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Activated leukocyte cell adhesion molecule (CD166)—Its prognostic power for colorectal cancer patients

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Article info

Article history:
Received 17 December 2011
Received in revised form 3 February 2012
Accepted 7 February 2012
Available online 30 March 2012

Keywords:
ALCAM
CD166
Colorectal cancer
CRC
Cancer stem cell
CSC
Biomarker
Prognosis

ABSTRACT

Background: The activated leukocyte cell adhesion molecule (ALCAM, CD166) has been reported to be involved in tumorigenesis of colorectal cancer (CRC) and to function as a cancer stem cell marker. Controversial data exist regarding the prognostic power of ALCAM expression in CRC. Here, we evaluate the expression of ALCAM in a cohort of CRC patients and its usage as a prognostic marker for survival.

Materials and methods: Tissue specimens from 299 patients with CRC treated between 1993 and 2006 were analyzed via ALCAM immunohistochemistry (clone MOG/07) using a tissue microarray. Results were correlated with clinical, histopathological, and patient survival data (Chi-square test, Kaplan–Meier analysis, and log-rank test, respectively). Multivariate analysis also was performed (Cox regression).

Results: ALCAM is expressed in most primary (76%) and secondary (62%) CRC lesions (P = 0.014). Immunohistochemistry revealed an inverse association with tumor grading (P = 0.002) but not with any other clinical or histopathological data. Kaplan–Meier survival analysis revealed a significant overall survival benefit in the group of ALCAM-positive patients (P = 0.019). Multivariate analysis showed that ALCAM is an independent positive prognostic marker for overall survival (P = 0.023).

Conclusions: ALCAM expression is a positive prognostic marker for overall survival of CRC patients, and its detection might help to optimize the existing prognostic staging system. Elevated expression in higher differentiated tumors might indicate a potential role in the early steps of tumorigenesis, and its loss might be associated with reduced cellular adhesion, resulting in a higher metastatic potential of the tumor. Further studies must be conducted investigating these hypotheses.

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1. Introduction

Colorectal cancer (CRC) is the second most common malignant neoplasia worldwide, making it a disease with enormous medical and health-economic relevance. CRC occurs at a lifetime incidence rate of approximately 6%, and its incidence increases with age [1,2]. CRC in its early stages can be curatively treated with surgery alone. However, 20%–25% of patients present with metastatic disease at first diagnosis, and most of these patients must be treated palliatively [3,4].

Therapeutic decision making is based today on conventional histopathological factors, such as cell differentiation, tumor invasion, lymph node metastasis, and distant metastasis status. To optimize the reliability of recurrence prediction, several new molecular markers have been investigated in the last few years. In recent times, the theory of the hierarchical organization of tumor cells was extensively investigated, supporting the cancer stem cell hypothesis [5–8]. These cells might be potential therapeutic biologic targets and prognostic markers. Several authors have identified putative stem cell markers for CRC, namely CD133, CD44, and CD166, the activated leukocyte adhesion molecule (ALCAM) [6,9–11]. The latter is a highly conserved 110-kDa multidomain transmembrane type 1 glycoprotein of the immunoglobulin superfamily. ALCAM plays a role in the development of different tissues during embryogenesis and in adults, and it functions via homotypic and heterotypic interactions between the cells [12–14]. It is also expressed in various malignant lesions, such as melanoma and esophageal, gynecologic, prostate, and pancreatic cancers, and its expression is associated with diverse outcomes in different tumors [12–19].

As with other membrane proteins, ALCAM represents a potential target for therapy, and its utility as a drug target may be further enhanced by ligand-induced endocytosis [20]. Moreover, a recently described internalizing single-chain anti-ALCAM antibody has the potential to deliver therapeutic agents into prostate cancer cells [20,21]. Wiiger et al. [22] showed the inhibitory effect of an anti-ALCAM, single-chain antibody on CRC cell growth in a xenograft mouse model. ALCAM expression is reported to be significantly elevated in CRC compared with normal mucosa [23,24]. However, inconsistent data exist regarding the prognostic significance of ALCAM expression in CRC [10,24,25].

We conducted the present study to determine the level of ALCAM expression in tumor cells of primary lesions and metastatic sites, such as lymph nodes and distant organ metastases of patients with CRC. In addition, we evaluated the relationship between the expression of ALCAM and the most clinically relevant factors of CRC, and we determined its significance as a diagnostic and prognostic marker.

2. Materials and methods

2.1. Patients and clinical data

This study was approved by the Ethics Committee of the Chamber of Physicians in Hamburg, Germany. Written informed consent was obtained from all patients for use of the resected samples.

Tumor stage and grade were classified according to the tumor–node–metastasis (TNM) classification of the International Union Against Cancer. All clinical and histopathological data including sex, tumor size, lymph node metastasis, tumor type, and disease stage were obtained from the clinical and pathological records.

2.2. Tissue microarray

For this study, 576 specimens from 375 patients with CRC who underwent surgery in the Department of Surgery at University Medical Center Hamburg-Eppendorf between January 1991 and February 2005 were chosen retrospectively. Of these, samples of 375 primary tumors, 128 lymph node metastases, and 48 liver metastases were included on a previously described tissue microarray (TMA) [26]. Tissue samples were fixed in 4% buffered formalin, embedded in paraffin, and used for TMA construction as described previously [26]. Hematoxylin–eosin-stained sections were made from selected primary tumor blocks (donor blocks) to define representative tumor regions. Tissue cylinders (0.6 mm in diameter) were then punched from that region of the donor block using an experiment-specific semiautomated tissue arrayer. The punch biopsies were obtained from a peripheral or central area with a high number of vital tumor cells and little necrosis. Control samples included normal esophagus mucosa (n = 10), endometrium (n = 2), skin (n = 2), skeletal muscle (n = 2), heart muscle (n = 2), colon mucosa (n = 2), lung (n = 2), lymph node (n = 2), prostate (n = 2), and kidney (n = 2). An overview of the complete TMA is shown in Fig. 1G. Three-micrometer sections were made by use of the Paraffin Sectioning Aid System (Instrumentics, Hackensack, NJ).

2.3. Immunohistochemical staining for ALCAM and evaluation of expression

The ALCAM staining protocol was optimized in an extensive multistep procedure on various benign and malignant tissues, modifying the staining protocol until selective staining with the lowest background signals was established [17,18].

ALCAM expression was detected using a mouse monoclonal antibody (clone: MOG/07, 1:450; Novocastra, UK) after boiling the sections in an autoclave covered with citrate buffer, pH 7.8. The Envision system (DAKO, Denmark) was used to visualize the immunostaining. Only membranous staining was evaluated because cytoplasmic staining, if present, was always linked with stronger membranous staining. Spots without staining were classified as ALCAM negative, and spots with a membranous staining intensity present, was always linked with stronger membranous staining. Spots without staining were classified as ALCAM negative, and spots with a membranous staining intensity were classified as ALCAM positive. Immunohistochemical analysis of the sections was performed without knowledge of the patients’ identities or clinical statuses.

2.4. Survival data

Clinical follow-up data were obtained by reviewing the hospital records, through direct communication with the attending
physicians and from the Cancer Registry of Hamburg. Overall survival was calculated from the date of surgical excision of the primary tumor to the date of death or last follow-up.

2.5. Statistical analysis

SPSS Statistics for Windows (Version 17, SPSS Inc., Chicago, IL) was used for statistical analysis. Interdependence between the immunostaining and ELISA results, as well as the clinical data, was calculated using chi-squared and Fisher’s exact tests and displayed by cross tables.

Survival curves were plotted using the Kaplan–Meier method and analyzed using the log-rank test. Univariate and multivariate analyses were performed for prognostic factors for recurrence free and overall survival using the Cox regression model.

All tests were two sided. P-values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Characteristics of the patients

Patients’ characteristics and ALCAM expression of primary tumors, correlated with age, sex, histological grading (G), tumor invasion depth (pT), lymph node status (pN), presence of metastases (M), resection status (R), and distant metastases, and mortality, are listed in Table 1. Briefly, the median age of patients was 65 y (range 15–91 y); 178 male and 122 female patients were included. Median follow-up time of all CRC patients included into survival analysis was 39 mo (range 1–180 mo); median calculated overall survival was 88 mo (95% confidence interval [CI] 53.3–121.8 mo). Thirteen (3%) patients died within the first 30 d after surgery.

3.2. Expression of ALCAM in primary tumors, lymph nodes, and distant metastases and correlation with clinical and histopathological characteristics

A total of 300 (80%) primary CRC, 79 (62%) lymph node, and 35 (73%) liver metastases samples were interpretable in our TMA analysis. Reasons for noninformative cases included a complete lack of tissue samples or the absence of unequivocal cancer tissue in the TMA sections.

The ALCAM immunohistochemistry shows a predominant membranous expression of the ALCAM molecule (Fig. 1). Primary tumors were ALCAM positive in 76% (n = 229), lymph node metastases in 61% (n = 48), and distant metastases in 66% (n = 23) of the cases. ALCAM expression is significantly reduced in metastases compared with primary tumors (P = 0.014; Table 1, Fig. 1A–E). Correlation of ALCAM-positive primary tumors with corresponding lymph node and distant metastases showed no significant association (P = 0.084 and P = 1.000, respectively; Table 1). The tumors show a heterogeneous staining pattern inside the cancerous lesions (Fig. 1A, B, and D).

ALCAM expression showed no association with clinical and histopathological parameters, such as age, sex, TNM classification, resection margin status, and 30-d mortality (Table 1). It is significantly inverse correlated with tumor cell differentiation (grading; P = 0.002). Association of primary tumor ALCAM expression with ALCAM-positive lymph node metastasis and histopathological parameter showed no statistically significant results (n = 66; sex, P = 0.620; age, P = 0.191; pT, P = 0.865; pM, P = 0.622; resection margin status, P = 1.000). Tumor grading just failed statistical significance (G, P = 0.059).

Survival curves plotted by the Kaplan–Meier analysis showed the significantly reduced overall survival of ALCAM-negative patients (P = 0.019, Fig. 2).
3.3. Multivariate analysis

Multivariate analysis revealed that ALCAM expression in the primary tumor is a significant positive prognostic parameter for overall survival (Table 2; \( P = 0.023 \)). In this cohort, other significant independent prognosticators were the age of the patients (\( P = 0.009 \)), lymph node metastasis (\( P = 0.002 \)), distant metastasis (\( P < 0.001 \)), and resection margin involvement (\( P < 0.001 \)).

![Fig. 2 – Kaplan–Meier overall survival analysis of immunohistochemical ALCAM staining of primary CRC specimen.](image)

### Table 1 – Correlation between clinical and pathological parameters with immunohistochemical ALCAM status.

<table>
<thead>
<tr>
<th>Total</th>
<th>ALCAM expression</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>primary tumor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ALCAM negative</td>
<td>ALCAM positive</td>
</tr>
<tr>
<td>Median age, y (range)</td>
<td>67 (37–89)</td>
<td>65 (15–91)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>178</td>
<td>43 (24%)</td>
</tr>
<tr>
<td>Female</td>
<td>122</td>
<td>28 (23%)</td>
</tr>
<tr>
<td>Disease stage (UICC 6th edition)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>2</td>
<td>61</td>
<td>14 (23%)</td>
</tr>
<tr>
<td>3</td>
<td>194</td>
<td>48 (25%)</td>
</tr>
<tr>
<td>4</td>
<td>33</td>
<td>9 (27%)</td>
</tr>
<tr>
<td>pN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>146</td>
<td>31 (21%)</td>
</tr>
<tr>
<td>1</td>
<td>74</td>
<td>16 (22%)</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>24 (30%)</td>
</tr>
<tr>
<td>M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>221</td>
<td>53 (24%)</td>
</tr>
<tr>
<td>1</td>
<td>79</td>
<td>18 (23%)</td>
</tr>
<tr>
<td>G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 and 2</td>
<td>250</td>
<td>50 (20%)</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>21 (42%)</td>
</tr>
<tr>
<td>R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>287</td>
<td>67 (23%)</td>
</tr>
<tr>
<td>1 and 2</td>
<td>6</td>
<td>2 (33%)</td>
</tr>
<tr>
<td>Mortality (30 d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>288</td>
<td>68 (24%)</td>
</tr>
<tr>
<td>Yes</td>
<td>12</td>
<td>3 (25%)</td>
</tr>
<tr>
<td>ALCAM immunohistochemistry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>26</td>
<td>10 (39%)</td>
</tr>
<tr>
<td>Positive</td>
<td>40</td>
<td>7 (17%)</td>
</tr>
<tr>
<td>Met</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>10</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>Positive</td>
<td>23</td>
<td>5 (22%)</td>
</tr>
<tr>
<td>Tumor localization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary tumor</td>
<td>300</td>
<td>71 (24%)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>79</td>
<td>31 (39%)</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>35</td>
<td>12 (34%)</td>
</tr>
</tbody>
</table>

UICC = International Union Against Cancer; LN = lymph node metastasis; Met = distant metastasis.

4. Discussion

The present study was performed to retrospectively evaluate the expression of ALCAM in CRC tissues of primary and metastatic sites and to determine whether ALCAM could serve as a diagnostic and prognostic marker. The results showed that ALCAM was expressed in most CRC lesions of primary and metastatic origins. Lymph node and distant metastases are significantly less often ALCAM positive compared with primary tumors. Correlation with histopathologic factors revealed an inverse association with tumor cell differentiation grade. In univariate and multivariate Cox regression analyses, ALCAM expression functions as a positive prognosticator for overall survival.

In recent years, three different groups have investigated the expression of ALCAM (with the same antibody for immunohistochemical methods, clone: MOG/07, Novocastra, UK, but different concentrations) in CRC with three different results [10,24,25]. Weichert et al. [24] did not see any correlation with the investigated histopathological factors, but they did observe the independent prognostic power of ALCAM expression for shortened overall survival in their cohort of 111 patients, which is contradictory to our findings.

### Table 2 – Independent prognostic factors analyzed by multivariate Cox regression analysis are shown.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.729</td>
<td>1.146/2.608</td>
<td>0.009</td>
</tr>
<tr>
<td>Disease stage (UICC 6th edition)</td>
<td>1.353</td>
<td>1.055/1.735</td>
<td>0.002</td>
</tr>
<tr>
<td>pN</td>
<td>4.610</td>
<td>2.964/7.171</td>
<td>0.000</td>
</tr>
<tr>
<td>M</td>
<td>2.661</td>
<td>1.676/4.226</td>
<td>0.000</td>
</tr>
<tr>
<td>ALCAM expression</td>
<td>0.610</td>
<td>0.398/0.934</td>
<td>0.023</td>
</tr>
</tbody>
</table>

HR = hazard ratio with 95% CI. P refers to significance according to Cox regression hazard model comparing specified variables; age (≥65 y versus <65 y), pN (positive versus negative), M (positive versus negative), resection margin (incomplete versus complete resection), ALCAM expression (positive versus negative).
Interestingly, they found a membranous expression of the molecule of only 31%. In 2009, Horst et al. [10] stained 110 primary CRC specimens for ALCAM and detected expression in 64% of the samples. They found neither the clinical or pathological data correlated with the expression of ALCAM nor a significant association with survival prognosis [10]. The most comprehensive study regarding ALCAM expression in CRC was published by Lugli et al. They used a TMA with 1,420 CRC specimens, and their study revealed a significant association between the loss of ALCAM expression and larger tumor size, nodal metastasis, infiltrating tumor border configuration, and a lower overall survival in univariate analysis [25]. Furthermore, they investigated the invasiveness of ALCAM negative compared with ALCAM-positive CRC cell lines and found significantly higher invasive potential in ALCAM-negative cells.

Although the data from our study showed an association with tumor cell grading and a reduced expression in metastatic sites only, they confirm the results of Lugli et al. because we have found the independent prognostic power of ALCAM expression for overall survival. This might help to optimize the existing staging of CRC patients, helping to tailor the best individual treatment to each patient.

In the present study, only membranous immunostaining was analyzed because ALCAM staining occurred predominantly at the cell membrane. Cytoplasmic staining intensity was related to the intensity of the membrane staining and did not occur in the absence of membrane staining. In contrast to this, Weichert et al. [24] observed cytoplasmic staining without membranous expression and therefore analyzed both membranous and cytoplasmic staining. Why are there discrepancies in the morphologic and statistical results of studies investigating ALCAM in CRC? Of course, multiple factors influence the staining intensity and specificity of immunohistochemistry, and antibody concentration is only one of them. The dilution ranges from 1:100 to 1:450 in the studies, which reflects the problem with the comparability of immunohistochemical studies. Unfortunately, general guidelines for optimal immunohistochemistry protocol development are lacking. Recently, in a study investigating the clinical significance of p53 alterations in prostate cancer, an immunohistochemistry protocol that was deliberately designed to be "oversensitive" resulted in a much higher rate of positive immunohistochemical findings (2.5% positivity with the standard protocol compared with greater than 90% positivity with the "oversensitive" protocol) [27].

In the large bowel, ALCAM is expressed at the surface of the healthy colonic crypt epithelium and might define the normal intestinal stem cell niche [23,24]. Its physiological role, which seems to be a general involvement in the morphogenesis of tubular structures via cell–cell and cell–matrix interactions, gets lost during the dedifferentiation of the tumor cells [23]. Normal tissue and low-grade tumors can form these structures, whereas higher-grade tumors lose the potential for producing cell formations and their metastatic potential is enhanced [28]. In accordance with this theory, our results showed a significantly reduced ALCAM expression in metastatic sites compared with the primary tumors. However, the biological role of ALCAM is not clear and future in vitro and in vivo studies should focus on the physiological and pathological molecular mechanisms and interactions of the molecule.

In vitro and in vivo experiments have shown an inhibitor effect of anti-ALCAM antibodies in CRC and prostate cancer [20–22]. Because of the frequent ALCAM expression in normal tissues, such as colonic mucosa, severe effects might result and will be taken into account when an application of systemic specific cancer therapies is considered [23].

Although during the last decades several molecular biomarkers for CRC were identified, there is a lack of prognostic and predictive markers with proven utility [29]. The only marker implemented in clinical routine, is KRAS mutational analysis and selection for epidermal growth factor receptor-specific therapy [30]. Unfortunately, many published studies are limited by their retrospective character, have too small and heterogeneous cohorts of patients, or show deficits in the standardization of methodologies, resulting in data, which are not comparable [31]. Nevertheless, in our opinion, studies similar to the present one, in spite of their shortcomings, have a scientific worth and might provide indications of a prognostic potential and also physiologic and pathologic role of the molecule. Of course, before clinical conclusions can be drawn, they must be validated in prospective studies with larger patient collectives. Besides the validation of the most promising predictive and prognostic biomarkers in clinical trials, future studies should focus on the systematical identification of novel diagnostic and therapeutic targets for CRC [32,33].

In conclusion, the expression of the adhesion molecule ALCAM in primary CRC lesions is a prognostic marker for the prolonged survival of the patient cohort of the study in both univariate and multivariate analyses. ALCAM might be one potential biomarker in combination with others, such as micrometastases status or TNM staging, which could help to identify and predict survival in CRC and help to tailor the best individual treatment to each patient. Further trials must validate the prognostic utility of ALCAM in CRC patients.

Acknowledgments

Financial support for this study was provided by a Young Investigator Research Grant from the University Medical Center Hamburg-Eppendorf (Forschungsförderungsfond der Medizinischen Fakultät—Nachwuchsforung 2011). The authors thank the patients who willingly and generously provided data and samples for research purposes, as well as Christina Koop for her excellent technical support.

Conflicts of interest: No conflicts of interest exist.

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