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Biological role of Activated Leukocyte Cell Adhesion Molecule (ALCAM) overexpression in breast cancer cell lines and clinical tumour tissue.

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Abstract

Purpose: The Activated Leukocyte Cell Adhesion Molecule (ALCAM) is overexpressed in many mammary tumours, but controversial results about its role and prognostic impact in breast cancer have been reported. Therefore, we evaluated the biological effects of ALCAM expression in two breast cancer cell lines and a larger cohort of mammary carcinomas.

Methods: By stable transfections, MCF7 cells with ALCAM overexpression and MDA-MB231 cells with reduced ALCAM levels were generated and analyzed in functional assays and cDNA microarrays. Additionally, an immunohistochemical study including 347 breast cancer patients with long-term follow-up and analysis of disseminated tumour cells (DTC) was performed.

Results: In both cell lines, high ALCAM expression was associated with reduced cell motility. Additionally, ALCAM silencing in MDA-MB231 cells resulted in lower invasive potential, whereas [high ALCAM expression was associated with increased apoptosis in both cell lines](#). Among genes which were differentially expressed in clones with altered ALCAM expression, there was an overlap of 15 genes between both cell lines, among them cathepsin D, keratin 7, gelsolin and ets2 whose deregulation was validated by western blot analysis. In MDA-MB231 cells, we observed a correlation with VEGF expression [which was validated by enzyme-linked immuno sorbent assay \(ELISA\)](#).

Our IHC results on primary breast carcinomas showed that ALCAM expression was associated with an estrogen receptor-positive phenotype. Additionally, strong ALCAM immunostaining correlated with nodal involvement and the presence of tumour cells in bone marrow. By Kaplan-Meier analysis, strong ALCAM expression in ductal carcinomas correlated with shorter recurrence-free intervals ($p=0.048$) and overall survival ($p=0.003$).

Conclusion: Our results indicate that the biological role of ALCAM in breast cancer is complex, but overexpression might be relevant for outcome in ductal carcinomas.

Introduction

Adhesion molecules enable cancer-related biological processes like survival, migration, extravasation, homing and metastasis and thus play a crucial role in cancer progression. Many adhesion molecules are characteristically dysregulated in human cancer, and the development of therapeutic anti-adhesion strategies is ongoing. Therefore, better insights into the function of proteins involved in cell adhesion are necessary.

The activated leukocyte cell adhesion molecule (ALCAM) is a member of the immunoglobulin superfamily, consisting of five extracellular IgG domains (2 V-type and 3 C₂-type), a transmembrane anchoring region and a short cytoplasmic tail. By homophilic (ALCAM-ALCAM) or heterophilic (ALCAM-CD6) ligand binding mediated by the distal domain (D1) and by lateral oligomerization mediated by the proximal C₂-type domains (D3-D5), a clustering of ALCAM molecules and formation of an ALCAM network is achieved [1]. In normal tissues, ALCAM is expressed on activated lymphocytes, neuronal cells, hepatocytes, pancreatic cells, selected epithelia (i.e. in mammary ducts and acini) as well as in embryonic cells, i.e. bone marrow, endothelial and yolk sac cells [1-3]. Moreover, it is localized at the lateral membranes of endothelial cells where it facilitates transmigration of ALCAM-positive monocytes [4].

Similar to other adhesion molecules, ALCAM binding can activate signalling pathways regulating various biological functions. Thus, ALCAM expression has been implicated in apoptosis [5], angiogenesis [6], migration and invasion [7] of various cell types. Concerning the role of ALCAM in human cancer, the results are partly controversial: In many tumours, ALCAM expression is associated with high invasive and metastatic potential and poor prognosis, i.e. in melanoma [8, 9], colorectal cancer [10], oral cancer [11], pancreatic carcinoma [12] and esophageal carcinoma [13]. In addition, metastatic clones of a mouse fibrosarcoma cell line are characterized by overexpression of ALCAM [14]. In contrast, tumour progression in prostate cancer is associated with ALCAM down-regulation

[15], and in an immunohistochemical study, loss of membrane staining was associated with an unfavourable prognosis in ovarian cancer [16].

Investigations on breast cancer samples revealed contradictory results: ALCAM mRNA overexpression was associated with better prognosis in one study [17], whereas ALCAM protein immunostaining (mainly cytoplasmic) was associated with poor survival in another cohort [18]. The lowest transcript levels were found in patients who developed bone metastasis [19]. Our own analyses suggested that ALCAM might also have a predictive value in patients treated with conventional chemotherapy [20].

In the light of these controversial results, we evaluated the role of ALCAM retrospectively in a larger cohort on a tissue microarray including 347 patients with analysis of disseminated tumour cells (DTC) in bone marrow and a median follow-up of 54.3 months, and by experimental studies using two breast cancer cell lines. Therefore, we generated stable clones with enhanced (MCF7) or reduced (MDA-MB231) ALCAM expression, which were compared to control cells in functional assays and by microarray analysis.

Materials and Methods

Cell lines and generation of stable transfectants

MDA-MB231 and MCF7 breast cancer cell lines were cultivated in DMEM/F-12 medium (Gibco/Invitrogen, Darmstadt, Germany) supplemented with 10% FCS, antibiotics and, for MCF7 cells, 10 µg/ml insulin. The authenticity of our cells was validated by DSMZ GmbH (Braunschweig, Germany) before the beginning of the experiments. For generation of stable MCF7 cell clones with increased ALCAM expression, the OmicsLink ORF Expression Clone EX-H0002-M02 (GeneCopoeia, Germantown, Maryland) which contains the full-length ALCAM ORF was transfected

into the cells, and stable transfectants were selected by addition of G418. For generation of MDA-MB231 cells with diminished ALCAM expression, the following sequences were synthesized and cloned into the pSilencerTM 3.1-H1 puro vector (Applied Biosystems / Ambion, Austin, TX, USA): 5'-GATCCAGTGTTCCTGATAGACAATTCAAGAGATTGTCTATCAGGGAACACTGGTTTTTTG-GAAA-3' (top strand) and 5'-AGCTTTTCCAAAAACCAGTGTTCCTGATAGACAAT-CTCTTGAATTGTCTATCAGGGACACTG-3' (bottom strand). After transfection and selection of puromycin-resistant clones with reduced ALCAM expression as shown in western blots, the sequences of the constructs were verified by direct sequencing. As controls, stable transfections with the appropriate control vectors EX-EGFP-MO2 (GeneCopoeia; for MCF7) or the pSilencer 3.1-H1 puro negative control vector containing a hairpin siRNA with limited homology to any known human sequences (Ambion; for MDA-MB231) were performed, and the resultant, antibiotic-resistant cells were used as negative controls for our experiments (MCF7-NC and MDA-shNC).

Immunocytochemistry

For immunocytochemistry, cells were grown on chamber slides for two days, fixed at -20°C in methanol for 10 min, then in acetone for 1 min. After rinsing in PBS, the sections were incubated with 10% horse serum (HS) containing 0.2% bovine serum albumin (BSA) for 15 min to block nonspecific binding sites, and incubated with the CD166/ALCAM specific antibody (1:300; Mab NCL-CD166, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) in PBS containing 1% HS and 0.2 % BSA for 1 h at room temperature. After washing, bound antibodies were visualized using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and counterstained with hematoxylin. Alternatively, the secondary antibody Alex488 (1:1000; Invitrogen-Molecular Probes, Karlsruhe, Germany) was applied for 60 min. Specimens were viewed and photographed either with a confocal microscope (Olympus Fluoview 1000, Olympus Deutschland GmbH Hamburg) or on a Zeiss Axioscope 40. Sections were coverslipped in ProLong Gold antifade reagent with DAPI (Invitrogen).

Functional assays

The invasive potential of the cells was tested with MatrigelTM Invasion Chambers (24-well plates; BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions with 25,000 cells per insert as described previously [21]. After 48 h incubation, the cells which had passed the 8 µm pores of the membranes were counted under a microscope in 7 identical square fields which together included most of the membrane area without the periphery. As controls, inserts without matrigel provided by the same manufacturer were used.

Motility was measured using a wound healing assay. After grown to confluence in 6-well plates, a band of cells was removed using a sterile pipet tip. Gap closure was monitored and the percentage of gap closure was estimated regarding the photographs made in regular intervals.

Apoptosis was shown by enrichment of nucleosomes in the cytoplasm of cells after addition of different concentrations of camptothecin (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions, and by M30 CytoDeath immunostaining. For the latter assay, cells were incubated with or without 0.4 µg/ml camptothecin for 16 hours. After trypsinization, cytopins with 200,000 cells per slide were prepared and fixed in solution B (Epimet, Micromet, Munich, Germany) for 10 min, washed in TBST and blocked with Protein Block (Serum-free, Dako, Glostrup, Denmark) for 20 min, before the monoclonal anti-M30 antibody (Cyto DEATH, Fluorescein; 1:200, Roche, Mannheim, Germany) was applied at RT for 60 min. After washing with PBS, slides were again incubated with Protein Block (Serum-free, Dako) for 20 min. Subsequently, the monoclonal antibody A45-B/B3 labelled with Cy3 (1:300, Micromet) was applied at RT for 45 min followed by washing with PBS. Finally slides were mounted with DAPI mounting medium. M30 staining was evaluated using a Zeiss Axioplan 2 fluorescence microscope and a 40x objective as number of apoptotic cells in at least 12 visual fields. All functional assays were performed at least 3 times.

RNA isolation and microarray analysis from cell lines

Isolation of total RNA, cDNA synthesis, labeling and hybridization to Affymetrix Human Genome GeneChip U133 Plus 2.0 were carried out as described [22]. To compare samples and experiments, the trimmed mean signal of each array was scaled to a target intensity of 200. Absolute and comparison analyses were performed with Affymetrix GCOS (version 1.4, Affymetrix) software using default parameters. For comparison analysis the implemented Wilcoxon's Signed Rank Test was used. From Wilcoxon's Signed Rank test one-sided p-values were computed for each probe set (cut off p-value: 0.003). To assist in the identification of genes that were positively or negatively regulated in the experiment, we selected genes that were increased or decreased at least 1.74 fold (SLR \neq >0.8) compared to the control.

Western blot analysis and ELISA tests

General western blot conditions have been described [22]. For ALCAM detection and validation of differentially expressed genes, the following antibodies were used: ALCAM Mab NCL-CD166 (1:200; Novocastra), rabbit anti-Ets-2 antibody C20 (1:100; Santa Cruz Biotechnology, Heidelberg, Germany), mouse anti-NCAM-L1 (1:200, Santa Cruz), mouse anti-cathepsin D (1:500; Santa Cruz), mouse anti-CK7 clone OV-TL 12/30 (1:30000, Dako, Glostrup; Denmark), and mouse anti-gelsolin antibody (1:30000; DAKO). As secondary antibodies, peroxidase-conjugated anti-mouse-IgG (1:2000) or anti-rabbit-IgG (1:4000; all from Santa Cruz) were used, which were visualized by chemiluminescence reagents (Super Signal West Pico kit, Pierce, Rockford, Ill.) with Hyperfilm ECL films (Amersham, Braunschweig, Germany). VEGF-165 in cell lysates and supernatants was quantified by commercially available ELISA (Siemens Healthcare Diagnostics, Tarrytown, USA) according to the manufacturer's instructions.

Patients and Immunohistochemistry

In a retrospective study, immunohistochemistry was performed on a Tissue Microarray (TMA) containing samples of 411 breast cancer patients who were treated at the Hamburg University Medical School between 1999 and 2006. Included were all patients from this time period, from whom bone marrow had been taken and analysed for the presence of disseminated tumour cells (DTCs) as described [23], based on availability of tumour material. The diagnosis including evaluation of histological type and grading had been performed by pathologists on whole formalin-fixed paraffin-embedded tissue sections, and routine immunohistochemical determination of ER and PR status had been performed as described [24]. For TMA generation, 0.6mm cores were taken from invasive parts of the tumours which had been selected by a pathologist. After exclusion of all cases without remaining tissue on TMA sections after IHC or without tumour cells in the 0.6mm-core, 347 cases could be evaluated.

Patient data and tumour characteristics are described in table 3. The median age of the patients was 58.1 years (range 27.5 – 85.0 years), and the median follow-up time was 54.3 months (range 0.1 – 103.5 months). Patients received endocrine treatment and/or taxane-free chemotherapy according to national guidelines. No therapy like radiotherapy or neoadjuvant chemotherapy had been performed prior to operation. Before surgery, informed consent for the scientific use of tissue materials had been obtained from the patients in coordination with the local ethics committee according to the principles of the declaration of Helsinki. REMARK criteria were used through this report [25].

Serial section of 8 µm were cut from the TMA blocks using an adhesive coated tape system (Tape-Transfer System, Instrumedics, Hackensack, New Jersey, USA), dewaxed with xylene and gradually hydrated. Heat-mediated antigen retrieval was achieved in hot 10mM citrate buffer (pH 6.0) for thirty minutes, washed and incubated in 0.5% H₂O₂-methanol solution for 30 min. For immunohistochemistry, the Vectastain Elite ABC mouse Kit (Vector Laboratories, Burlingame, CA, USA) was used according to the manufacturer's instructions with the monoclonal antibody to CD166 (ALCAM) clone MOG/07 (1:400; Vector Lab.) incubated overnight at 4°C. As negative control, the primary antibody was omitted in parallel sections, resulting in negative ALCAM results (not shown). The ALCAM immunostaining was evaluated independently in a blinded fashion by two people using the immunoreactive score (IRS) as product of staining intensity (graded between 0 and 3) and

percentage of positive cells (graded between 0 and 4; 1: 1-20%, 2: 21-50%; 3: 51-80%, 4:>80%) resulting in a score of 0-12. No differentiation between membranous and cytoplasmic ALCAM staining was done. In case of differences between results, the samples were re-viewed and a consensus was achieved.

Statistical analysis

Correlations between ALCAM protein expression and histological or clinical tumour characteristics were calculated by Chi-square tests using the SPSS 15.0 software using groups described in Table 4. For Kaplan-Meier analysis, overall survival (OAS) was computed from the date of diagnosis to the date of death due to distant metastasis. Kaplan-Meier survival curves were compared with the logrank test. Cox regression analysis was used to estimate Hazard ratios (HR) in uni- and multivariate analysis. Results of functional assays were compared by T-tests. All tests were performed at a significance level of $p=0.05$, and all p values are two-sided.

Results

ALCAM expression inhibits migration, but enhances invasion of breast cancer cells

In order to evaluate the impact of high ALCAM expression on the biological properties of mammary cancer cells, experimental studies with two breast cancer cell lines were performed. In contrast to the highly invasive MDA-MB231 cells which are characterized by strong ALCAM expression as shown in Western blots, ALCAM amounts in untransfected, weakly invasive MCF7 cells are relatively low. Therefore, MCF7 transfectants with ALCAM overexpression (clone # 1.3) as shown in western blots and two MDA-MB231 clones with strongly reduced ALCAM expression (# 14 and 9) were analyzed and compared to their respective controls. The localization of ALCAM proteins was demonstrated by immunocytochemistry. In MCF7-ALCAM cells, the adhesion molecule was localized in the

cytoplasmic membranes in zones of cell-cell contacts, but not in membranes at the outer margins of cell clusters (Fig. 1a-b). In untransfected MDA-MB231 cells, ALCAM expression was also mainly found in regions of cell-cell contacts. Only little cytoplasmic staining was observed in both cell lines (Fig. 1a-b).

For all functional tests, at least 3 experiments were performed. With respect of proliferation, the transfected clones did not differ from their parental cells (not shown). In wound healing assays, migration of MDA-MB231 cells was enhanced 2-3 fold after ALCAM silencing by shRNA transfection (Fig. 1c). Accordingly, motility of MCF7 cells was diminished by 20-30% after ALCAM overexpression (Fig. 1d). In contrast, the invasive potential of MDA-MB231 cells as shown in matrigel invasion assays was strongly reduced in cells with ALCAM downregulation leading to a 80-90% reduction of invasive tumour cells, whereas there was no significant impact of ALCAM expression on cell invasion in MCF7 cells (Fig. 1e-f). **In control inserts without matrigel, no significant influence of ALCAM expression on the number of cells passing the pores was found (not shown).**

ALCAM expression correlates with apoptosis in breast cancer cells

Apoptosis was monitored by enrichment of nucleosomes after addition of different concentrations of camptothecin and by M30 CytoDeath immunoreactivity. The M30 antibody recognizes a specific caspase cleavage site within cytokeratin 18 that is not detectable in normal epithelial cells. With both methods, the basal rate of apoptotic MCF7 cells was reproducibly higher in ALCAM transfectants compared to the control, although this difference did not reach statistical significance (Fig. 2a,c). The difference in the number of nucleosomes diminished after addition of higher concentration of camptothecin ($\geq 0.4 \mu\text{g/ml}$) (Fig. 2a) whereas the number of M30-positive cells in the presence of camptothecin was significantly higher in ALCAM transfectants (Fig. 2c). In MDA-MB231, ALCAM downregulation was associated with a reduced percentage of apoptotic cells as shown by both methods. This difference persisted after addition of different concentrations of camptothecin (Fig.2 b, d).

ALCAM influences expression of genes involved in cell invasion, adhesion and angiogenesis

For evaluation of gene expression data, a threshold expression value of 50 and a signal log ratio (SLR) of ≥ 0.8 or ≤ -0.8 was used. Using these parameters, 233 probesets (182 genes) with known function were up-regulated, and 65 genes (85 probesets) were down-regulated in MCF7-ALCAM cells. In MDA-MB231 cells, only genes that were regulated in both clones with diminished ALCAM expression were included, resulting in 156 genes (203 probesets) with decreased expression and 18 genes (20 probesets) with increased expression. Fifteen genes were **both significantly up-regulated in MCF7-ALCAM and down-regulated in MDA-shALCAM cells**. The encoded proteins include factors involved in proteolysis (cathepsin D, PRSS23), signal transduction and transcriptional regulation (amphiregulin, Runx2, Ets2, Stmn3), components of the cytoskeleton (filamin A, cytokeratin 7, gelsolin) and extracellular matrix (fibronectin), and others (Plac1, SH3BGRL, IDS, PRICKLE2, SLC16A4; tables 1-2).

Interestingly, overexpression of ALCAM results in up-regulation of additional adhesion molecules, namely NRCAM and CEACAM6 in MCF7 and L1-CAM and integrin $\beta 5$ in MDA-MB231 cells (table 2). In addition, ALCAM expression correlates with higher levels of some components of the extracellular matrix (ECM), i.e. fibronectin and different collagen isoforms. In MDA-MB231 cells, proteins involved in angiogenesis were down-regulated by ALCAM silencing (i.e. VEGF-A), and in both cell lines, regulators of apoptosis were affected by ALCAM expression. A list of the most important deregulated genes is shown in table 2.

The regulation of some ALCAM target genes was validated on protein level. By western blot analysis, the down-regulation of cytokeratin 7, cathepsin D, gelsolin, Ets2 and L1-CAM could be shown in MDA-MB231 cells, whereas only gelsolin and cytokeratin 7 up-regulation was validated in MCF7 cells (Fig. 3a). In MDA-MB231 clones with reduced ALCAM expression, both intracellular and secreted VEGF protein levels were diminished as shown by ELISA experiments (Fig. 3b). Similar to our RNA results, this could not be shown in MCF7 cells (Fig. 3c).

ALCAM overexpression is associated with poor prognosis in breast cancer patients

Since prior results concerning the prognostic impact of ALCAM expression were partly controversial, we decided to analyze ALCAM protein expression in a larger, independent cohort. The clinical and histological characteristics of the cohort (n=347) are shown in table 3. Since the subcellular localization of the immunoreactivity was not always clearly definable, we did not differentiate between membranous and cytoplasmic staining. In 74 cases (21%), no ALCAM staining was observed in tumour cells. Weak staining (immunoreactive score 1-3) was shown in 136 tumours (39%), moderate staining (IRS 4-6) in 95 cases (27%) and in 42 patients (12%), strong staining (IRS 8-12) was observed (Fig. 3a-c).

For statistical analysis, three groups of ALCAM-negative cases, tumours with weak/moderate immunoreactivity (IRS 1-6) and cases with strong staining (IRS 8-12), respectively, were generated, since tumours with weak and moderate expression behaved similar (no significant differences). Using these groups, correlations with clinical and histological parameters were evaluated. There were highly significant positive associations with estrogen and progesterone receptor status (table 4). In contrast, the correlation with histological grading showed a bimodal distribution, with an over-representation of negative or strong ALCAM staining in poorly differentiated tumours. No significant associations with tumour stage, age, or histological type were found (not shown).

In addition to lymph node involvement, the presence of disseminated tumour cells in bone marrow is a potential risk factor for metastatic relapse in breast cancer [26]. In our cohort, ALCAM overexpression correlated significantly with nodal involvement ($p=0.006$), and there was a tendency pointing to a higher rate of tumour cells in bone marrow in these patients ($p=0.084$; table 4).

By Kaplan-Meier analysis (median follow-up time 54.3 months), high ALCAM staining (IRS 8-12) was associated with shorter overall survival compared to patients with low/moderate ALCAM scores ($p=0.021$, Fig. 4). ALCAM-negative tumours had an intermediate position. In terms of recurrence-free survival, tumours with negative or weak/moderate results (ALCAM score 0-6) had a better prognosis compared to those with ALCAM overexpression, but this difference was statistically not significant ($p=0.279$).

By stratification according to histological tumour type, we could show that the prognostic impact of ALCAM overexpression was more clear-cut in ductal carcinomas (OAS: $p=0.003$; RFS: $p=0.048$; Fig.

4), whereas in other types, no significant prognostic value was found (not shown). After further stratification according to endocrine or chemotherapy, no significant differences in RFS or OAS according to ALCAM levels in the respective subgroups were found (not shown).

For univariate Cox regression analysis, patients with negative and weak/moderate ALCAM staining were combined and compared to cases with ALCAM overexpression. Thus, a significant prognostic effect of overexpression on RFS (HR = 2.47, 95% CI: 1.17 – 5.22, p=0.018) and OAS (HR = 4.32, 95% CI: 1.61 – 11.56, p=0.004) was seen in ductal carcinomas. In the total cohort, the prognostic effect was not (RFS) or only marginally significant (OAS: HR = 2.34, 95% CI: 1.01 – 5.39, p=0.047). By multivariate analysis including stage, nodal involvement, grading, ER status and the presence of DTCs, the prognostic effect of ALCAM on RFS in ductal carcinomas **curtly missed significance** (p=0.050; table 5).

Since in most studies, the median expression level was used as cut-off value, we performed the same analysis with two groups of similar size (IRS 0-2: n=174; IRS 3-12: n=173). By this approach, no significant correlations with clinical or histological parameters and disease outcome were found except an association of high ALCAM expression with nodal involvement (p=0.006; not shown).

Discussion

Although ALCAM expression has been shown in various tumour types, there is only limited knowledge about the biological role of this adhesion molecule in cancer cells. In order to evaluate the effects of ALCAM expression in breast cancer cells in more detail, we performed functional assays with two cell lines. In both MDA-MB231 and MCF7 cells, high ALCAM levels were associated with a reduced motility in wound healing assays, which corresponds to results obtained with other experimental systems: In endothelial-like yolk sac cells and human microvascular endothelial cells, ALCAM inhibited migration, but enhanced tube formation on matrigel [6]. Inhibition of ligand binding and of network formation by overexpression of a short secreted (s)ALCAM variant reverted

this effect. Thus, escape of tumour cells from the tumour mass might be facilitated by loss of ALCAM-mediated cell-cell contacts.

Interestingly, ALCAM enhanced the invasive potential of the hormone-receptor-negative MDA-MB231 cells. This corresponds to the aggressive nature of tumours with strong ALCAM immunostaining in our cohort of 347 patients, shown by lymph node involvement and shorter RFS and OAS. A role of ALCAM in cell invasion has been reported in melanoma cells where inhibition of ALCAM clustering by (s)ALCAM overexpression reduced invasion in a reconstituted skin model. Moreover, cells with (s)ALCAM expression were attenuated in metastasis frequency in a xenograft mouse model [7]. Similar to melanomas [27], ALCAM might function as a cell surface sensor in breast cancer cells which regulates dynamic responses at high cell densities leading to enhanced invasion into the surrounding tissue.

Apoptosis was positively influenced by ALCAM in both cell lines. This might result in an increased chemoresistance in these cells and corresponds well with our observations on clinical tumour tissues where high ALCAM levels are associated with better prognosis in chemotherapy-treated patients [20]. In order to identify changes in gene expression which are induced by high ALCAM levels, cDNA microarrays were performed. Among the genes which are deregulated in both MDA-MB231 and MCF7 cells, there was an overlap of 15 genes. Among those, cathepsin D might be involved in enhanced invasiveness of ALCAM-expressing MDA-MB231 cells. This aspartic protease is an independent predictor of early metastasis and shorter survival in mammary carcinomas [28, 29]. Most other genes which were deregulated in both cell lines have also been shown to be associated with tumorigenesis and tumour progression, e.g. PLAC1 [30], amphiregulin (AREG) [31], cytokeratin 7 [32], RUNX2 [33-35], STMN3 [36], gelsolin [37] and fibronectin [38, 39]. Another target gene, the transcription factor Ets2, is frequently up-regulated in breast cancer [40] [41]. The enhanced apoptotic potential of cells with high ALCAM expression is in accordance with the increased expression of some pro-apoptotic genes in both cell lines (table 2). In contrast to others [5], we did not observe an influence of ALCAM on BCL2 expression. Thus, the influence of ALCAM on apoptosis and chemoresistance should be further analyzed.

A high number of genes was deregulated in only one cell line. In MDA-MB231 cells, ALCAM silencing leads to reduced VEGF expression. A positive influence of ALCAM on angiogenesis has been shown in studies on endothelial cells [6] and in other experimental systems [42]. Moreover, comparison of gene expression profiles of ALCAM-positive, metastatic melanoma cell lines versus ALCAM-negative cell lines showed overexpression of VEGF [27]. The influence of ALCAM on cell invasion in MDA-MB231 cells also corresponds to its effect on expression of the adhesion molecule L1-CAM, which has been shown to enhance invasiveness of other cells [43, 44] and is a negative prognostic parameter in mammary carcinomas [45].

The signalling pathways which lead to changes in expression of these genes are still [unclear, and further studies are required to elucidate these mechanisms](#). In other investigations, most of the biological functions of ALCAM could be blocked by inhibition of ALCAM network formation which implies the presence and importance of membraneous ALCAM protein [7]. Whether similar pathways can also be activated by intracellular ALCAM which is observed in many tumours [18], should be further analysed.

In prior studies on the role of ALCAM in breast cancer, contradictory results indicating associations with good prognosis [17, 46] or poor outcome [18, 20] have been reported. This might be partially due to different technical or statistical approaches. In our immunohistochemical study, we differentiated between negative, weak/moderate and strong ALCAM expression, and found the worst outcome in cases with strong ALCAM staining (Fig. 3). If only two groups were analysed with the median ALCAM expression as cut-off value, no significant differences were found indicating that the results of ALCAM studies might be influenced strongly by the selected cut-off for statistical analysis.

By our approach, we could show the previously reported correlation of ALCAM expression with an ER-/PR-positive phenotype which suggests an association with a more differentiated phenotype. On the other hand, strong ALCAM expression was associated with nodal involvement and shorter recurrence-free and overall survival, especially in ductal carcinomas. The presence of disseminated tumour cells (DTC) is a surrogate parameter of hematogenous tumour cell spread [47]. We observed an association of the presence of DTC with ALCAM expression which was not significant, probably

due to the low incidence of DTC in patients with primary breast cancer applying restrictive evaluation criteria.

A potential limitation of our immunohistochemical study is that the patients were not uniformly treated and that we have no untreated cohort. Therefore, we are not able to differentiate between prognostic and predictive effects of ALCAM expression in this cohort. [In addition, the analysis of additional breast cancer cell lines would be interesting since the role of ALCAM is probably dependent on the tumour cell type.](#)

In the present study, the first functional analysis of the role of ALCAM in breast cancer cells is presented and correlated with data from human tumour tissues. In conclusion, our results indicate that the biological role of ALCAM in breast cancer cells is complex and partially dependent on the tumour cell type. The effects of strong ALCAM overexpression on the biology of breast cancer cells might include decreased motility, enhanced invasive potential, [and increased apoptosis. This might partly explain the contradictory results obtained with breast cancer samples where ALCAM was associated either with a positive or a negative prognosis \[17, 18, 20, 46\].](#) In a recent study, a human antibody phage library was screened for cancer-specific antibodies, resulting in an ALCAM-binding scFV antibody which inhibited invasiveness of MDA-MB231 cells and showed anti-tumour activity in an [in-vivo mouse colorectal carcinoma model \[48\].](#) In the light of the possible importance of ALCAM as target for cancer therapies, our results allow further insights into the biology of ALCAM in breast cancer.

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Conflict of interest

The authors declare that there are no conflicting interests.

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Figure legends

Fig. 1 Characterization of stably transfected MCF7 and MDA-MB231 cells with enhanced or down-regulated ALCAM expression, respectively. a-b Immunocytochemistry showing ALCAM expression in regions of cell-cell contacts in MDA-MB231 control cells (MDA-NC; arrows) compared with a clone with ALCAM silencing (MDA-shALC14) and membranous ALCAM staining in stably transfected MCF7 cells (MCF7-ALC1.3) compared to the negative control (MCF7-NC). Mag. 400x. c-d Influence of ALCAM expression on motility of breast cancer cells (representative experiment). Gap closure during wound healing assays was monitored at different time points in MCF7 and MDA-MB231 cells. e-f Influence of ALCAM expression on invasive potential in matrigel invasion assays..

Fig. 2 Influence of ALCAM expression on apoptosis in MCF7 and MDA-MB231 cells with enhanced or down-regulated ALCAM expression. a-b, amount of nucleosomes after treatment of the cells with camptothecin at different concentrations. Mean mean values after three independent experiments are shown. c-d, M30-positive cells with or without treatment with 0.4 $\mu\text{g/ml}$ camptothecin. Mean values of at least 12 visual fields (v.f.) in a representative experiment. In the insert in c one M30-positive apoptotic MCF7 cell (green) among several M30-negative cells is shown (800x). *, significant difference from control ($p < 0.05$)

Fig. 3 Validation of differentially expressed genes in MCF7-ALCAM and MDA-MB231shALCAM clones. a, Western blot analysis of cytokeratin 7 (CK7), cathepsin D (CSTD), gelsolin (GSN), L1-CAM and Ets2 expression. Usb, unspecific 35 kB band used as internal control, which was detected on membranes after incubation with anti-Ets2 antibodies. b-c, VEGF protein levels in cytosols and supernatants of MDA-MB231 (b) and MCF7 (c) transfectants

Fig. 4 Results of ALCAM immunostaining in 347 breast cancer samples in a tissue microarray. a-c, examples of ALCAM immunostaining. a, strong ALCAM expression (IRS 12). b, moderate staining

(IRS 6). c, negative ALCAM staining (IRS 0). d-g, Kaplan-Meier analysis showing overall survival (OAS) and recurrence-free intervals (RFS) of patients with strong ALCAM expression (IRS 8-12), weak/moderate ALCAM expression (IRS 1-6) and ALCAM-negative tumour phenotype (IRS 0). Groups were compared by log-rank tests. d-e, all patients. f-g, ductal carcinomas

Table 1. Genes deregulated by ALCAM overexpression in MCF7 and/or ALCAM silencing in MDA-MB231 cells: subdivision into functional groups

Function of gene products	MCF7-ALCAM		MDA-shALCAM	
	Increase	Decrease	Increase	Decrease
adhesion	5	1	0	11
angiogenesis	1	0	0	7
apoptosis	7	3	1	8
cell cycle	4	5	3	3
cytoskeleton	9	0	1	8
development	6	0	0	4
ECM	7	4	0	3
immune response	4	3	1	7
kinase/phosphatase	2	0	0	6
metabolism	21	11	0	20
proteolysis	9	0	0	3
signal transduction	31	6	1	14
transcriptional regulation	18	15	3	13
transport	17	9	4	16
others	41	8	4	33
total	182	65	18	156

Table 2. Differentially regulated genes in MCF7-ALCAM and MDA-MB231-shALCAM clones.

Probe Set ID	Gene Symbol	Gene Title	function	SLR MCF7-ALCAM	SLR MDA-shALCAM
Genes deregulated in both cell lines					
202458_at	PRSS23	protease, serine, 23	proteolysis	2.9 ^a	-0.9
219702_at	PLAC1	placenta-specific 1	development	1.8	-1.3
200766_at	CTSD	cathepsin D	proteolysis	1.4	-0.8
205234_at	SLC16A4	solute carrier family 16, member 4	transport	1.4	-1.1
232231_at	RUNX2	runt-related transcription factor 2	transcriptional reg. signal	1.3	-0.8
205239_at	AREG	amphiregulin /// amphiregulin B	transduction	1.3	-1.1
211719_x_at	FN1	fibronectin 1	ECM signal	1.3	-1.5 ^d
222557_at	STMN3	stathmin-like 3	transduction	1.1	-1
225968_at	PRICKLE2	prickle homolog 2 (Drosophila) SH3 domain binding glutamic acid-rich	ion binding	1.0	-0.9
201312_s_at	SH3BGRL	protein like v-ets erythroblastosis virus E26 oncogene	others transcriptional	1.0 ^a	-1.5
201328_at	ETS2	homolog 2	reg.	0.9 ^a	-0.8
202439_s_at	IDS	iduronate 2-sulfatase	metabolism	0.9 ^a	-1.0 ^a
214752_x_at	FLNA	filamin A, alpha	cytoskeleton	0.8 ^a	-0.8
209016_s_at	KRT7	keratin 7	cytoskeleton	0.8	-0.8
200696_s_at	GSN	gelsolin	cytoskeleton	0.8	-0.9
Genes involved in proteolysis					
208683_at	CAPN2	calpain 2, (m/II) large subunit	proteolysis	1.7	
201666_at	TIMP1	TIMP metalloproteinase inhibitor 1	proteolysis	1.4	
201117_s_at	CPE	carboxypeptidase E	proteolysis	1.2 ^a	
Genes involved in angiogenesis					
202510_s_at	TNFAIP2	tumour necrosis factor, alpha-induced protein 2	angiogenesis		-1.0
210512_s_at	VEGFA	vascular endothelial growth factor A	angiogenesis		-1.1 ^a
231183_s_at	JAG1	Jagged 1 (Alagille syndrome)	angiogenesis		-1.3 ^c
200878_at	EPAS1	endothelial PAS domain protein 1	angiogenesis		-1.3
Genes involved in adhesion					
204105_s_at	NRCAM	neuronal cell adhesion molecule carcinoembryonic antigen-related cell	adhesion	1.9	
211657_at	CEACAM6	adhesion molecule 6	adhesion	1.7	
203953_s_at	CLDN3	claudin 3	adhesion	-1.0 ^a	
203323_at	CAV2	caveolin 2	adhesion		-0.9
212070_at	GPR56	G protein-coupled receptor 56	adhesion		-0.9
204584_at	L1CAM	L1 cell adhesion molecule	adhesion		-0.9
201124_at	ITGB5	integrin, beta 5	adhesion		-1.1
201506_at	TGFBI	transforming growth factor, beta-induced, 68kDa	adhesion		-1.4
227566_at	HNT	neurotrimin	adhesion		-3.6
Genes involved in apoptosis					
213373_s_at	CASP8	caspase 8, apoptosis-related cysteine peptidase	apoptosis	1.4	
209406_at	BAG2	BCL2-associated athanogene 2	apoptosis	1.3	
215719_x_at	FAS	Fas (TNF receptor superfamily, member 6)	apoptosis	0.9	
204595_s_at	STC1	stanniocalcin 1	apoptosis		-1.0 ^b
201849_at	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	apoptosis		-1.1 ^a
221666_s_at	PYCARD	PYD and CARD domain containing	apoptosis		-1.3
202887_s_at	DDIT4	DNA-damage-inducible transcript 4	apoptosis		-1.3
205207_at	IL6	interleukin 6 (interferon, beta 2)	apoptosis		-1.7

206569_at	IL24	interleukin 24	apoptosis	1.5	1.4
Genes involved in extracellular matrix					
208096_s_at	COL21A1	collagen, type XXI, alpha 1	ECM	2.1	
202291_s_at	MGP	matrix Gla protein	ECM	1.4	
1553602_at	MUCL1	mucin-like 1	ECM	1.4	
227059_at	GPC6	glypican 6	ECM	1.2 ^a	
211651_s_at	LAMB1	laminin, beta 1	ECM	-1.4 ^a	
201438_at	COL6A3	collagen, type VI, alpha 3	ECM		-1.2
212488_at	COL5A1	collagen, type V, alpha 1	ECM		-1.4 ^b
204136_at	COL7A1	collagen, type VII, alpha 1	ECM		-1.0
209082_s_at	COL18A1	collagen, type XVIII, alpha 1	ECM		-1.1
Others					
223836_at	FGFBP2	fibroblast growth factor binding protein 2	signal transduction	1.3	
209687_at	CXCL12	chemokine (C-X-C motif) ligand 12	signal transduction	1.2 ^a	
211421_s_at	RET	ret proto-oncogene	signal transduction	1.0	
209956_s_at	CAMK2B	Ca/calmodulin-dependent protein kinase II beta	signal transduction	0.9	
212977_at	CXCR7	chemokine (C-X-C motif) receptor 7	signal transduction	0.9	
211237_s_at	FGFR4	fibroblast growth factor receptor 4	signal transduction	-1.0 ^b	
211959_at	IGFBP5	insulin-like growth factor binding protein 5	signal transduction	-1.4	
1555340_x_at	RAP1A	RAP1A, member of RAS oncogene family	signal transduction	-11.2 ^a	
219622_at	RAB20	RAB20, member RAS oncogene family	signal transduction		-1.0
217764_s_at	RAB31	RAB31, member RAS oncogene family	signal transduction		-1.1 ^a

SLR, signal log ratio. ^a, two probesets. ^b, three probesets. ^c, four probesets. ^d, five probesets. Differentially regulated genes which were validated by western blot analysis or ELISA are shown in bold.

Table 3. Cohort characteristics of breast cancer patients analyzed for ALCAM expression by IHC

		n=a	%
histological Type	ductal	246	70.9
	lobular	61	17.6
	ductulolobular	20	5.8
	others/unknown	20	5.8
stage	T1	180	51.9
	T2	135	38.9
	T3	17	4.9
	T4	12	3.5
nodal involvement	negative	210	60.5
	positive	136	39.2
bone marrow status	no tumour cells	256	73.8
	1-2 tumour cells	64	18.4
	> 2 tumour cells	21	6.1
distant metastasis	M0	328	94.5
	M1	13	3.7
grading	G1	23	6.6
	G2	187	53.9
	G3	130	37.5
ER status	negative	75	21.6
	positive	270	77.8
PR status	negative	116	33.4
	positive	228	65.7
type of surgery	breast conserving surgery	244	70.3
	mastectomy	97	29.0
adj. Chemotherapy	yes	223	64.3
	no	117	33.7
radiotherapy	yes	275	79.3
	no	64	18.4
hormone therapy	yes	249	71.8
	no	87	25.1
recurrence	no	277	79.8
	yes	55	15.9
died of disease	no	308	88.8
	yes	33	9.5

a: missing values to n=347 or 100%: unknown

Table 4. ALCAM protein expression in breast cancer patients: correlations with clinical and histological tumour characteristics

		n ^a	ALCAM IRS 0	ALCAM IRS 1-6	ALCAM IRS 8-12	p=
n (%)			74 (21)	231 (67)	42 (12)	
ER status	negative	75	29 (38)	34 (45)	12 (16)	<0.001
	positive	270	45 (17)	196 (73)	29 (11)	
PR status	negative	116	37 (32)	63 (54)	16 (14)	0.001
	positive	228	37 (16)	166 (73)	25 (11)	
grading	G1-2	210	37 (18)	151 (72)	22 (10)	0.066
	G3-4	130	35 (27)	78 (60)	17 (13)	
stage	T1	180	41 (23)	123 (68)	16 (9)	0.196
	T2	135	25 (19)	91 (67)	19 (14)	
	T3-4	29	8 (28)	15 (52)	6 (21)	
lymph node involvement						
	negative	210	48 (23)	146 (70)	16 (8)	0.006
	positive	136	26 (19)	84 (62)	26 (19)	
bone marrow status						
	no tumour cells	256	60 (23)	170 (66)	26 (10)	0.084
	>= 1 tumour cells	85	13 (15)	57 (67)	15 (18)	

^a, missing patients to n=347: no data. Tumours with missing values were excluded from the single analysis.

Table 5: Cox regression analysis including conventional prognostic markers, presence of DTC in bone marrow and ALCAM overexpression in ductal breast cancer

<i>Overall survival</i>			
	<i>HR</i>	<i>95% CI</i>	<i>p value</i>
ALCAM ¹	2.98	0.94-9.53	0.065
grading	1.72	0.59-5.07	0.322
stage	0.79	0.35-1.75	0.556
ER status	0.45	0.15-1.33	0.149
Lymph node status	2.62	0.88-7.81	0.084
Bone marrow status	3.68	1.40-9.67	0.008
<i>Recurrence-free survival</i>			
ALCAM ¹	2.30	1.00-5.27	0.050
grading	1.77	0.87-3.59	0.115
stage	1.12	0.67-1.87	0.673
ER status	1.57	0.68-3.66	0.294
Lymph node status	1.99	0.98-4.03	0.057
Bone marrow status	2.94	1.50-5.77	0.002

¹, two groups corresponding to IRS 0-6 and 8-12 were compared. Other groups used for analysis: see table 3.

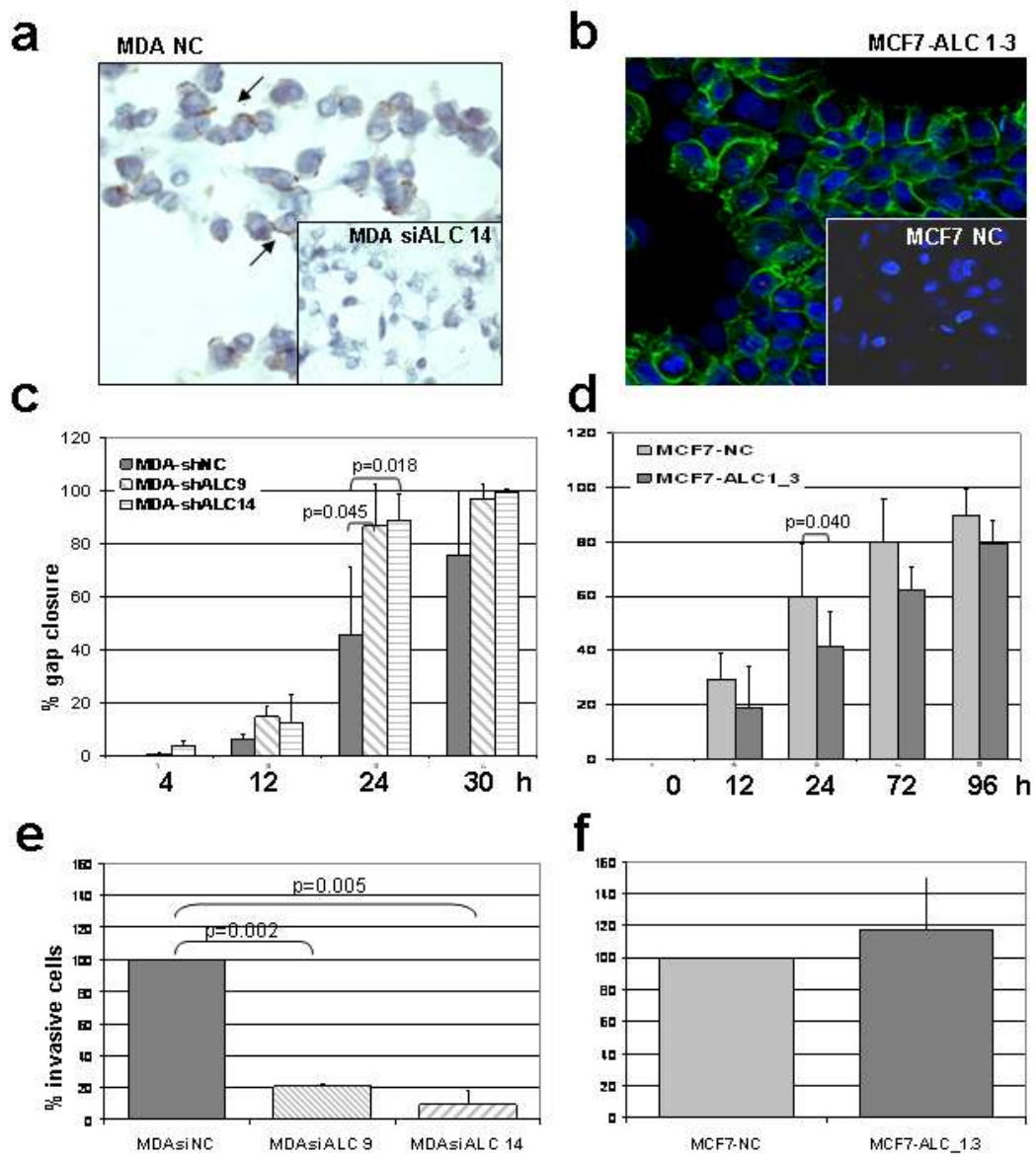


Fig.1, Hein et al.

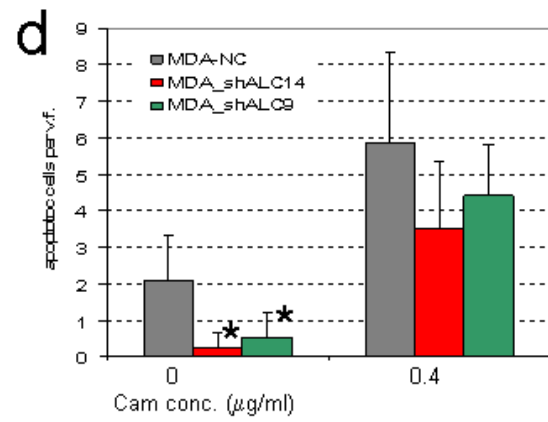
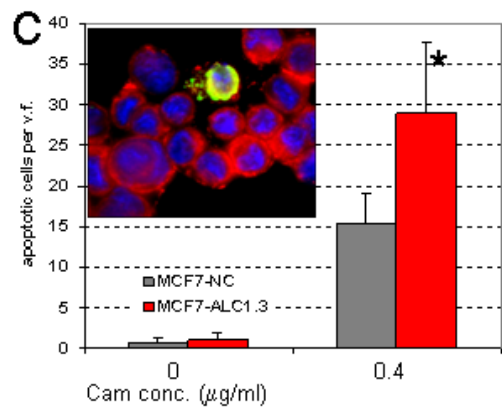
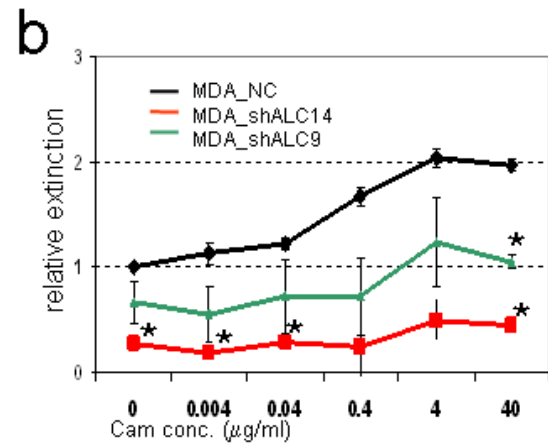
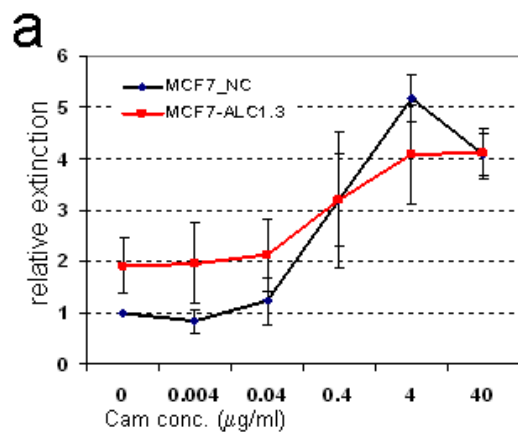


Fig. 2, Hein et al.

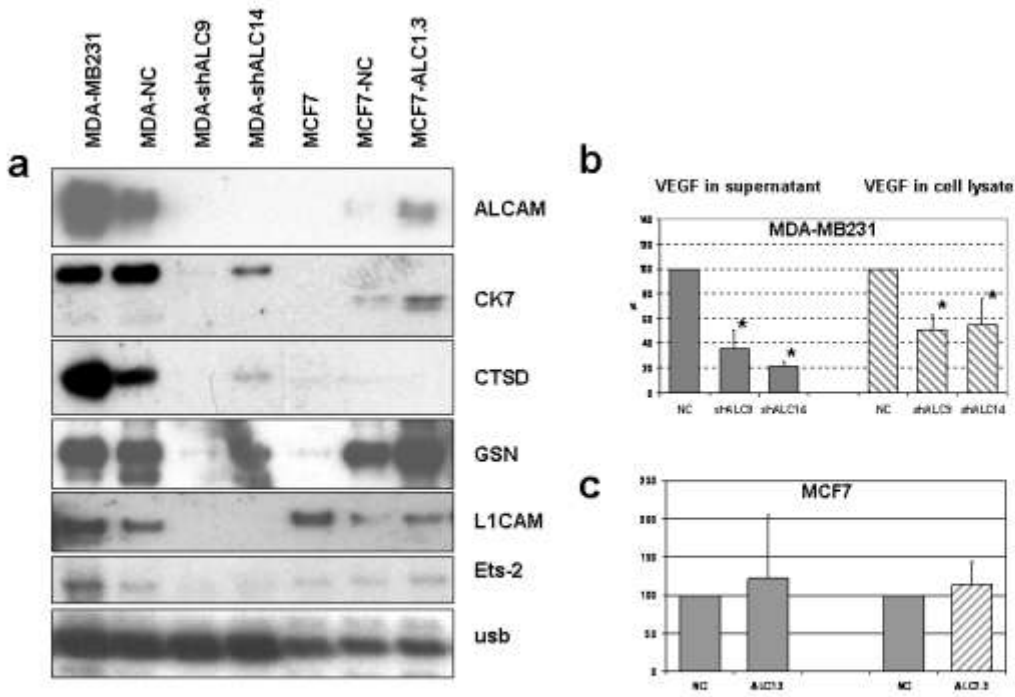


Fig. 3, Hein et al.

