficiency of PCR amplification and relative copy numbers of L-form C-CAM1 and S-form C-CAM1 using the corresponding primer sets. We constructed plasmids containing either L-form C-CAM1 or S-form C-CAM1 and used them as templates. A panel of samples with different ratios of L-form C-CAM1 and S-form C-CAM1 was used for PCR amplification. We found that the primers used could amplify L-form C-CAM1 and S-form C-CAM1 DNA fragments with similar efficiency, and reliable ratios could be obtained when different ratios of DNA were applied (Fig. 3). These results suggest that the ratios of L-form C-CAM1 and S-form C-CAM1 observed in the normal-appearing lung tissues reflect the actual differences of the L-form C-CAM1 and S-form C-CAM1 expression levels.

**C-CAM1** Expression Pattern Is Altered in Lung Cancer.

In the corresponding primary lung tumors, we found that 43 (84%) of the 51 NSCLCs expressed predominantly S-form C-CAM1 (Table 1 and Fig. 2), which is in contrast to 13 of 51 or 25% in the corresponding normal-appearing lung tissues. Among the 43 primary tumors expressing predominantly S-form C-CAM1, 32 (74%) of the corresponding normal-appearing lung tissues expressed predominantly L-form C-CAM1. Among eight tumors expressing predominantly L-form C-CAM1, six had the same expression pattern in the corresponding normal-appearing lung tissues, and the other two expressed predominantly S-form C-CAM1. The association of predominant S-form C-CAM1 expression pattern and primary NSCLC was statistically significant ($P < 0.0001$ by the McNemar test).

One complexity in analyzing primary tumor tissues is the contamination of normal cells in specimens, which might result in underestimation of the degree of abnormality in these tissues. Although gene expression profiles in cancer cell lines may not always reflect gene expression patterns in vivo, cancer cell lines are not contaminated with normal cells. We therefore analyzed 24 NSCLC cell lines and 20 SCLC cell lines for the expression patterns of L-form C-CAM1 and S-form C-CAM1. We found 19 (79%) of the 24 NSCLC cell lines, and 17 (85%) of the 20 SCLC cell lines expressed predominantly S-form C-CAM1. These results are consistent with our findings in primary lung tumor tissues and further support a potential role of L-form C-CAM1 in lung tumorigenesis.

**Association between C-CAM1 Expression Profiles and Clinical Parameters.**

We looked at whether there were associations between the expression patterns of L-form C-CAM1 and S-form C-CAM1 and clinical and histopathological parameters. We did not find an obvious association between C-CAM1 expression patterns and any of the clinical or pathological parameters analyzed in tumor tissues (Table 1). However, we found that the predominantly S-form C-CAM1 expression pattern was more frequent in normal-appearing lung tissues from patients with late-stage NSCLC (stages III and IV) than it was in normal-appearing lung tissues from patients with earlier stages of NSCLC ($47\% \text{ versus } 12\%, P = 0.02$; Table 1).

Although there was no statistically significant difference between the expression patterns of C-CAM1 and the sex of the patients, it was notable that the frequency of predominant S-form C-CAM1 expression was higher in men than in women, both in normal-appearing lung tissues ($34\% \text{ versus } 14\%$) and tumor tissues ($90\% \text{ versus } 77\%$; Table 1). It will be interesting to see whether these trends become statistically significant when a large number of samples are analyzed. Additionally, in current smokers, 31% (11 of 35) of normal-appearing lung tissues expressed predominantly S-form C-CAM1, whereas only 17% (2 of 12) of lung tissues from former smokers or nonsmokers did so.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Association of C-CAM1 expression patterns and clinical parameters</th>
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<tr>
<td></td>
<td>Age (years)</td>
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<td>≤60</td>
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<td>&gt;70</td>
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<td></td>
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<td>Smoking status</td>
<td>Non smoker</td>
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<td>Former smoker</td>
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<tr>
<td></td>
<td>Smoker</td>
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<tr>
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<tr>
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<td>Well/moderately differentiated</td>
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<td>Adenocarcinoma</td>
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<td>Large cell carcinoma</td>
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*a Fisher’s exact test: $P = 0.02$.
DISCUSSION

In previous studies, S-form C-CAM1 was found to be the dominant form in most adult tissue (10). However, most of the normal-appearing lung tissues analyzed in this study expressed predominantly L-form C-CAM1. The ratio of L-form C-CAM1 and S-form C-CAM1 was measured by quantitative RT-PCR. Because we have tested amplification efficiencies of both L-form C-CAM1 and S-form C-CAM1 and obtained reliable and reproducible results, we are confident that the expression ratios observed in this study reflect the in vivo expression status of the C-CAM1. Because expression patterns of the two forms of C-CAM1 were not extensively examined in the previous studies, our results provide an important new look at the C-CAM1 expression patterns in human normal-appearing lung tissue. That the L-form C-CAM1 is predominantly expressed in these tissues suggests the importance of this isoform in lungs and is consistent with the role of L-form CAM1 as a tumor suppressor reported in previous studies (10–15).

It was reported previously that the level of C-CAM1 expression was increased in primary lung tumors when Northern blot analysis and RNA in situ hybridization were used (17). However, it was not clear whether the results represent an increased expression of L-form C-CAM1, S-form C-CAM1, or both. In this study, we have demonstrated that most of the primary NSCLCs expressed predominantly S-form C-CAM1 in contrast to most of the corresponding normal-appearing lung tissues, which expressed predominantly L-form C-CAM1. This result suggests that the alternative splicing of C-CAM1 gene was altered in lung tumorigenesis. Because the L-form C-CAM1 possesses tumor-suppressive functions, the decreased expression of this isoform or the increased expression of S-form C-CAM1 might play an important role in lung cancer development. Our data support the possibility that the previously observed stable or increased C-CAM1 expression in lung cancers was the result of an increased level of S-form C-CAM1 expression.

It was noticed that some of the normal-appearing lung tissues from patients with NSCLC expressed predominantly S-form C-CAM1. One explanation for this phenomenon is that the abnormal expression might occur in the early tumorigenic process, and some premalignant lesions might have already developed in what appeared to be normal-appearing lung tissues of patients with lung cancer attributable to carcinogenic damage occurring in the whole field of the airway. This idea is supported by our finding the S-form C-CAM1 expression pattern to be predominant in bronchial epithelial cells from some smokers who show no evidence of lung cancer (data not shown). In addition, dysregulation of C-CAM1 is also found to be an early event in the carcinogenic process (6, 18).

One of the interesting findings was that normal-appearing lung tissues from patients with advanced NSCLC showed a significantly high rate of presenting predominant S-form C-CAM1 than those from patients with earlier stage NSCLC. It is possible that the normal-appearing lung tissues from patients with more advanced lung cancer contained more premalignant lesions in the surrounding normal lungs than those tissues from patients with earlier stages of the disease. Alternatively, the advanced tumors might produce certain factors that could influence expression patterns of C-CAM1 in surrounding tissues.

C-CAM1 shares high homology with CEA, particularly in terms of extracellular structure. However, in contrast to CEA, L-form C-CAM1 has a 73-amino acid cytoplasmic domain that may be capable of transmitting signals and may play a role in suppression of tumorigenicity (14, 15). Because of the deletion of exon 7, S-form C-CAM1 uses a different reading frame at the 3′ end of the gene and does not contain a functional cytoplasmic domain. Upon activation of the insulin receptor, the tyrosine residues in the cytoplasmic domain of L-form C-CAM1 can be phosphorylated by protein kinases (19–21), suggesting that L-form C-CAM1 may have a role in signal transduction. In prostate cancer cells as well as breast cancer cells, L-form C-CAM1 can strongly suppress tumorigenicity in both in vitro and in vivo experiments (14, 15). In contrast, S-form C-CAM1 does not exhibit such tumor-suppressive activity (14, 15). Although the mechanism of L-form C-CAM1-induced tumor-suppressive function is unclear, a C-CAM1-associated protein, CAP-80, has been found to bind to L-form C-CAM1 and may play an important role in L-form C-CAM1-mediated growth inhibition (22). The discovery of the reduced expression level of L-form C-CAM1 or increased level of S-form C-CAM1 in most of the lung cancer tissues in this study supports these previous experimental findings and suggests that L-form C-CAM1 is important in lung tumorigenesis.

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