Expression of CEACAM-1 in Pulmonary Adenocarcinomas and their Metastases

INA THÖM[1], OLAF SCHULT-KRONEFELD[1], IRIS BURKHOLDER[2], GUNTER SCHUCH[1], BIRTE ANDRITZKY[1], HARTWIG KASTENDIECK[4], LUTZ EDLER[2], CHRISTOPH WAGENER[5], CARSTEN BOKEMEYER[1], UDO SCHUMACHER[3] and ECKART LAACK[1]

[1]Department of Oncology and Hematology, Bone Marrow Transplantation and Pneumology Sections, [3]Institute of Anatomy II: Experimental Morphology, Center of Experimental Medicine, and [5]Center of Clinical Pathology, University Hospital Hamburg-Eppendorf;
[2]Department of Biostatistics, German Cancer Research Center, Heidelberg;
[4]Institute of Pathology, General Hospital Hamburg-Harburg, Hamburg, Germany

Abstract. *Background: CEACAM-1 is involved in intercellular adhesion and is expressed in a variety of human tissues. In cases of malignant transformation, a down-regulation or loss of CEACAM-1 has been shown. In contrast, CEACAM-1 is not expressed in normal lung tissue or melanocytes. It has been demonstrated that an expression in these tissues is associated with the development of metastatic disease. The aim of the present investigation was to analyze a possible association between the expression of CEACAM-1 in pulmonary adenocarcinomas and their lymph node and hematogenous metastatic cells. Patients and Methods: CEACAM-1 expression was immunhistochemically evaluated in primary tumors, lymph nodes and distant metastases of 96 patients with metastatic pulmonary adenocarcinoma who had undergone surgery between 1999 and 2002. Results: Expression of CEACAM-1 was shown in 78 out of 96 primary tumors (81.3%). A significant positive correlation was found between CEACAM-1 expression on cells of the primary tumor, lymph node metastases ($p<0.005$) and hematogenous metastases ($p=0.03$). CEACAM-1 expression did not correlate with stage, gender, grading or patients' age. Compared to patients with tumors not expressing CEACAM-1, patients with a CEACAM-1-expressing tumor had a shorter median overall survival (21 vs. 28 months) and progression-free survival (11.7 vs. 16.3 months). Conclusion: CEACAM-1 is expressed in most primary pulmonary adenocarcinomas. This investigation demonstrates that its expression is preserved in lymph node and hematogenous metastases, indicating that its expression is of functional significance for both metastatic sites. These results support the prognostic relevance of the expression of CEACAM-1 in pulmonary adenocarcinoma.*

Among all malignant diseases, lung cancer is the most frequent cause of death, both in Europe and in the United States, with smoking as the predominant risk factor. For a male population, this has been a fact for decades, but due to increasing numbers of women smoking, females have been catching up in recent years (1). Histologically, lung cancer comprises a heterogeneous group of tumors, with about three quarters being non-small cell lung cancer (NSCLC) and one quarter being small cell lung cancer (SCLC). Based on histomorphology, the former group can be subdivided into adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Recent epidemiological studies have shown an increasing incidence of adenocarcinoma in women and the younger population, whereas the incidence of SCLC is slightly decreasing (2).

At the time of diagnosis of NSCLC, about 40% of the patients show locally advanced disease with lymph node involvement. Though surgical resection is the therapy of choice in localized stages, the relapse rate of over 50% after complete resection indicates that the tumor has already spread beyond its anatomical site. This is a particular challenge to the oncologist, since the tumor-node-metastasis classification (TNM) is still the gold standard concerning prognosis and stratification of this disease (3). However, this merely anatomical description of tumor spread does not allow for further insight into the biological diversity and metastatic potential of lung cancer.

Cell adhesion plays a key role in tumor invasion and metastasis. Compared to normal tissues, malignant tumors are characterized by a disruption of tissue architecture and a dysfunctional differentiation. Changes in cell-to-cell and cell-

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Correspondence to: Ina Thöm, MD, Department of Oncology and Hematology with Bone Marrow Transplantation and Pneumology Sections, University Hospital Hamburg-Eppendorf, Martinistraße 52, 20246 Hamburg, Germany. Tel: +49 40428033962, Fax: +49 40428038054, e-mail: i.thoem@uke.uni-hamburg.de

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to-matrix interactions lead to the derangement of differentiation, enabling the cancer cells to cross tissue boundaries and to disseminate to distant sites (4). Many different cell adhesion molecules have been identified. Members of cell adhesion molecules belong to the immunoglobulin superfamily. One of these is the carcinoembryonic antigen (CEA)-related cell adhesion molecule (CEACAM-1), a cell-to-cell adhesion molecule that is known to mediate both homophilic and heterophilic adhesion (5). It is expressed in a variety of cell types, including epithelial cells, T-cells, natural killer (NK) cells and dendritic cells (6).

Down-regulation of CEACAM-1 expression is often observed in epithelial tumors, such as breast (7), prostate (8), colorectal (9), liver, renal cell (10) and endometrial cancer (11). Restoration of expression in tumor cell lines often abolishes their oncogeneity *in vivo*, consequently, CEACAM-1 is regarded as a tumor suppressor (12, 13). However, in contrast to these cited tumor entities, *de novo* expression of CEACAM-1 is observed in malignant melanoma and NSCLC. Contrary to the expectations for a tumor suppressor protein, *de novo* expression of CEACAM-1 actually increases the risk of metastasis in melanoma patients and is an independent predictor of survival in NSCLC patients (14, 15). These findings raised the possibility that CEACAM-1 expression might facilitate metastatic tumor spread by exhibiting properties of an angiogenic factor and acting as a major effector of vascular endothelial growth factor (VEGF), promoting metastasis by the induction of angiogenesis at the metastatic site.

The aim of the present investigation was to analyse a possible association between the expression of CEACAM-1 of primary adenocarcinoma cells of the lung and their metastatic deposits in lymph node and hematogenic sites.

Patients and Methods

Patients. Tumor tissue blocks from 96 patients with metastatic adenocarcinomas of the lung who had undergone surgery between 1999 and 2002 in the General Hospital Hamburg-Harburg, Germany, were investigated.

Histology and histochemistry. To determine the expression and localization of CEACAM-1, immunohistochemistry was performed on paraffin-embedded sections with the monoclonal antibody 4D1/C2. Details of the production and characterization of the monoclonal antibody have been published elsewhere (16). Routinely fixed and paraffin-embedded 5 μm-thick sections were deparaffinized in xylene and rehydrated in a series of graded ethanols to Tris-buffered saline (TBS; pH 7.6) with 0.1% Tween-20 added. The slides were microwaved at 500 W, five times for 2 minutes in 10 mmol/l citrate buffer (pH 6.0). After cooling for 20 minutes, the slides were washed three times in TBS plus 0.1% Tween-20 for 5 minutes, blocked for 30 minutes at room temperature with normal rabbit serum (Dako, Glostrup Denmark), diluted 1:10 in TBS, and incubated in a humid chamber overnight at 4˚C with the monoclonal CEACAM-1 antibody 4D1/C2 8 μg/ml (in-house preparation by C. Wagener). The sections were then first washed three times in TBS for 5 minutes and then incubated with a 1:40 diluted biotinylated rabbit anti-mouse antibody (Dako) for 20 minutes at room temperature. After further washes in TBS, the sections were incubated with an avidin-alkaline phosphate complex (Vectastain ABC kit; Vector, Burlingame, CA, USA) for 30 minutes followed by additional washes in TBS. Enzyme reactivity of the alkaline phosphate complex was visualized using Naphthol-AS-bisphosphate as substrate, and hexatozised new fuchsin was used for simultaneous coupling. Slides were counterstained with Mayer's hemalum diluted 1:1 with distilled water for 10 seconds, blued under running tap water, and mounted with Crystal Mount (Biomeda, Foster City, CA, USA). Negative controls were treated the same way, omitting the incubation with the primary antibody.

The percentage of staining of the cancer cells was recorded as follows: negative indicated no or weak staining of single tumor cells (<5%), while positive staining indicated that at least 5% of the tumor cells were stained. The slides were examined under a Zeiss Axioplan photomicroscope and photographed with Kodak Ektachrome 64T color film.

Statistical analysis. Overall survival time was defined as the interval from the date of diagnosis to death or to last contact for living patients. The duration of progression-free survival was calculated from the date of diagnosis to date of relapse or death, whichever first occured, or to last follow-up information for living patients.

Overall survival and progression-free survival were graphically presented using the Kaplan-Meier method and were compared with respect to gender, grading, tumor stage and age at diagnosis using the log-rank test. Median overall survival, median progression-free survival and the 1-year survival rates were obtained from the Kaplan-Meier curves.

Detection of staining of the primary tumor cells and the metastatic cells in the lymph node were statistically analysed using methods for contingency tables and Fisher's Exact test as a test for significance. In this analysis, lymph node metastases were considered to be negative only if all investigated lymph node locations (N1, N2, N3) were negative. An association was stated as significant if the p-value of Fisher's Exact test was <0.05.

Statistical analyses were performed using the statistical packages SAS (SAS Institute, Cary, NC, USA) for Windows Version 9, R Version 2.1.1 and StatXact Version 6.0 (Cytal Software Corporation, Cambridge, MA, USA).

Results

Patients characteristics. The tumor tissues of 96 patients were investigated; patient characteristics are given in Table I. The median age at time of surgery was 62 years (range 37-82 years). Fifty-eight (60.4%) of the patients were male. At diagnosis 10 patients (10.4%) had hematogenous metastases (stage IV disease), of which eight were pulmonary and two brain metastases. Eighty-six N1, 64 N2 and 26 N3 lymph node metastases, and nine hematogenous metastases (comprising three lung metastases at the time of diagnosis and five lung metastases and one muscle metastasis at the time of relapse) were investigated. From the brain metastases and the other lung metastases at diagnosis, tissue blocks were not available. In two patients, only one hematogenous metastasis each but no lymph node metastasis were investigated.

Table I. *Patient characteristics*.

Characteristic	No. of patients
Total	96
Gender	
Male	58 (60%)
Female	38 (40%)
Stage	
IIA	1 (1%)
IIB	22 (23%)
IIIA	26 (27%)
IIIB	37 (39%)
IV	10 (10%)
Grading	
Well-differentiated	3 (3%)
Moderately differentiated	16 (17%)
Poorly differentiated	56 (58%)
Undifferentiated	4 (4%)
Unknown	17 (18%)
Postoperative status	
R0	79 (82%)
R1	12 (13%)
R2	3 (3%)
Rx	2 (2%)

At the time of diagnosis 7 patients (7.3%) received neoadjuvant chemotherapy and one patient (1%) received neoadjuvant radiotherapy. All patients then underwent surgery of their primary tumor and their lymph node metastases. In the majority of patients (82.3%), an R0 resection was achieved, while in two cases the R situation remained unclear (2%). Histologically, the majority of tumors were moderately (G2) or poorly differentiated (G3). In 17 of the cases (17.7%), the grading was unknown or could not be identified (Gx). Two patients (2.1%) received additional chemotherapy, while 64 patients (66.7%) received additional radiotherapy.

Expression of CEACAM-1 and survival analysis. The primary tumors of 18 patients (19.1%) among the 94 patients with lymph node metastases showed no expression of CEACAM-1, whereas the primary tumors of 76 patients (80.9%) showed CEACAM-1 expression. There was a positive association between CEACAM-1 positive primary tumors and positivity of lymph node metastases ($p<0.005$) (see Figure 1): of the 76 patients with a CEACAM-1 expressing primary tumor, 75 (98.7%) had CEACAM-1 expressing tumor cells in local lymph nodes. Of the 18 patients without CEACAM-1 expression in the primary tumor, 13 (72.2%) showed no CEACAM-1 expression, whereas 5 (27.8%) patients did.

As explained above, nine hematogenous metastases were investigated. Seven metastases (77.8%) showed CEACAM-1 expression of the tumor cells. In these cases, the primary tumor also showed CEACAM-1 expression. In the two cases in which the metastases showed no CEACAM-1 expression, the primary tumor was also CEACAM-1 negative. These results were statistically significant ($p=0.03$).

The median time-to-follow-up was 53.4 months [95% confidence interval (CI) 44.3-63.9 months]. The median survival time of all 96 patients was 21.0 months (95% CI 18.9-28.7 months) (Figure 2). The 1-year survival rate was 70.8%, the 2-year survival rate 42.6% and the 3-year survival rate 27.8%. The median progression-free survival time was 11.8 months (95% CI 10.5-15.8 months) with a 1-year progression-free survival rate of 49.0% (Figure 3). In our study population, tumor stage (log-rank test: $p=0.95$), grade of tumor differentiation ($p=0.23$) and gender ($p=0.18$) had no significant influence on overall survival. The patients with a CEACAM-1-expressing tumor had a median overall survival of 21.0 months; the patients with tumors which did not express CEACAM-1 had a median overall survival of 27.9 months ($p=0.84$). The patients with a CEACAM-1-expressing tumor also had a shorter median progression-free survival of 11.7 months than did the patients with a CEACAM-1 negative tumor, who had a median progression-free survival of 16.3 months ($p=0.55$). CEACAM-1 expression did not correlate with tumor stage, grading or gender in our study population.

Discussion

The aim of the present investigation was to analyse a possible association between the expression of CEACAM-1 of pulmonary adenocarcinoma cells at the primary site and their lymph node and hematogenous metastatic cells. For this purpose, expression of CEACAM-1, which is strongly associated with metastatic spread in malignant melanoma and a poor prognosis in patients with pulmonary adenocarcinoma (14, 15), was assessed in the primary tumors and their corresponding lymph node and hematogenous metastases. To our knowledge, this is the first report investigating the expression of CEACAM-1 in primary adenocarcinomas of the lung and their lymph node as well as their hematogenous metastases.

In our study 81% of the primary tumors showed CEACAM-1 expression, which was lower in the studies of Laack *et al*. and Sienel *et al*. [66% and 62% respectively; (15, 17)]. This discrepancy is most likely due to sampling differences between the two studies. While Laack *et al*. looked at a patient sample with more than 50% stage I disease without lymph node involvement, in the present study we investigated only patients with local lymph node and/or distant metastases, resulting in higher tumor stages. In the study of Laack *et al*., the expression of CEACAM-1 was significantly correlated with the tumor stage (15). Most of the patients (72%) with a primary tumor which did not express CEACAM-1 had a stage I disease, 19% had a stage II disease and only 9% had a stage III/IV disease (15). In our study, only stage II, III and IV disease patients were included because of the necessary investigation of lymph node and/or hematogenous metastases.

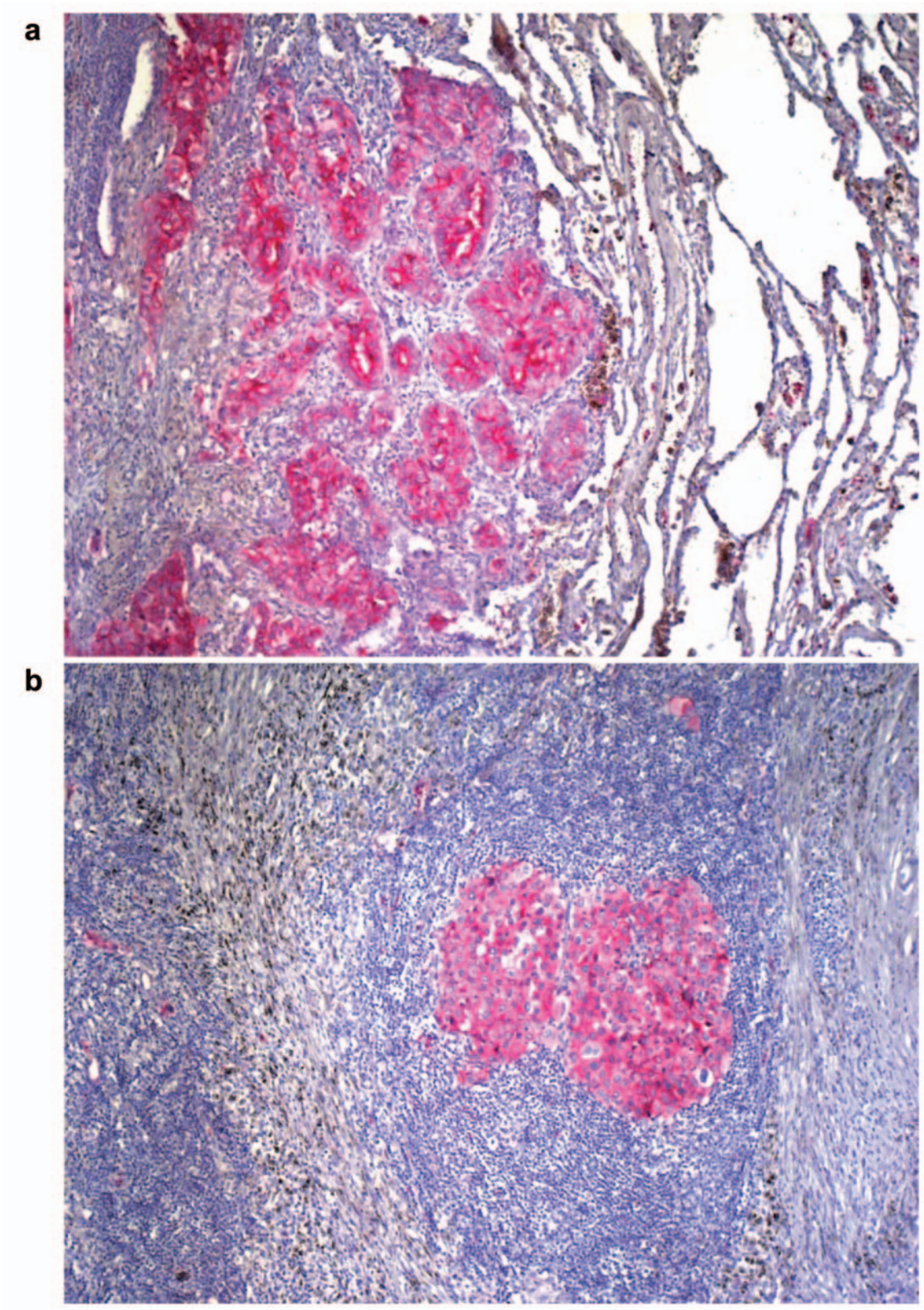

Figure 1. *CEACAM-1 expressing adenocarcinoma. a, Primary tumor of the lung, and b, lymph node metastasis.*

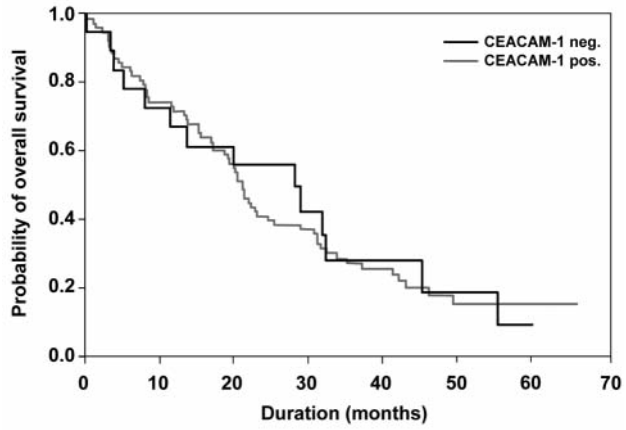

Figure 2. *Kaplan-Meier plot of overall survival of 96 patients with resected adenocarcinoma of the lung.*

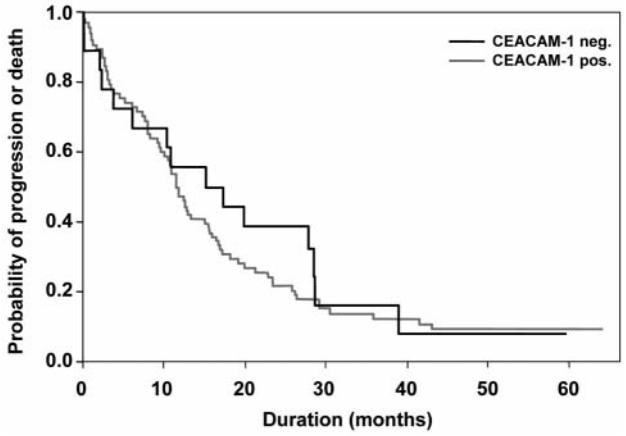

Figure 3. *Kaplan-Meier plot of progression or death of 96 patients with resected adenocarcinoma of the lung.*

We found a highly significant positive correlation between the expression of CEACAM-1 of the cells of the primary tumor and of their lymph node and hematogenous metastases: 98.7% of the CEACAM-1-expressing tumors showed CEACAM-1-expressing tumor cells in their lymph node metastases and conversely, 72.2% of the primary tumors without CEACAM-1 expression did not contain CEACAM-1-expressing tumor cells in their lymph node metastases. This also applied to the hematogenous metastases. Metastatic cells with CEACAM-1 expression were derived from CEACAM-1-positive primary tumors, whereas metastases originating from CEACAM-1-negative primary tumors were also negative. Thus, CEACAM-1 expression is highly conserved in the tumor cells in lymph node and hematogenous metastases, no loss or gain in CEACAM-1 expression during lymphogenic and hematogenic tumor spread was observed.

Thies *et al*. demonstrated that CEACAM-1 expression in the primary tumor of malignant melanoma was significantly associated with the subsequent development of metastatic disease (14). In another study, Thies *et al*. demonstrated that the CEACAM-1 expression was preserved in both lymphatic and hematogenous metastases of malignant melanomas (18). The studies suggested that CEACAM-1 is involved in the motility and invasiveness of the tumor cells, being up-regulated in the invading front of the tumor with an interaction of CEACAM-1 and the surrounding matrix (14, 18). This might facilitate the invasion of blood vessels and so of the metastatic hematogenous route. As the melanoma cells express CEACAM-1 on their cell surface as well, it has been postulated that they may be able to influence the extracellular matrix in a proangiogenic way.

In several other studies, it has been demonstrated that CEACAM-1 is a potent angiogenic factor being the major effector of VEGF in the early microvessel formation of several tumor types (19). CEACAM-1 is expressed and up-regulated in endothelia of adjacent microvessels of *in situ* carcinomas of the urinary bladder and prostate (20, 21). With tumor angiogenesis a noninvasive and nonvascularized tumor switches to an invasive and vascularized tumor. In these studies, the prognostic value of CEACAM-1 expression was not investigated. In another study, the angiogenic activity of CEACAM-1 and microvessel density was investigated in NSCLC (22). The study showed that high CEACAM-1 expression was associated with an increased angiogenic activity and higher metastatic potential. The authors postulated that the prognostic influence of CEACAM-1 might be derived from this association.

That CEACAM-1 is an independent prognostic factor in patients with resectable adenocarcinoma of the lung was demonstrated in a study by Laack *et al*. (15). In this study, only the primary tumors were investigated. During follow-up there was no significant correlation between CEACAM-1 expression and the site of recurrence (distant metastases *vs*. local relapse). Sienel *et al*. also investigated tumor tissue of resected NSCLC (17). They postulated an unfavorable outcome for patients with high CEACAM-1 expression on their tumor cells. Almost half of the patients had a stage I tumor and several histological tumor types besides adenocarcinoma were included. The authors discriminated a low, intermediate and high CEACAM-1 expression rate. They did not use a cut-off level as in our and other studies. High CEACAM-1 expression was associated with a tendency toward distant metastasis; there was no influence on local recurrence.

In our study, the patients with a CEACAM-1-expressing tumor had a shorter median overall survival (21.0 *vs*. 27.9 months) and progression-free survival (11.7 *vs*. 16.3 months) than did the patients without CEACAM-1 expressing tumors. However, the log-rank test showed no statistically significant differences in overall survival (p=0.84) or progression-free survival (p=0.55). This might be because of the shorter follow-up in our study.

In a study of metastatic malignant melanoma, CEACAM-1 expression of the primary tumor did not predict for hematogenous *versus* lymphatic spread of the tumor cells (14). In melanoma, CEACAM-1 was mainly expressed on the invading front of the tumor hence the authors postulated that CEACAM-1 may be functionally involved in the motility and invasiveness of the tumor cells. Furthermore, as seen by others, CEACAM-1 expression was associated with a poor prognosis.

In conclusion, this study is the first to show that CEACAM-1 expression is preserved during lymphatic and hematogenous tumor spread of adenocarcinoma of the lung. Our results support the prognostic relevance of the expression of CEACAM-1 in pulmonary adenocarcinoma and might allow classification of patients into prognostic groups, which could be important for future application of targeted therapy. Furthermore, as the expression of CEACAM-1 is preserved in lymphatic and hematogenous metastases, it may be an interesting molecular target for treatment strategies.

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