ORIGINAL ARTICLE

ALCAM/CD166 is overexpressed in colorectal carcinoma and correlates with shortened patient survival

W Weichert, T Knösel, J Bellach, M Dietel, G Kristiansen

J Clin Pathol 2004;**57**:1160–1164. doi: 10.1136/jcp.2004.016238

See end of article for authors' affiliations

Correspondence to: Dr G Kristiansen, Institute of Pathology, Charité Hospital, Campus Mitte, Schumannstr. 20/21, D-10117 Berlin, Germany; glen.kristiansen@charite.de

Accepted for publication 19 April 2004

Background: Activated leucocyte cell adhesion molecule (ALCAM) has been implicated in tumorigenesis and tumour progression of malignant melanoma and prostate cancer.
Aims: To clarify the expression patterns of ALCAM in colon cancer and to correlate these with clinicopathological parameters, including patient survival.
Methods: One hundred and eleven colorectal carcinomas were immunostained for ALCAM (clone MOG/07) using a standard detection system. Cytoplasmic and membranous immunoreactivity were scored semiquantitatively. Fisher's exact test, χ^2 test for trends, Kaplan–Meier analysis, and Cox's regression were applied.
Results: In colorectal cancer, 58.6% and 30.6% of cases showed strong cytoplasmic and membranous expression of ALCAM, respectively. No significant correlation with patient age, tumour grade, stage, or nodal status was apparent. In survival analyses, membranous ALCAM expression correlated significantly (Cox's regression, p = 0.028; relative risk, 2.3) with shortened patient survival.
Conclusions: ALCAM is frequently upregulated in colorectal cancer and is a new independent prognostic marker, underscoring the importance of ALCAM in tumour progression in this disease.

Colorectal cancer is the third most common cause of death from cancer in both sexes in the Western world, a high incidence that has remained stable over the past 20 years.[1] Even though this disease is surgically curable in the early stages, the tumour is often not symptomatic until the metastatic stage, which is associated with a high mortality. Therefore, increasing efforts are being made to develop more effective screening and prevention strategies for colorectal cancer and to enhance our ability to predict the disease's course. Currently, the most important conventional prognostic factors for patients' survival are histological tumour grade and tumour stage of disease at the time of diagnosis (pTNM (UICC), Astler-Coller, or Dukes's), including depth of tumour invasion, involvement of regional lymph nodes, and metastatic spread to distant organs. In addition to these clinicopathological parameters, molecular markers are being sought and established for a wide variety of tumours including colon cancer.[2] These new markers are being examined for their diagnostic and prognostic impact, and even therapeutic implications, particularly immunotherapeutic approaches.

> ''Increasing efforts are being made to develop more effective screening and prevention strategies for colorectal cancer and to enhance our ability to predict the disease's course''

Human cancers often show altered expression of genes associated with cell proliferation and cell cycle control, genetic repair and apoptosis, and finally cell adhesion.[3] The activated leucocyte cell adhesion molecule (ALCAM; CD166) is a cell adhesion molecule that belongs to the immunoglobulin superfamily and is widely expressed in a variety of normal tissues.[4] It functions as a ligand to CD6 and also mediates homophilic (ALCAM–ALCAM) interactions.[5–7] ALCAM expression correlates with the invasiveness of malignant melanoma and has been proposed as a prognostic marker in this disease.[8 9] Recently, ALCAM overexpression

Table 1 Distribution of clinicopathological characteristics, cytoplasmic (c), and membranous (m) ALCAM expression

Characteristic	Number of patients	Per cent of patients
Astler-Coller stage		
A	6	5.4
B1	21	19.0
B2	36	32.4
C1	5	4.5
C2	34	30.6
D	9	8.1
Tumour stage		
T1	6	5.4
T2	26	23.4
T3	67	60.4
T4	12	10.8
Lymph node status		
Nx	3	2.7
N0	63	56.8
N1	22	19.8
N2	23	20.7
Metastasis		
Mx	102	91.9
M1	9	8.1
Grading		
G1	5	4.5
G2	87	78.4
G3	19	17.1
Age at diagnosis		
<65	52	46.8
>65	59	53.2
cALCAM		
Negative	46	41.4
Positive	65	58.6
mALCAM		
Negative	77	69.4
Positive	34	30.6

ALCAM, activated leucocyte cell adhesion molecule.

Abbreviations: ALCAM, activated leucocyte cell adhesion molecule; WHO, World Health Organisation

was detected in prostate cancer.[10–12] To date, ALCAM expression has not been investigated in colon cancer. We aimed to investigate the expression patterns of ALCAM in colorectal carcinoma and to correlate our findings with clinicopathological parameters, including patient survival times.

MATERIAL AND METHODS

Patients

One hundred and eleven consecutive patients (age, 41–87 years; median, 65; table 1) who were diagnosed with colon cancer at the institute of pathology, Charité University Hospital, Berlin, Germany between 1996 and 1998 took part in our study. Only patients with primary colonic adenocarcinomas and no other known malignancies at the time of diagnosis and during follow up were studied. The histological diagnosis was established on standard haematoxylin and eosin stained sections, according to the guidelines of the World Health Organisation (WHO). Clinical follow up data were available for all patients. The median follow up time was 47 months. In addition, five adenomas of the colon were included in our study.

Immunohistochemistry

Formalin fixed, paraffin wax embedded tissue specimens were freshly cut (4 μm). The sections were mounted on superfrost slides (Menzel Gläser, Braunschweig, Germany), dewaxed with xylene, and gradually hydrated. Antigen retrieval was achieved by pressure cooking in 0.01M citrate buffer for five minutes. The primary anti-ALCAM antibody (clone MOG/07; Novocastra, Newcastle upon Tyne, UK) was diluted 1/100 using a background reducing dilution buffer (Dako, Hamburg, Germany). The primary antibody was incubated at room temperature for one hour. Detection was carried out according to the manufacturer's instructions, using a streptavidin–biotin system (LSAB kit; Dako) with alkaline phosphatase as the reporting enzyme. Fast-Red (Sigma-Aldrich, Munich, Germany) served as chromogen. Afterwards, the slides were briefly counterstained with haematoxylin and aqueously mounted.

Immunostaining of tissue slides was independently evaluated by three clinical pathologists who were unaware of patient outcome. Because ALCAM shows cytoplasmic and membranous staining, we scored both types of staining as either positive or negative. More precisely, negative staining

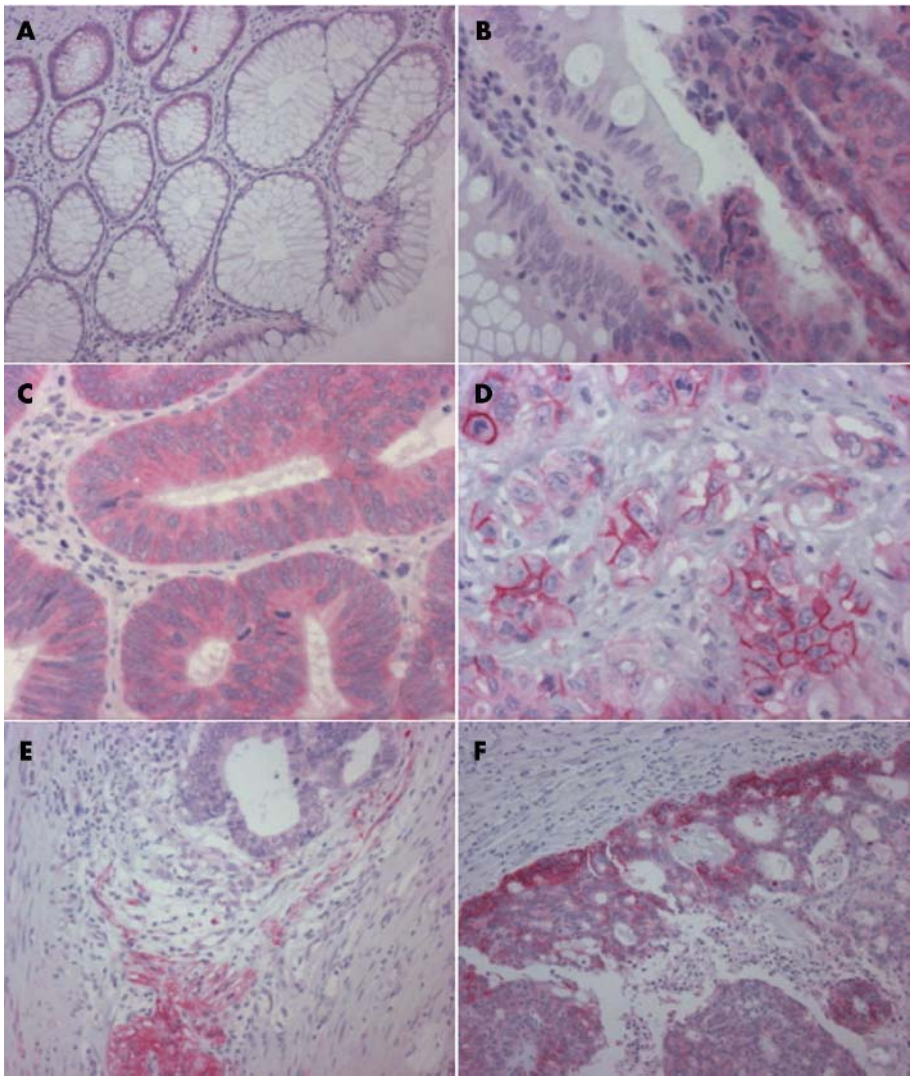

Figure 1 Activated leucocyte cell adhesion molecule (ALCAM) immunohistochemistry in normal colon and colorectal cancer. (A) Normal mucosa of the colon with minimal cytoplasmic staining of the basal parts of the crypts. (B) Transition of normal colon mucosa to dysplastic epithelium of high grade adenoma with abrupt onset of ALCAM expression. (C) Colon cancer with strong cytoplasmic immunoreactivity. (D) Strong membranous ALCAM staining of colon cancer. (E) Colon carcinoma with weak ALCAM expression invading a strongly ALCAM positive nerve, which served as a positive control in all immunostainings. (F) ALCAM positive colon carcinoma, with pronounced expression at the front of invasion.

Table 2 Univariate survival analysis for cytoplasmic and membranous ALCAM expression and several clinicopathological parameters

Characteristic	Mean survival (months)	SE	p Value
Age at diagnosis			
<65 years	60.28	2.83	0.0438
⩾65 years	46.25	3.12	
Astler-Coller stage			
A	Not reached	—	
B1/B2	65.62	1.69	<0.0001
C1/C2	42.59	4.01	
D	12.13	4.02	
Tumour stage			
T1/T2	65.67	2.18	0.0087
T3/T4	46.33	2.69	
Lymph node status			
N0	65.40	1.62	<0.0001
N1/N2	36.65	3.93	
Metastasis			
Mx	58.66	2.18	<0.0001
M1	12.13	4.02	
Grade			
G1/2	59.21	2.25	0.0001
G3	31.23	5.39	
ALCAM cytoplasmic			
Negative	56.16	3.58	0.6908
Positive	49.62	2.72	
ALCAM membranous			
Negative	58.74	2.53	0.0133
Positive	43.33	4.23	

The log rank test was used to estimate the p values.
ALCAM, activated leucocyte cell adhesion molecule.

was defined by a complete absence of immunoreactivity or, in the case of cytoplasmic expression, by at most a scattered staining pattern, which was not unequivocally positive.

Statistical analysis

The data were compiled with the software package SPSS, version 10.0. Fisher's exact test and the χ^2 test for trends were used to assess the significance of associations between the expression of ALCAM and the clinicopathological parameters. Univariate survival analysis was performed according to the Kaplan–Meier method and differences in survival curves were assessed with the log rank test. Multivariate survival analysis was performed on all parameters that were found to be significant on univariate analysis using Cox's regression model. In a visual evaluation of log(−log(survival)) plots no violation of the proportional hazards assumption became apparent. p Values < 0.05 were considered significant. All statistics were accredited by the head biostatistician of the tumour centre, Charité University Hospital (JB), Berlin, Germany.

RESULTS

ALCAM immunohistochemistry

Ganglia of the submucosal and myenteric plexus and nerves stained strongly and served as internal positive controls (fig 1E). Stromal expression was seen only in the capillaries. In normal colon mucosa, mature epithelia showed minimal cytoplasmic staining for ALCAM at the base of colonic crypts (fig 1A). All five adenomas of the colon showed homogeneous cytoplasmic ALCAM staining, which was stronger than that of adjacent normal mucosa (fig 1B). Strong cytoplasmic ALCAM overexpression was seen in 65 of the invasive colon cancer cases (58.6%; fig 1C) and pronounced membranous expression was seen in 34 cases (30.6%; fig 1D). Membranous ALCAM expression was seen only in tumours that also showed cytoplasmic ALCAM positivity, and was often distributed non-homogeneously throughout the tumour epithelium. In general, ALCAM expression was pronounced at the invasive edges of the tumour (fig 1F).

Statistical analysis

Neither the cytoplasmic nor the membranous ALCAM expression pattern correlated significantly with patient age, WHO tumour stage, Astler-Coller stage, lymph node status, status of metastasis, or histopathological tumour grade. A trend of association was seen for tumours with membranous ALCAM expression towards a positive nodal status ($p = 0.091$) and higher patient age ($p = 0.063$).

Kaplan–Meier survival analysis for established prognostic factors in colon carcinoma confirmed a significant impact of patient age ($p = 0.04$), WHO tumour stage ($p < 0.01$), Astler-Coller stage ($p < 0.01$), lymph node status ($p < 0.01$), status of distant metastases ($p < 0.01$), and histopathological tumour grade ($p < 0.01$) on patient survival time (table 2).

Patients whose tumours showed cytoplasmic expression of ALCAM did not have a significantly shortened survival time compared with those whose tumours were ALCAM negative (mean 49.6 *v* 56.2 months; table 2; fig 2A).

In contrast, patients with membranous ALCAM positivity showed a significantly shortened mean survival time compared with those whose tumours showed no membranous ALCAM expression (43.3 months *v* 58.7 months; $p = 0.01$; fig 2B).

In multivariate survival analysis, membranous ALCAM expression remained a significant prognostic factor ($p = 0.03$), with a relative risk of 2.3 (95% confidence interval, 1.1 to 5.0). Other independent prognostic factors in multivariate survival analysis were lymph node status, status of distant metastases, and tumour grade (table 3).

DISCUSSION

ALCAM (CD166) is a 65 kDa cell adhesion molecule of the immunoglobulin superfamily, which is physiologically expressed in activated leucocytes, neural cells, epithelial cells, and haemopoietic progenitor cells.[4–6 13] ALCAM functions as a ligand for CD6 and is thought to be involved in the interaction between thymic epithelial cells and thymocytes.[7] In neoplasia, the expression of ALCAM was first described in

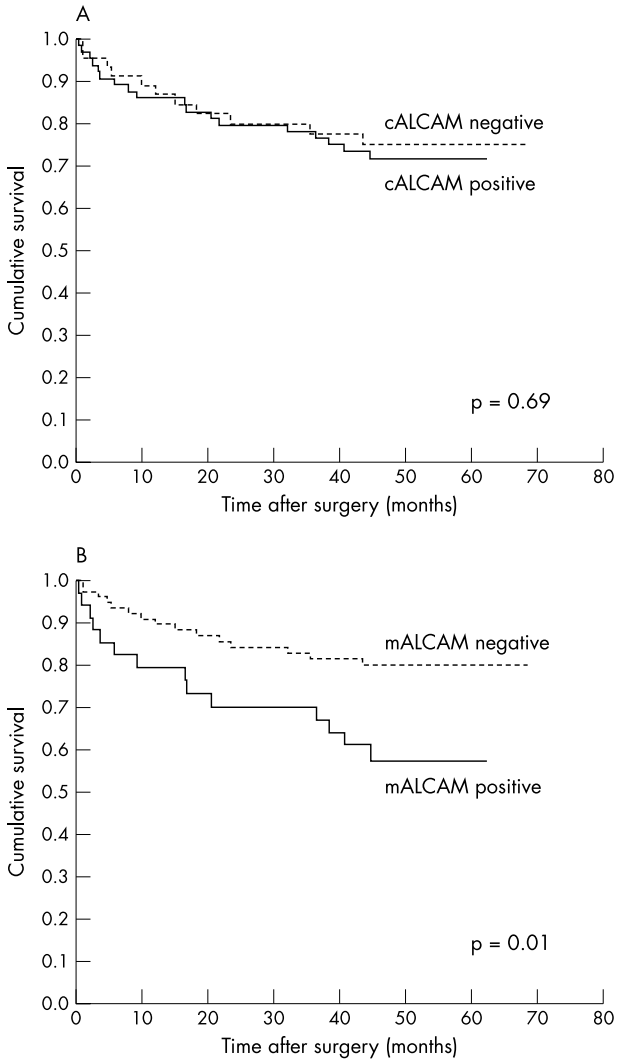

Figure 2 Univariate survival analysis according to Kaplan–Meier for (A) cytoplasmic (c) and (B) membranous (m) activated leucocyte cell adhesion molecule (ALCAM) expression. Note the significantly shorter survival time in the mALCAM positive patients compared with the mALCAM negative patients (p = 0.01). In contrast, expression of cALCAM had no significant impact on survival (p = 0.69).

malignant melanoma and has been proposed as a marker of disease progression.[8 9] ALCAM is also found in fibrosarcoma cell lines and is associated with chemoresistance.[14] Stamey *et al* performed a prostate cancer expression profiling study and found that ALCAM was overexpressed in these tumours,[11] a finding that we could confirm in a frozen section based immunohistochemistry study.[12] To date, the expression of ALCAM in colon cancer has not been investigated. In our study, we used a monoclonal antibody that was suitable for use in paraffin wax embedded specimens to examine the expression patterns of ALCAM in colon cancer.

> ''We hypothesise that the upregulation of ALCAM is an early event in malignant cell transformation in colon carcinogenesis, because it was found in all adenomas of the colon, which are considered to be precursor lesions''

Immunohistochemically, 59% of colon cancers showed upregulation of cytoplasmic ALCAM expression and 31% showed upregulation of membranous ALCAM compared with normal colonic mucosa. In our previous study on prostate cancer, we used a different antibody on frozen sections, but this did not allow a distinction to be made between cytoplasmic and membranous staining. We found focal overexpression of total ALCAM in 81% of prostate cancer cases, with a partial loss of ALCAM expression in high grade tumours. In contrast, we failed to show a significant correlation between ALCAM expression and tumour grade in colon carcinomas. We hypothesise that the upregulation of ALCAM is an early event in malignant cell transformation in colon carcinogenesis, because it was found in all adenomas of the colon, which are considered to be precursor lesions.

In malignant melanoma, ALCAM expression was associated with the vertical growth phase and thus with a more aggressive phenotype. This appears to match our data in colorectal cancer, where we found a significantly shorter survival time in patients whose tumours exhibited membranous ALCAM expression. Importantly, this finding remained significant in Cox's regression model considering all conventional prognostic factors, which suggests that this marker should be investigated further.

We found two types of ALCAM expression in colon carcinomas, cytoplasmic and membranous. The biological relevance of these patterns is largely unclear. Tomita *et al* examined the expression of ALCAM and α catenin in prostate cancer cell lines and found cytoplasmic staining exclusively

Table 3 Multivariate survival analysis (Cox's regression)

	Overall survival		
	RR	95% CI	p Value
Age at diagnosis			
Each year	1.033	0.995 to 1.073	0.088
Tumour stage			
T1/T2	1.000		
T3/T4	1.574	0.426 to 5.820	0.497
Lymph node status			
N0	1.000		
N1/N2	3.977	1.395 to 11.338	0.010
Metastasis			
MX	1.000		
M1	7.264	2.674 to 19.737	<0.001
Grade			
G1/G2	1.000		
G3	2.546	1.081 to 6.010	0.032
ALCAM membranous			
Negative	1.000		
Positive	2.337	1.096 to 4.983	0.028

ALCAM, activated leucocyte cell adhesion molecule; CI, confidence interval; RR, relative risk.

Take home messages

- Activated leucocyte cell adhesion molecule (ALCAM) is frequently overexpressed in the neoplastic colonic epithelia of adenomas and carcinomas
- Membranous ALCAM expression was associated with shortened patient survival times in both univariate and multivariate analysis
- Further studies are needed to clarify the biological role of ALCAM in colon cancer

in cell lines that had lost α catenin expression.[10] Therefore, they concluded that membranous staining reflected a physiological condition of ALCAM expression. Interestingly, we found that staining for cytoplasmic ALCAM in colon cancer and basal parts of colonic crypts of normal mucosa was almost ubiquitous, whereas strong membranous staining was restricted to a fraction of cancers. Importantly, those patients with strong membranous staining had a significantly shorter survival time, suggesting that membranous ALCAM expression is biologically relevant in these tumours. Therefore, we suggest membranous ALCAM expression as a new prognostic marker gene in colon cancer, although confirmatory studies are required.

In conclusion, we found ALCAM overexpression in the neoplastic colonic epithelia of adenomas and carcinomas. Moreover, membranous ALCAM expression was associated with shortened patient survival times in both univariate and multivariate analyses. Further studies are needed to clarify the biological role of ALCAM in colon cancer.

ACKNOWLEDGEMENTS

The excellent technical assistance of B Beyer and the helpful comments of AE Neumann and M-C Koll are gratefully acknowledged.

Authors' affiliations

W Weichert, T Knösel, M Dietel, G Kristiansen, Institute of Pathology, Charité University Hospital, D-10117 Berlin, Germany
J Bellach, Tumour Centre, Charité University Hospital

REFERENCES

1 **Jemal A**, Murray T, Samuels A, *et al*. Cancer statistics, 2003. *CA Cancer J Clin* 2003;**53**:5–26.
2 **McLeod HL**, Murray GI. Tumour markers of prognosis in colorectal cancer. *Br J Cancer* 1999;**79**:191–203.
3 **Hesketh R**. Introduction. In: *The oncogene and tumour suppressor gene facts book*. New York: Academic Press, 1997:2–9.
4 **Swart GW**. Activated leukocyte cell adhesion molecule (CD166/ALCAM): developmental and mechanistic aspects of cell clustering and cell migration. *Eur J Cell Biol* 2002;**81**:313–21.
5 **Bowen MA**, Aruffo A. Adhesion molecules, their receptors, and their regulation: analysis of CD6-activated leukocyte cell adhesion molecule (ALCAM/CD166) interactions. *Transplant Proc* 1999;**31**:795–6.
6 **Bowen MA**, Patel DD, Li X, *et al*. Cloning, mapping, and characterization of activated leukocyte-cell adhesion molecule (ALCAM), a CD6 ligand. *J Exp Med* 1995;**181**:2213–20.
7 **Patel DD**, Wee SF, Whichard LP, *et al*. Identification and characterization of a 100-kD ligand for CD6 on human thymic epithelial cells. *J Exp Med* 1995;**181**:1563–8.
8 **Degen WG**, van Kempen LC, Gijzen EG, *et al*. MEMD, a new cell adhesion molecule in metastasizing human melanoma cell lines, is identical to ALCAM (activated leukocyte cell adhesion molecule). *Am J Pathol* 1998;**152**:805–13.
9 **van Kempen LC**, van den Oord JJ, van Muijen GN, *et al*. Activated leukocyte cell adhesion molecule/CD166, a marker of tumor progression in primary malignant melanoma of the skin. *Am J Pathol* 2000;**156**:769–74.
10 **Tomita K**, van Bokhoven A, Jansen CF, *et al*. Coordinate recruitment of E-cadherin and ALCAM to cell–cell contacts by alpha-catenin. *Biochem Biophys Res Commun* 2000;**267**:870–4.
11 **Stamey TA**, Warrington JA, Caldwell MC, *et al*. Molecular genetic profiling of Gleason grade 4/5 prostate cancers compared to benign prostatic hyperplasia. *J Urol* 2001;**166**:2171–7.
12 **Kristansen G**, Pilarsky C, Wissmann C, *et al*. ALCAM/CD166 is up-regulated in low-grade prostate cancer and progressively lost in high-grade lesions. *Prostate* 2003;**54**:34–43.
13 **Ohneda O**, Ohneda K, Arai F, *et al*. ALCAM (CD166): its role in hematopoietic and endothelial development. *Blood* 2001;**98**:2134–42.
14 **Choi S**, Kobayashi M, Wang J, *et al*. Activated leukocyte cell adhesion molecule (ALCAM) and annexin II are involved in the metastatic progression of tumor cells after chemotherapy with Adriamycin. *Clin Exp Metastasis* 2000;**18**:45–50.