Elevated Expression of Carcinoembryonic Antigen-related Cell Adhesion Molecule 1 Promotes Progression of Non-Small Cell Lung Cancer

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ABSTRACT

Purpose: Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1) has recently been implicated in cancer development and progression. This study was performed to assess whether CEACAM-1 expression in primary tumors is correlated to long-term survival in patients with operable non-small cell lung cancer (NSCLC).

Experimental Design: Primary tumors of 145 consecutive patients with completely resected NSCLC (pT1-4 pN0-2 M0 R0) were stained immunohistochemically using the monoclonal anti-CEACAM-1 antibody 4D1/C2. The prognostic relevance of CEACAM-1 expression was evaluated by univariate Kaplan-Meier and multivariate Cox regression analysis. The median follow-up period was 72 months (range, 10–130 months).

Results: Normal bronchiolar epithelium present in all sections exhibited no immunostaining. In contrast, 73 tumors (50.4%) showed between 1 and 66% CEACAM-1 positive tumor cells, and 72 tumors (49.6%) exhibited even a higher percentage of positive tumor cells. A high CEACAM-1 expression rate (i.e., $\geq$66% positive tumor cells) was more frequent in adenocarcinomas than in squamous cell carcinomas (61.9 versus 35.7%, respectively). Multivariate Cox regression analysis demonstrated that CEACAM-1 represents an independent prognosticator for cancer-related survival ($P = 0.018$; relative risk, 1.8; 95% confidence interval, 1.1–2.8). Subgroup analysis revealed that a high CEACAM-1 expression rate was of significant prognostic impact in pN1-pN2 patients ($n = 60$; $P = 0.024$), pT2-pT4 patients ($n = 22$; $P = 0.009$), and stage IIa-IIIa patients ($n = 69$; $P = 0.012$).

Conclusions: The absence of CEACAM-1 in normal lung tissue and its expression in tumor cells argues against a tumor-suppressive role of CEACAM-1 in NSCLC. The correlation between elevated CEACAM-1 expression and an unfavorable prognosis indicates rather that CEACAM-1 might promote lung cancer progression.

INTRODUCTION

Lung cancer remains the most common cause of cancer-related death in Europe and in the United States (1–3). NSCLC affects $\sim$80% of all lung cancer patients (3, 4). The standard treatment for early-stage NSCLC is surgery resulting in a 5-year survival of only 50–60% in stage I and II (5, 6). Patients with the same stage of disease have markedly different rates of disease progression. Thus, there’s an urgent need for better prognostic parameters in operable NSCLC. Better understanding of the molecular mechanisms of lung cancer progression may reveal new prognostic markers and suggest subgroups that could benefit from adjuvant therapy after surgical resection.

Cell adhesions play a key role in tumor invasion and metastasis (7). Compared with normal tissues, malignant tumors are characterized by a disruption of tissue architecture and a derangement in differentiation. It has been postulated that changes in cell-cell and cell-matrix interactions account for the ability of cancer cells to cross tissue boundaries and to disseminate to distant sites. The loss of cell-cell binding that closely correlates with differentiation and the invasive potential of malignant tumors is accompanied, by a loss of, or alteration in expression of cell adhesion molecules (8). To date, many cell-cell adhesion molecules and cell-matrix adhesion molecules have been identified (7). A number of adhesion molecules belong to the immunoglobulin superfamily (9). One interesting member of the immunoglobulin superfamily is the CEACAM-1, formerly known as biliary glycoprotein I or CD66a (10). It is a member of the carcinoembryonic antigen family and combines structural features of the immunoglobulin superfamily with functional properties of cadherins (9, 11). Furthermore,
CEACAM-1 is the major antigen of the CD66 cluster of granulocyte differentiation antigens.

CEACAM-1 is known to mediate both homophilic and heterophilic adhesion and is expressed in a variety of normal human tissues (12). CEACAM-1 seems to play a role in various human diseases, (13), and it has been suggested as putative tumor suppressor (14–17). Expression of CEACAM-1 was found to be reduced in malignant tissues as compared with corresponding normal tissues deriving from breast, (14) prostate, (15) colon, (16), and endometrium (17). These findings indicated that CEACAM-1 might suppress carcinogenesis.

In contrast, Thies et al. (18) recently provided evidence that CEACAM-1 expression in primary tumors of malignant melanoma patients is associated with subsequent development of metastatic relapse. This finding raised the possibility that CEACAM-1 might facilitate metastatic tumor spread. In endothelial cells, CEACAM1 exhibits properties of an angiogenic factor and acts as a major effector of VEGF (19), suggesting that CEACAM-1 expression might promote metastasis by the induction of angiogenesis.

Thus far, CEACAM-1 expression in operable lung cancer has not been investigated. We examined CEACAM-1 expression in primary NSCLC immunohistochemically and analyzed the clinical outcome after a follow-up period of more than 10 years. This study provides the first evidence that CEACAM-1 promotes lung cancer progression.

PATIENTS AND METHODS

Patients. Specimens of 145 consecutive patients with completely resected NSCLC were collected after approval by the ethical committee of the University of Munich and after written informed consent. The tumors were classified according to the international union against cancer (UICC) TNM-classification (6). The preoperative staging of all patients had resulted to the CD66 cluster of granulocyte differentiation antigens. The expression of angiogenesis.

Immunohistochemical Staining of Primary Tumors. The expression of CEACAM-1 protein was analyzed by immunohistochemical staining using the LSAB method. Briefly, paraffin sections were dewaxed, rehydrated, and subsequently incubated with Pronase (Sigma, Taufkirchen, Germany) at a final concentration of 1 mg/ml for 5 min at 25°C. Endogenous peroxidase activity was blocked by treating the specimens with 30% hydrogen peroxide for 10 min. Nonspecific antibody binding was prevented with a commercial blocking agent (LSAB-kit; Dako, Hamburg, Germany). Excess blocking agent was drained, and the sections were incubated overnight at 25°C with the monoclonal CEACAM-1 antibody 4D1/C2. The antibody was kindly provided by C. Wagener (Institute of Clinical Chemistry, University Hospital Hamburg-Eppendorf, Hamburg, Germany).

Immunohistochemical staining was continued by incubating the slides with biotinylated antimouse/antirabbit secondary antibody solution (LSAB-kit; Dako Corp.) for 30 min at 25°C. Peroxidase was introduced using a streptavidine conjugate (LSAB-kit; Dako Corp.). Between each step of the procedure, the specimens were thoroughly rinsed with 0.1M Tris-HCl buffer (pH 8.2). Peroxidase reactivity was visualized using aminoethyl carbazole (AEC; Sigma) dissolved in dimethylformamide and 0.1 M acetate buffer (pH 5.2) creating a red-brown staining with 30% hydrogen peroxide. Finally, the sections were counterstained with hematoxylin and were mounted in Kaiser's glycerol gelatin.

Evaluation of Immunohistochemical Staining. Immunohistochemical staining of tumor sections was examined independently by two observers who were unaware of the clinical data. The slides with discrepant evaluations were re-evaluated and a consensus was reached (n = 6). The slides were examined under light-microscopes using objectives with ×10 and ×40 magnification. The staining intensity for CEACAM-1 in tumor cells was assessed in comparison with granulocytes and normal lung tissue in the sections. CEACAM-1 is the major antigen of the CD66 cluster of granulocyte differentiation antigens. Therefore, granulocytes are suitable to serve as an internal positive control of CEACAM-1 immunoreactivity. CEACAM-1 immunoreactivity of normal lung tissue and granulocytes was analyzed.

Statistical Analyses. Statistical analysis was performed using the SPSS software package, version 11.0 (SPSS, Inc, Chicago, IL). The two-tailed Pearson χ² test was used to analyze the association between clinicopathological variables. All of the variables were dichotomized. For analysis of follow-up data, life table curves were calculated using the Kaplan-Meier method.
and survival distributions were compared by log-rank statistics. The primary end point was cancer-related survival, as measured from the date of surgery to the time of the last follow-up or cancer-related death. Data of patients who were still alive and without evidence of disease at the end of the study were censored. The joint effects with already recognized prognostically relevant variables were examined via Cox proportional hazards analysis. pT status, pN status, and patient age were entered stepwise forward into the model to test these covariables for possible prognostic joint effects with CEACAM-1 expression rate. The threshold for statistical significance was chosen at $P = 0.05$.

**RESULTS**

**Immunohistochemical Staining of Primary Tumors.** Normal lung tissue, normal bronchiolar epithelium, and stromal cells of all sections exhibited no immunostaining. Staining intensity of tumor cells ranged between no staining and a staining intensity comparable with the immunoreactivity of granulocytes (Fig. 1). Granulocytes served as an internal positive control for CEACAM-1 immunoreactivity because CEACAM-1 is the major antigen of the CD66 cluster of granulocyte differentiation antigens. Staining intensity was not considered a parameter for the assessment of CEACAM-1 expression because staining intensity varies between different batches of immunohistochemical staining. Three patients with unspecific staining of the negative controls had to be excluded before further analyses, resulting in 145 eligible patients.

Primary tumors were divided into three groups according to the percentage of stained tumor cells in the tumor center. Tumors of 31 patients (21.4%) exhibited a low CEACAM-1 expression rate (i.e., $<33\%$ positive tumor cells) and specimens of 42 patients (29.0%) showed an intermediate expression rate (i.e., $\geq 33\%$ and $<66\%$ positive tumor cells). A high CEACAM-1 expression rate (i.e., $\geq 66\%$ positive tumor cells) was observed in 72 patients (49.6%; Fig. 1; Table 1). No significant correlation was observed between the results of CEACAM-1 immunostaining and pT status, pN status, grading, or sex (Table 1). Interestingly, a high CEACAM-1 expression rate was significantly more frequent in adenocarcinomas (61.9%) than in squamous cell carcinomas (35.7%; $P = 0.017$, $\chi^2$ test; Table 1).

**Survival Analysis.** An exclusion of 14 patients with incomplete follow-up or cancer-unrelated death was necessary,
resulting in a total of 131 patients eligible for survival analysis. The median follow-up duration was 72 months (range, 10–130 months). Within this observation period, distant metastases developed in 29 (22.1%) patients, local recurrence occurred in 15 (11.5%) patients, and an additional 35 (26.7%) patients suffered developed in 29 (22.1%) patients, local recurrence occurred in 15 (11.5%) patients, and an additional 35 (26.7%) patients suffered.

Univariate survival analysis revealed a relationship between high CEACAM-1 expression by tumor cells and unfavorable outcome. Patients with a high CEACAM-1 expression rate in tumor cells showed a tendency toward reduced cancer-related survival (P = 0.063, log-rank test). Additional Kaplan-Meier analyses of local recurrence and occurrence of distant metastases demonstrated that, comparable with cancer-related survival, high CEACAM-1 expression was associated with a tendency toward distant metastasis in the total population (P = 0.063, log-rank test). No influence of CEACAM-1 expression rate concerning local recurrence became apparent (P = 0.68, log-rank test).

The association with cancer-related death was statistically significant in advanced tumor stages. The group of patients with pT2–pT4 tumors (n = 22) exhibited a significant association between a high CEACAM-1 expression rate and shortened cancer-related survival (P = 0.009, log-rank test). Patients with pN1–pN2 tumors (n = 60) also showed a significantly reduced cancer-related survival in case of high CEACAM-1 expression (P = 0.024, log-rank test). A high expression rate of CEACAM-1 was also significantly associated with poor cancer-related survival in stage Ila-IIla patients (n = 69; P = 0.012, log-rank test; Fig. 2d). Stratification according to the tumor type showed that CEACAM-1 had only weak prognostic influence in the resulting subgroups of adenocarcinoma (P = 0.11, log-rank test), squamous cell carcinoma (P = 0.17, log-rank test), and other histologies (P = 0.61, log-rank test).

A multivariate analysis was conducted to evaluate whether the correlation between high CEACAM-1 expression and shortened cancer-related survival resulted from a possible association of CEACAM-1 expression with other prognostically relevant factors or whether CEACAM-1 could maintain its own prognostic value (Table 2). Expression rate of CEACAM-1 and standard prognostic parameters such as tumor extension, lymph node status, and patient age (6, 21) were tested for possible prognostic joint effects in the total population of 133 eligible patients. A preceding univariate analysis revealed that pT status and pN status were significant prognostic parameters (P < 0.05) and showed that CEACAM-1 expression rate and patients age tended toward an association with unfavorable prognosis (P < 0.10) in the total population (Table 2). Because CEACAM-1 expression was associated with tumor histology (Table 1), the prognostic impact of tumor histology itself was analyzed to evaluate a possible prognostic joint effect of both parameters. Univariate analysis demonstrated that the tumor histology did not influence cancer-related survival (P = 0.63, log-rank test). Therefore, tumor histology was excluded from multivariate analysis. The multivariate Cox regression analysis demonstrated that a high CEACAM-1 expression rate was a significantly
independent prognostic parameter of reduced cancer-related survival ($P = 0.018$). The relative risk for cancer-related death was 1.8-fold increased in patients with high CEACAM-1 expression rate (95% confidence interval, 1.1 – 2.8).

**DISCUSSION**

The present study was performed to investigate the possible role of CEACAM-1 in the progression of NSCLC. Previous studies reported that CEACAM-1 was down-regulated in malignant tissues when compared with corresponding normal tissues deriving from breast, (14) prostate, (15) colon, (16), and endometrium (17), indicating that the reduction of CEACAM-1 expression is involved in carcinogenesis of various tumor types. Therefore, a growth-suppressive effect of CEACAM-1 was postulated in the past. Although the mechanism of its suppressive action is largely unresolved, several reports suggested that CEACAM-1 participates in signal transduction by interacting with other membrane or cytoplasmic proteins via its cytoplasmic domain. The cytoplasmic domain (CEACAM1cyt) is phosphorylated by multiple protein kinases, and the phosphorylation on one or both of its two tyrosine residues (Tyr-488 and Tyr-515) is triggered by several physiological events.
Counter to our expectations, no progression-suppressive effect of CEACAM-1 became apparent in the present study. In contrast, elevated expression of CEACAM-1 was associated with severe disease progression. High CEACAM-1 expression was associated with a tendency toward reduced cancer-related survival in the total population and with a significant association with unfavorable outcome in advanced disease such as ptT3-ptT4 tumors, pN1-pN2 tumors and stage IIa-IIIa tumors. A multivariate Cox regression analysis of the total population confirmed that CEACAM-1 expression was an independent predictor for reduced cancer-related survival. Interestingly, this Cox regression analysis of CEACAM-1 and cancer-related survival resulted in a significant P, whereas the P of the preceding univariate analysis of the total population was not statistically significant. This phenomenon can occur in cases of highly independent prognostic parameters (22). The predictive power of CEACAM-1 expression was highlighted by including known prognostic factors such as ptT status, pN status, or patient age into the multivariate analysis. In this analysis, only CEACAM1 expression and pN status remained as independent predictors for survival-related cancer.

It is noteworthy that CEACAM-1 immunoreactivity was significantly more frequently observed in adenocarcinoma of the lung (61.9%) than in squamous cell carcinoma of the lung (37.9%; Table 1). Similar observations have not been reported thus far because NSCLC represents a unique model for cancer with a potential for both glandular and squamous cell differentiation. In mammary epithelial cell lines, the loss of CEACAM-1 expression is causally related to the loss of glandular differentiation (23). Thus, CEACAM-1 might play an active role in the initialization of differentiation pathways in epithelial tumors. A loss of glandular differentiation via down-regulation of CEACAM-1 might explain the observed lower CEACAM-1 immunoreactivity of squamous cell lung carcinomas compared with adenocarcinomas of the lung. In contrast to the present study, a recent cDNA microarray analysis that investigated differential expression of CEACAM-1 in adenocarcinomas of different origins demonstrated that high CEACAM-1 expression was present in 12 lung metastases from colon cancer and not in 125 primary lung adenocarcinoma (24). It was suggested that CEACAM-1 expression be analyzed to facilitate the discrimination of a primary lung adenocarcinoma from a distant metastasis to the lung (24). Another cDNA microarray also revealed contrasting results, showing that high expression of CEACAM1 was significantly associated with an improved 5-year survival in 27 patients with adenocarcinoma of the lung (25). These differences to the present study may result from the small number of cases analyzed in the array studies, but may also reflect the potential difficulties in interpreting microarray data deriving from an analysis of RNA that was extracted from whole tumor samples that contained variable proportions of tumor cells, stroma, blood vessels, and inflammatory cells. However, these microarray studies were not designed to investigate the prognostic effect of a single protein and show their huge advantages in expression profiling that may elucidate molecular comechanisms of up- and down-regulation.

A recent study on malignant melanoma patients has challenged the concept of a tumor-suppressive effect of CEACAM-1. The same antibody as in the present study was used for immunohistochemical evaluation of CEACAM-1 expression rate, and the results revealed an increased metastatic relapse in patients with CEACAM1-positive tumors (18). A possible explanation for the observed correlation between CEACAM-1 expression and tumor progression in both malignant melanoma and NSCLC might be a recently reported angiogenic potential of CEACAM-1. In detail, it has been reported that CEACAM-1 is expressed in microvessels of proliferating tissues such as endometrium, healing wounds, and solid tumors (12, 19). In microvascular human endothelial cells, purified native and recombinant CEACAM-1 stimulated proliferation, chemotaxis, and tube formation (19). Moreover, in the choriovitallantoic membrane of the chicken, CEACAM-1 induced angiogenesis (19). Recently, it has been shown that the angiogenic effects of CEACAM1 are additive to those of the VEGF: The expression of CEACAM-1 was up-regulated by VEGF, and VEGF-induced in vitro tube formation was completely blocked by a monoclonal anti-CEACAM-1 antibody (19). However, the present study did not investigate the possible reasons for an unfavorable outcome of CEACAM1-positive NSCLC because this prognostic impact was not expected according to the former literature. Therefore, the mechanistic basis of the observation of the present study remains unresolved, and additional studies need to be performed to clarify the causes of unfavorable outcome in patients with CEACAM1-positive NSCLC. Such

### Table 2 Univariate and multivariate analysis of cancer-related survival in the total population

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Univariate analysis P&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Relative risk</th>
<th>95% confidence interval</th>
<th>P&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>High vs. intermediate and low expression rate of CEACAM-1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.0667</td>
<td>1.8</td>
<td>1.1–2.8</td>
<td>0.018</td>
</tr>
<tr>
<td>pT&lt;sub&gt;1&lt;/sub&gt; vs. pT&lt;sub&gt;2&lt;/sub&gt; vs. pT&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.0198</td>
<td>1.6</td>
<td>0.9–2.9</td>
<td>0.109</td>
</tr>
<tr>
<td>pN&lt;sub&gt;0&lt;/sub&gt; vs. pN&lt;sub&gt;1&lt;/sub&gt; vs. pN&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.0003</td>
<td>2.3</td>
<td>1.4–3.8</td>
<td>0.001</td>
</tr>
<tr>
<td>Age ≤60 vs. &gt;60 years</td>
<td>0.0697</td>
<td>1.0</td>
<td>0.8–2.5</td>
<td>0.054</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cancer-unrelated death or incomplete follow-up resulted in the exclusion of 12 patients leaving 133 patients available for the analysis of joint effects of prognostic parameters.

<sup>b</sup> P of univariate analyses were determined by log-rank test.

<sup>c</sup> Stepwise multivariate analysis was performed using the Cox proportional-hazard model.

<sup>d</sup> CEACAM-1 expression was categorized according to the percentage of stained tumor cells into low (<i>i.e.</i>, <33% positive tumor cells), intermediate (<i>i.e.</i>, ≥33% and <66% positive tumor cells), and high (<i>i.e.</i>, ≥66% positive tumor cells).
additional studies should also investigate the biological basis of CEACAM-1 expression. Because CEACAM-1 expression has a significant clinical impact in advanced tumor stages, the inhibition of CEACAM-1 may be a new therapeutic strategy for patients with advanced operable NSCLC, as soon as the biological basis of the unfavorable prognosis of CEACAM-1-positive NSCLC has been clarified. A recent report has shown that the in vitro effects of CEACAM-1 on angiogenesis can be completely blocked by a monoclonal antibody against CEACAM-1 (19). The present study may provide a rationale for the preselection of patients to be included in future trials investigating adjuvant systemic CEACAM-1 antibody therapy after surgical resection of NSCLC.

REFERENCES

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