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Elevated ALCAM shedding in colorectal cancer correlates with poor patient outcome

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Abstract

Molecular biomarkers of cancer are needed to assist histological staging in the selection of treatment, outcome risk stratification, and patient prognosis. This is particularly important for patients with early-stage disease. We demonstrate that shedding of the extracellular domain of ALCAM (Activated Leukocyte Cell Adhesion Molecule) is prognostic for outcome in patients with colorectal cancer (CRC). Previous reports on the prognostic value of ALCAM expression in CRC have been contradictory and inconclusive. This study clarifies the prognostic value of ALCAM by visualizing ectodomain shedding using a dual stain that detects both the extracellular and the intracellular domains in formalin-fixed tissue. Using this novel assay, 105 primary colorectal cancers patients and 12 normal mucosa samples were evaluated. ALCAM shedding, defined as detection of the intracellular domain in the absence of the corresponding extracellular domain, was significantly elevated in CRC patients and correlated with reduced survival. Conversely, retention of intact ALCAM was associated with improved survival, thereby confirming that ALCAM shedding is associated with poor patient outcome. Importantly, analysis of stage II CRC patients demonstrated that disease-specific survival is significantly reduced for patients with elevated ALCAM shedding ($p=0.01$, HR 3.0) suggesting that ALCAM shedding can identify patients with early stage disease at risk of rapid progression.

Keywords

colorectal; cell adhesion; tumor markers and detection of metastasis; molecular diagnosis and prognosis; novel assay technology

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The authors have no potential conflicts of interest to disclose.

COMPETING INTERESTS

The authors have no competing interests.

INTRODUCTION

Colorectal cancer (CRC) is the third most frequently diagnosed cancer, and second leading cause of cancer-related deaths in the US(1). Current prognosis for CRC patients predominantly rely on pathologic UICC/AJCC tumor node metastasis (TNM) staging classification (2). Although TNM staging successfully stratifies high-risk patients, there is significant variability in the rate of disease progression within each stage. Particular concern exists for early stage disease (Stage I and II) where patients can progress more rapidly than expected. It is well known that approximately 30% of stage II CRC patients die of recurrent and metastatic disease. Identification of patients at risk of recurrence/progression could inform clinicians on adjuvant chemotherapeutic treatment decisions. Biomarkers can assist in identifying those patients that require more aggressive intervention or patients at risk of relapse after initial treatment. Promising clinical tests including Oncotype DX™ and Coloprint™ evaluate possible disease progression by assessing gene expression. These tests are not yet widely applied possibly because their epigenetic evaluation reflects on gene expression which does not always reliably predict actual cellular behavior. Thus, existing prognostic tests would be enhanced with the addition of biomarkers that report on cancer progression.

While clinical trials have demonstrated less than 5% 5-year survival benefit from adjuvant therapy for unselected stage II colon cancer patients (3), it is clear that a subset of these patients are at high risk for poor outcome and would likely benefit from adjuvant therapy (4; 5). Those high-risk stage II patients have similar outcomes to patients with stage III tumor status (6), highlighting the need for molecular stratification parameters to identify high-risk patients with apparent early stage disease. Attempts made to stratify patients using gene expression profiles have experienced some success but have not been translated to the clinic (7; 8). Molecular indicators capable of identifying subgroups of patients with poor prognosis and beneficence of therapy include microsatellite instability and 18q loss of heterozygosity (LOH), however, 18qLOH has not been translated to a useful prediction tool for clinical use (9).

ALCAM (Activated Leukocyte Cell Adhesion Molecule) has been highlighted as a putative biomarker for the progression of many cancers, including CRC (10–16). ALCAM is a cell-cell adhesion protein that has been identified in a broad array of biological processes including inflammatory responses, neuronal outgrowth and epithelial migration (17). Unlike most candidate biomarkers, ALCAM expression is not tissue-restricted and it is commonly found in most epithelia and related carcinomas. ALCAM contributes to tumor progression by controlling migration and the molecular activity of ALCAM appears to be regulated through shedding of its extracellular domain. Consequently, advanced disease tissues continue to express ALCAM but exhibit an elevated level of ALCAM shedding.

ALCAM has been evaluated for CRC in five published reports. Unfortunately, the findings from these studies are contradictory (10; 11; 18–20). In a study characterizing the expression of ALCAM in the gastrointestinal tract and colorectal cancer, ALCAM was found to be highly expressed in the colon crypts of normal tissue and heterogeneously expressed in tumor sections (20). In a study of 299 CRC patients, membranous ALCAM expression was a positive prognostic indicator for overall survival (11). Similarly, a previous study by Lugli *et al.* also found the loss of membranous ALCAM to be indicative of worse patient prognosis (10). In contrast, Weichert *et al.* reported that membranous ALCAM expression is associated with decreased patient survival (18). A subsequent study by Horst *et al.* (19) found ALCAM not to be correlated with CRC patient outcome.

Although these studies are contradictory, ALCAM has significant potential as a biomarker for CRC because it is not only readily detected in CRC but is also functionally and clinically associated with a large number of cancers including: colorectal (10; 11; 18; 19), prostate (21; 22), breast (13; 23), gastric (24), thyroid (14), pancreatic (25), melanoma (26), and ovarian (15). ALCAM consists of five extracellular IgG-like domains, a transmembrane domain and a short cytoplasmic domain (27). ALCAM can be proteolytically processed by ADAM17, thereby generating a soluble ALCAM component and a truncated membrane-bound ALCAM containing the transmembrane and cytoplasmic domain (28). Functional importance of this shedding was emphasized by the laboratory of Dr. Guido Swart who demonstrated that the truncated, trans-membrane fragment of ALCAM increased lung metastasis *in vivo* (26) while over-expression of a soluble extracellular ligand-binding fragment diminished metastasis. At the clinical level, shed ALCAM is detectable in the serum of breast, thyroid, ovarian and pancreatic cancer patients, and the loss of cell surface ALCAM is associated with poor prognosis (13–14; 29; 42). These data suggest that the proteolytic cleavage of ALCAM is functionally important in tumorigenesis, and detection of ALCAM shedding may function as a prognostic biomarker.

In this study, we sought to determine if ALCAM shedding in human primary colorectal cancers reflects a unique molecular progression of the tumor and consequently acts as a prognostic biomarker. For this purpose we developed a unique dual stain to detect both the extracellular and the intracellular domain of ALCAM within the same tissue. We find that ALCAM shedding in the primary tumor correlates strongly with a poor clinical outcome. This was particularly striking in stage II patients in which disease-specific survival was significantly worse when the tumor tissue exhibited high ALCAM shedding.

MATERIALS AND METHODS

Cell lines and mice

The continuous cell lines for cancer of the breast (MDA-MB-231 and MCF-7), prostate (PC3 and Du145) and colon (RKO, DLD, LOVO, LS174t, HCT116, HCA7, Scko1, Caco2, HT29, KM12c and KM12) were cultured in their appropriate basal media (DMEM or RPMI) with 10% FBS to confluence before lysis with 1% Triton-X 100 in PBS. ALCAM knockout mice (c57bl/6 ALCAM^{-/-}) were purchased from Jackson Laboratories. Mouse tissues were surgically resected, snap frozen and subsequently extracted with 1% Triton-X 100 lysis buffer.

Western Blot Analysis

SDS-PAGE under non-reducing conditions and transfer of proteins to a PVDF membrane has been described previously (31). After blocking with 5% skimmed milk in PBS/0.05% Tween-20, blots were probed with primary antibodies for extracellular ALCAM (Clone 105902; R&D Systems) and selected hybridoma clones, followed by peroxidase-conjugated secondary antibody and ECL (Perkin-Elmer) detection.

Lentivirus-delivered RNA Interference

Four individual constructs containing shRNAs for human ALCAM and a negative control (scrambled sequence) were purchased from Sigma (Mission shRNA). Constructs were packaged for viral production and infection and tested for target knockdown. For viral packaging, constructs were co-transfected into 293T cells using Fugene HD (Roche Applied Science). Media containing viruses were collected 48hr after transfection. PC3 cells were infected with the viruses in the presence of Polybrene (8 µg/ml) for 24hr and then subjected to selection by 5 µg/ml puromycin. Two constructs with 90% knockdown efficiency as determined by immunoblotting and flow cytometry were used for further studies.

Human material

The protocols and procedures for this study were approved by the institutional review boards at the University of Alabama-Birmingham Medical Center, Vanderbilt Medical Center (VMC), the Veterans Administration Hospital (Nashville, TN), and the H. Lee Moffitt Cancer Center (MCC; Tampa, FL). Tissue specimens from 250 colorectal cancer patient enrolled at Vanderbilt Medical Center (VMC, n = 55) and Moffitt Cancer Center (MCC, n = 195) were used for gene-expression microarray analyses as described previously (8). All patients had a diagnosis of colorectal adenocarcinoma. Each cancer specimen was staged according to American Joint Commission on Cancer (AJCC) guidelines (stages I-IV), and 10 normal adjacent specimens were deemed to contain only normal colonic tissue by a certified gastro-intestinal pathologist. VMC 55 includes 14 patients from the University of Alabama-Birmingham Medical Center (8). Microarray data for the NCI cell lines was obtained through the NCBI Gene Expression Omnibus (GEO data set GDS1761).

A tissue microarray containing 75 primary colorectal carcinomas and 12 normal age and sex-matched colorectal mucosa was constructed using 2 mm cores in triplicate. Specimens from 69 CRC patients and 12 normal colonic mucosa were suitable to be used in the dual staining analysis. Subsequent expansion of this dataset was accomplished by selection of 36 stage II patients under IRB #120063 providing analysis for a total of 105 CRC and 12 normal mucosa with triplicate representation of each patient. Collection of serum from control (n=6), non-cancer patients (n=48) and colorectal patients immediately before surgery (pre-op, n=71) or after treatment (followup, n=20) at was accomplished at Vanderbilt Medical Center under IRB# 121365.

ALCAM Dual Immunofluorescence stain

Immunofluorescent staining for ALCAM in tissues was performed with hybridoma HPA010926 (Sigma Prestige Antibodies) directed against the extracellular domain and clone 1G3A1 (obtained from our fusion) directed against the intracellular domain. Sections cut from patient tissue and tissue microarrays were deparaffinized in xylene and rehydrated. Sections were blocked in 20% Aqua Block™ after pressure cooker antigen retrieval in citrate buffer (pH 6.0). Samples were immunostained with mouse monoclonal intracellular ALCAM antibody, 1G3A1, (3 µg/ml) and rabbit monoclonal extracellular ALCAM antibody, HPA010926 (1:250 dilution). The arrays were incubated with Alexa-546 Goat anti-rabbit (1:500) and Alexa-647 Goat anti-mouse secondary antibody (1:500, LifeTechnologies). The sections were counterstained with 2 µg/ml of Hoechst for 2 mins, and mounted with Prolong® Gold Anti-fade.

Image acquisition and quantitative analysis of ALCAM shedding

Tissue microarrays were imaged using the Ariol® SL-50 platform from Genetix. Image analysis and quantitation were performed using the open-source software ImageJ (FIJI). The analysis pipeline was designed as follows: a) The tumor area was selected using the free-hand selection tool. b) The color image was split into its red, green and blue component channels. c) Image thresholding was used to generate the detectable region of intracellular ALCAM staining (red channel) and extracellular ALCAM staining (green channel). d) Intact ALCAM was determined as the area of co-localized intracellular and extracellular ALCAM (red and green channel) while ALCAM shedding was determined as the area of intracellular ALCAM that lacks extracellular ALCAM (red but no green). The sum of these two represents total ALCAM expression.

Statistical analysis

Descriptive statistics were applied to show patient's basic characteristics stratified by ALCAM shedding score. Wilcoxon rank sum test and Kruskal-Wallis test were applied to exam the mRNA expression difference between normal tissues and cancer tissues or the ALCAM shedding percentage among normal patients and cancer patients in all different stages. Kaplan-Meier curve was used to estimate the survival probability for each group, with corresponding p-value and hazard ratio calculated from log-rank test. Receiver Operating Characteristic (ROC) curves were used to identify the optimal specificity and sensitivity for patient stratification. For survival analysis the patient population was dichotomized across a value of ALCAM shedding or intact ALCAM as defined by the ROC curves. For ALCAM shedding this was 0.75 and for intact ALCAM this was 0.15. For shedding the p-values of all statistical tests were two-sided and considered significant when $p < 0.05$ where * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$. All statistics were completed using either R, SPSS or GraphPad Prism. Multivariable analysis using logistic regression was performed on stage II patients ($n=66$; median follow-up, 70 months; median age of diagnosis, 67 years). The variables included were ALCAM shedding, age at time of diagnosis, race and gender with an incidence of 51.5% (34 events) for overall survival and 26.9% (18 events) for disease specific survival.

RESULTS

Correlation of ALCAM and ADAM17 expression with survival of colorectal cancer patients

In normal colorectal tissue, immunohistochemical staining for the extracellular domain of ALCAM using HPA10926 reveals the protein at areas of cell-cell contact within the epithelial cells of the colonic crypts and in hemopoietic cell populations of the stroma (Fig. 1Ai and ii). In contrast to normal colon, the concomitant staining of colorectal cancer tissue reveals a very heterogeneous staining. Within the same tumor, some regions exhibit elevated ALCAM (Fig. 1Aiii) while others exhibit irregular staining (iv) or lack ALCAM staining all-together (v). Similar heterogeneity of ALCAM staining is observed in a publicly available tissue microarray (proteinatlas.org (34)). ALCAM protein expression is detectable in 12/14 CRC cell lines (Fig. 1B and Suppl. Fig. 2). Expression of the ALCAM mRNA in CRC cell lines among the NCI60 (Col) is intermediate between the low expressing leukemia (Leu) cell lines and the high expressing breast cancer (Br) cell lines (Fig. 1B).

To evaluate ALCAM mRNA expression in colorectal cancer, a single cohort consisting of 250 patients obtained through a multi-institutional collection (VUMC, UAMC and Moffitt Cancer Center) was analyzed. ALCAM mRNA is elevated in cancer patients (Fig. 1C, $p < 0.001$) and univariate analysis revealed that high ALCAM expression was in fact associated with significantly decreased survival (Fig. 1C, $p < 0.0001$). Similarly, expression of ADAM17 (the sheddase of ALCAM) was also significantly elevated in colorectal cancer (Fig. 1D, $p < 0.0001$). The association of ADAM17 expression with patient survival was not statistically significant ($p=0.067$), but its elevated expression in CRC together with its established ability to cleave ALCAM is sufficient to suggest that ADAM17 is available to cleave ALCAM and increase its shedding within the tumor microenvironment. Indeed, previous studies reported heterogeneous staining of ADAM17 in colorectal cancer (35), which might be responsible for the variable detection of ALCAM extracellular domain within the tissue. The soluble extracellular domain can be detected in the serum of some cancer patients (13; 14). However, ALCAM-specific ELISA of serum from CRC patients did not reveal a correlation between disease progression and increase in circulating ALCAM when comparing serum obtained from cancer free patients and serum obtained from CRC patients prior to, and after therapy (Fig. 1E). Detailed comparison of cancer free patients and

normal healthy individuals versus increasing stages of CRC patients revealed no significant correlation with circulating levels of ALCAM (Fig. 1F).

The histological detection of membranous ALCAM had been found to correspond negatively with patient survival (18). Using an antibody to the extracellular domain of ALCAM, a histological evaluation of a 69-patient cohort was performed (Suppl. Fig. 3). While the presence or absence of membrane staining did not correspond with overall or disease specific survival, the loss of detectable cytoplasmic ALCAM corresponded with very poor prognosis. However, only 8/69 patients (12%) were negative for cytoplasmic ALCAM while 39/69 (56%) lacked membranous ALCAM. This loss of ALCAM from the membrane (with concomitant retention of cytoplasmic staining) is likely to be due to shedding of the ectodomain from the cell surface.

Production and validation of an antibody specific for the cytoplasmic domain of ALCAM

Since ALCAM shedding occurs on the surface of the tumor cells, we hypothesized that shedding might be detectable within the tumor tissue itself. In order to achieve this, we thought to develop an ALCAM dual-stain based on independent detection of the intracellular and extracellular domains with domain-specific antibodies (Suppl. Fig. 4A). Using these antibodies in histological staining of normal and tumor tissue sections should enable the detection of ALCAM shedding *in situ* (Suppl. Table I). To accomplish this, a unique antibody directed to the cytoplasmic tail of ALCAM was generated using a 14 AA sequence from the cytoplasmic tail (Suppl. Fig. 1 and Supplemental Methods) conjugated to KHL to immunize four A/J mice. Spleens from two seropositive mice were fused and 125 viable hybridomas selected from >8000 antigen-reactive clones were evaluated by comparing reactivity to native ALCAM and KLH using direct ELISA (Fig. 2A). ALCAM-specific hybridomas were validated by immunoblotting using whole cell lysate to confirm binding to intact ALCAM protein (Fig. 2A). Antibody specificity for ALCAM was verified by comparing reactivity with lysates from control and ALCAM knockdown cells (Fig. 2B) and mouse tissue from *wild type* and *ALCAM*^{-/-} mice (Suppl. Fig. 4B). Antigen specificity was confirmed by competitive blocking using the immunizing peptide during immunoblotting (0.1 or 1 µg/ml, Fig. 2C) and histological staining (1 µg/ml, Suppl. Fig. 4C). As expected, peptide competition with the immunizing peptide resulted in a loss of Intracellular ALCAM. The stable hybridoma 1G3A1 was selected as the most promising antibody based on its reactivity in ELISA, immunoblot, immunofluorescence and standard immunohistochemistry. Specificity of 1G3A1 for the cytoplasmic tail of ALCAM was defined by its ability to detect intact ALCAM in cell lysates but not shed ALCAM in conditioned medium (Fig. 2D). In contrast, the commercial antibody against the extracellular domain of ALCAM (R&D) detects intact as well as shed ALCAM which lacks the cytoplasmic domain.

Dual-staining for the intracellular and extracellular domains of ALCAM in normal and tumor tissue

Using the antibody 1G3A1 to specifically detect the cytoplasmic domain of ALCAM together with the commercial antibody HPA010926 specific for the extracellular domain, we developed a dual-staining procedure for ALCAM in human tissues (Fig. 3&4). Three-color staining (Nuclei: blue, Extracellular Domain: green, Intracellular Domain: red) was performed on tissues along the digestive tract including stomach (Fig. 3A) and colon (Fig. 3B). In normal tissues, detection of the extracellular and cytoplasmic domains of ALCAM coincided, thereby suggesting that ALCAM is expressed and present in its intact form (Fig. 3C, arrow). However, within tumor tissues the intracellular domain (Red) is often seen in the absence of the extracellular domain (Green) indicating that the extracellular domain of ALCAM was shed (Fig. 3C, arrow head). In gastric cancer, entire glandular structures

appear to lack staining for the extracellular domain while others are fully positive (Fig. 3Ci). This all-or-none staining for the extracellular domain suggests that ALCAM-shedding is activated at a macroscopic level within the tissue architecture of the stomach. In colorectal carcinoma tissue ALCAM staining is more heterogeneous with small populations of cells within the same histological structure exhibiting different levels of staining for the ALCAM extracellular domain (Fig. 3Cii). The irregular staining in CRC suggests that ALCAM shedding is occurring throughout the tumor but regulated at a cellular level.

Quantitative analysis of ALCAM shedding

Previous studies evaluating ALCAM as a biomarker for predicting colorectal cancer patient survival have published conflicting and inconclusive results (10–12; 18; 20). We postulate that this variability is due to ALCAM shedding, since these studies all evaluate ALCAM through detection of its extracellular domain. To visualize ALCAM shedding we used HPA010926 (Sigma, St. Louis) to detect the extracellular domain and 1G3A1 (Fig. 2) to detect the intracellular domain. Shedding of ALCAM was defined for the selected tumor area as the presence of the intracellular domain of ALCAM and the absence of the extracellular domain of ALCAM (Fig. 4). Immunofluorescent staining for each domain was completed simultaneously on sections from paraffin-embedded colorectal cancer tissue which were digitally scanned and quantitatively assessed using ImageJ (Suppl. Fig. 5, see Methods for details on procedure). Shedding was defined as the loss of the extracellular domain and retention of the cytoplasmic domain. Shedding data is presented graphically in Fig. 4A and B. Quantitatively ALCAM shedding is the fraction of detectable ALCAM from which the extracellular domain is absent ($[\text{Total ALCAM-Intact ALCAM}]/\text{Total ALCAM}$). In normal colonic mucosa, detection of the extracellular and intracellular domains overlap extensively, demonstrating the predominant presence of intact ALCAM (yellow) and little ALCAM shedding (teal) (Fig. 4A, last panel). Conversely, in tumor sections the intracellular domain of ALCAM remains detectable while the extracellular ALCAM is frequently absent, indicating that ALCAM is shed (Fig. 4B, last panel).

ALCAM shedding corresponds with reduced patient survival

Since ALCAM shedding is clearly elevated in tumors, we hypothesized that ALCAM shedding can be an accurate prognostic marker for colorectal cancer. To evaluate this, histological detection of ALCAM shedding was performed on specimens from 105 CRC patients and 12 healthy controls (see Supplemental Table II for patient demographics). For each specimen, ALCAM shedding was quantified as described for Fig. 4. For each patient, the mean value across three specimens was used to evaluate the correlation between ALCAM shedding and patient survival. ALCAM shedding is clearly elevated in tissue from CRC (Fig. 5A). Shedding is already increased in some stage I CRC patients and is significantly increased for stage II, III and IV patients. Indeed ANOVA analysis confirms significant elevation across the increasing stages (mean fraction shed = 0.64, 0.73, and 0.89, for stage II, III, and IV, Fig. 5B). Moreover, ALCAM shedding was elevated in patients that died during the course of their disease. The presence of elevated ALCAM shedding in stage II patients is particularly interesting. This led us to hypothesize that patients with elevated ALCAM shedding have a worse outcome.

To evaluate the correlation between ALCAM shedding and patient outcome, survival analysis was performed by segregating CRC patients based on ALCAM expression (Fig. 5D) using a shed fraction of 0.75 to delineate “High” vs “Low” ALCAM shedding. When analyzing the full population of CRC patients, high ALCAM shedding correlated positively with worse overall survival ($p=0.035$, Figure 5D). To determine if ALCAM shedding can be prognostic for early stage disease, a univariate survival analysis was performed specifically to stage II patients. While ALCAM shedding does not correlate with overall survival in

stage II patients (Fig. 5E), it correlates strongly with disease-related death showing significant survival benefit in the Stage II patients with low ALCAM shedding (Fig. 5F, $p=0.01$ HR=3.0).

Multivariable analysis was restricted to stage II patients since they were the emphasis of our investigation and the cohort biased to this population. Multivariable analysis with logistic regression of stage II patients found that ALCAM shedding and age at time of diagnosis were both independent predictors of overall survival after adjusting for gender and race (ALCAM shedding; adjusted odds ratio (OR), 9.972; 95% confidence interval (CI) 1.17–84.9; $p=0.035$. Age; OR, 1.079; 95% CI, 1.027–1.133; $p=0.003$). An analysis of disease-specific survival found that only ALCAM shedding was an independent predictor of survival after adjusting for age at time of diagnosis, race and gender (ALCAM shedding OR, 29.02; 95% CI, 2.165–389.08; $p=0.011$). Bootstrapping was performed as an internal validation to confirm these results and found that ALCAM shedding continued to be an independent predictor of survival in stage II patients (overall survival $p=0.002$; disease-specific survival $p=0.023$).

DISCUSSION

The main goal of this study was to determine if ALCAM shedding corresponds to patient outcome in colorectal cancer. As cancer treatments evolve towards individualized therapies, they rely increasingly on the availability of prognostic and predictive markers to determine the patient's status and facilitate treatment decisions. Some published studies evaluating ALCAM detection as a biomarker for CRC suggest clinical utility, but others have been inconclusive and contradictory (10; 11; 18–20). This ambiguity in the literature may be explained by ALCAM shedding via ADAM17-mediated cleavage (15; 29). We propose that ALCAM shedding, rather than its expression, indicates disease progression. Our dual stain reveals both intra- and extracellular epitopes of ALCAM and clarifies its prognostic value in CRC. ALCAM shedding in tissues was defined as the detection of the intracellular epitope in the absence of the extracellular domain. Using this novel assay, we demonstrate a strong correlation between elevated ALCAM shedding and poor patient outcome. Importantly, ALCAM shedding correlates with poor outcome in early stage disease (Stage II, Fig. 5). Thus, ALCAM is not merely a biomarker for disease progression but may also allow for outcome stratification among patients with early stage disease.

ALCAM was originally identified in the context of at least five distinct biologies: leukocyte activation, neuronal guidance, bone development, stem cell identification, and cancer progression (17). ALCAM expression is frequently elevated during oncogenesis. However, detection of the protein in tumor tissues is extremely variable. Knowing that ALCAM is shed by the protease ADAM17 (28), we speculated that in CRC, ALCAM is expressed and shed by ADAM17 into the circulation. Indeed, the irregular pattern of expression reported here for ALCAM was previously observed for ADAM17 in glandular tissue of early to advanced gastric cancer (36) and colorectal cancer patients (29). Unfortunately, serum levels of ALCAM in controls (cancer-free age + sex-matched individuals, 45–95 ng/ml) overlap with circulating levels in CRC patients pre- or post-therapy (45–125 and 50–110 ng/ml respectively, Fig. 1E). This compromises the accuracy and specificity of a blood test for ALCAM ((17; 37); Fig. 1). Nevertheless, the lack of specificity for tumor-derived ALCAM detection in the circulation does not negate the fact that ALCAM shedding within the tumor tissue corresponds with disease progression.

The detection of ALCAM shedding within the tumor tissue itself greatly increases the specificity of ALCAM as a prognostic factor. Our primary aim was to devise a method to stratify at-risk patients using ALCAM shedding as an indicator of disease progression and

poor patient outcome, and not as a diagnostic tool. Indeed, the strong correlation between ALCAM shedding and poor patient outcome in early stage disease suggests that molecular progression can occur in a cancer that appears histologically more benign.

An important disparity becomes apparent when we correlate clinical outcome (survival) with ALCAM gene transcription (mRNA), protein expression (based on detection of intact ALCAM) and ALCAM shedding. Elevated ALCAM transcription is associated with poor outcome yet elevated levels of intact ALCAM protein (through detection of co-localized extracellular domain and intracellular domain) is associated with improved outcome (Suppl. Fig. 6 A vs. B). This disparity could be rectified if we consider that the extracellular domain of ALCAM is shed leaving the mistaken impression that ALCAM protein is lost during tumor progression. Indeed, our analysis of ALCAM shedding (summarized in Suppl. Fig. 6C) demonstrates that ALCAM shedding rather than loss of expression corresponds with patient outcome. This observation can explain why five independent evaluations of ALCAM in CRC have given conflicting results (10; 11; 18–20).

Although ADAM17 is responsible for proteolytic cleavage of several tumor-associated proteins, few studies have analyzed ectodomain shedding for prognostic purposes. A rare study attempted to look at shedding used monoclonal antibodies specific for the cleavable form of ErbB4 (38) but was unable to look at shedding directly. The dual staining of ALCAM we presented here is then a novel approach to detect molecular behavior (shedding) rather than the molecular identity. Indeed, our results suggest that detection of molecular behavior correlates more specifically with the disease than gene expression itself. Given that the disruption of ALCAM-ALCAM interactions promotes tumor cell motility and metastasis (26), ALCAM shedding may predict malignant progression at a molecular level. The clinical correlation between ALCAM shedding and patient outcome (Fig. 5) suggests that detection of disease progression at a molecular level can predict long-term patient outcome. The presence of this correlation in early stage disease (stage II, Fig. 5) emphasizes that this molecular progression is present prior to pathological and clinical progression. Detection of this molecular progression allows for stratification of patients according to their risk for poor long-term outcome. Considering that ALCAM is altered in a number of malignancies (39–42), the clinical correlation of ALCAM shedding to patient outcome is likely to extend beyond CRC to other cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. ALCAM expression in colorectal carcinoma and its correlation to patient outcome
A) Representative immunohistochemical staining for ALCAM in normal and tumor colon sections using an antibody directed to the extracellular domain of ALCAM (HPA010926).
B) Expression of ALCAM in CRC cell lines evaluated by immunoblotting (left) and microarray analysis (right, GDS1761). Expression (mRNA) of ALCAM (**C**) and ADAM17 (**D**) in a cohort of 250 colorectal cancer patients was correlated to patient survival after dichotomizing the population across its mean expression into patients with “High ALCAM” and “Low ALCAM”. **E)** Using an ALCAM sandwich ELISA, circulating levels of shed ALCAM were evaluated in serum from cancer-free patients (n=48) and compared to serum from CRC patients at time of diagnosis (pre-op, n=42) and serum from patient at time of followup after treatment (post-op, n=19). **F)** CRC patients were stratified according to stage (I-IV) and compared to cancer-free patients (n=48) and healthy adults (n=6). Box plots show the mean, standard deviation and full range of the expression data. Survival is presented with Kaplan-Meier plots and log-rank test was used to evaluate significance.

Figure 2. Screening and validation of an antibody specific to the cytoplasmic domain of ALCAM
A) B) Individual hybridoma clones reactive to the immunizing peptide were screened by direct ELISA using whole cell lysate from a cell line expressing high levels of ALCAM (PC3). A 1.0 OD was used to select clones for further validation by immunoblotting cutoff. Three high-titre clones chosen for further validation were 1G3A1 (ii), 1G3B3 (iv), and 1G3CF (i). **B)** Hybridoma clones 1G3A1, 1G3B3, and 1G3CF were further for specificity to ALCAM by comparing their reactivity to ALCAM with a commercial antibody (directed against the extracellular domain) using lysates from parental PC3 cells and PC3 cells with shRNA-mediated knockdown of ALCAM. **C)** ALCAM specificity of individual clones was further evaluated by outcompeting antibody binding with the immunizing peptide in Immunoblots of whole cell lysates (PC3). **D)** Specificity of purified 1G3A1 against the cytoplasmic tail of ALCAM was confirmed by immunoblotting intact ALCAM (whole cell lysate; Lys.) and shed ALCAM (conditioned medium; C.M.) from PC3 cells.

Figure 3. Detection of ALCAM shedding in cancers of the stomach and colon

The ALCAM dual stain composed of the antibody HPA010926 directed against the extracellular domain (green) and antibody 1G3A1 directed against the intracellular domain (red) was used to identify locations within the tissue that exhibited ALCAM shedding in cancers of the stomach (**A**) and colon (**B**), scale bar = 64 μm . Images of nuclear stain (blue) were merged with images of HPA010926 (green) and 1G3A1 (red) staining. Insets (**C**) present magnified regions i and ii from stomach and colon carcinoma. Areas of ALCAM shedding are identified by the presence of 1G3A1 staining (red) and an absence of HPA010926 (green) staining. Arrow: intact ALCAM, Arrow Head: ALCAM shedding. Scale bar = 320 μm .

Figure 4. Quantitative analysis of ALCAM shedding in CRC

The ALCAM dual stain was used to detect the extracellular domain (green) and intracellular domain (red) of ALCAM in normal colonic mucosa (A) and colorectal cancer (B). Using computational analysis with ImageJ, intact ALCAM was defined by the co-localization of detectable intracellular domain (ICD) and extracellular domain (ECD). ALCAM shedding was defined as the detection of ALCAM-ICD in the absence of co-localized ALCAM-ECD. The results of this analysis present ALCAM shedding quantitatively as the fraction of total detectable ALCAM from which the extracellular domain is absent. This is shown graphically in the last panel with the colors yellow and teal representing intact ALCAM and ALCAM shedding respectively.

Figure 5. ALCAM shedding in CRC correlates with poor survival

ALCAM shedding was evaluated in tissue from 105 CRC patients and 12 healthy controls. Scatter plots demonstrate the differences in ALCAM shedding between normal colonic mucosa and CRC tumors as a single population (**A**), stratified across histological stage (**B**), or separated according to survival (**C**). Kaplan-Meier survival curves of CRC patients with high versus low ALCAM shedding were generated and log-rank tests performed to evaluate the correlation of ALCAM shedding with overall survival of patients at all stages (**D**), overall survival of stage II patients (**E**) or disease-specific survival of stage II patients (**F**). Scatter plots show the mean and SEM with statistical evaluation by Mann-Whitney for selected groups (A and C) or Kruskal-Wallis with Dunn's multiple comparison restricted to normal (B). Survival is presented as Kaplan-Meier plots with the log-rank test used to evaluate significance.