CEACAM1-3S drives melanoma cells into NK cell-mediated cytolysis and enhances patient survival

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CEACAM1 splice variants in melanoma

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CEACAM1 splice variants in melanoma

Abstract

CEACAM1 is a widely expressed multi-functional cell-cell adhesion protein reported to serve as a poor prognosis marker in melanoma patients. In this study we examine the functional and clinical contributions of the four splice isoforms of CEACAM1. Specifically, we present in vitro and in vivo evidence that they impact melanoma progression and immune surveillance in a negative or positive manner that is isoform-specific in action. In contrast to isoforms CEACAM1-4S and CEACAM1-4L, expression of isoforms CEACAM1-3S and CEACAM1-3L is induced during disease progression shown to correlate with clinical stage. Unexpectedly, overall survival was prolonged in patients with advanced melanomas expressing CEACAM1-3S. The favorable effects of CEACAM1-3S related to enhanced immunogenicity, which were mediated by cell surface upregulation of NKG2D receptor ligands, thereby sensitizing melanoma cells to lysis by natural killer cells. Conversely, CEACAM1-4L downregulated cell surface levels of the NKG2D ligands MICA and ULBP2 by enhanced shedding, thereby promoting malignant character. Overall, our results define the splice isoform-specific immunomodulatory and cell biological functions of CEACAM1 in melanoma pathogenesis.
Introduction

Melanoma is one of the most aggressive types of cancer, and its prevalence has risen faster than any other malignant disease in the western world. Tumor progression starts very early, resulting in a median survival of 6-12 months in patients with advanced melanoma (1). Melanoma progression is a complex multistep process orchestrated by a variety of cellular factors, including dysregulation of cell adhesion molecules (2). Evidence has amassed that expression of the multi-functional carcinoembryonic antigen (CEA)-related cell adhesion molecule 1 (CEACAM1) may be involved during this process (3). CEACAM1 belongs to the CEA family within the immunoglobulin superfamily (4) and is expressed in human epithelial (5, 6), endothelial (7) and hematopoietic cells (8, 9). Down-regulation of CEACAM1 has been reported in colon (10), prostate (11) and breast cancer (12). Melanocytes are CEACAM negative (13), while high expression levels of CEACAM1 have been detected in melanoma (14) and adenocarcinoma (15). CEACAM1 was reported to inhibit cell proliferation in several tumor entities, excepting melanoma (13, 16, 17). On the tumor cell surface the CEACAM1 protein has been shown to interact directly with CEACAM1 on immune cells, leading to functional inhibition of those cells (18-21), or to indirectly modulate cancer cell immunogenicity by down-regulating their cell surface expression of MHC class I-related molecule A and B (MICA/B) and UL-16-binding protein1 (ULBP) (22), both ligands for the natural killer (NK) gene complex group 2 member D (NKG2D) receptor expressed on malignant cells (23, 24).

Although 12 different splice variants of the human CEACAM1 gene have been reported, only four are shown to be expressed at mRNA level (25, 26). The CEACAM1-4 variants consist of four, CEACAM1-3 of three heavily glycosylated
CEACAM1 splice variants in melanoma extracellular domains. Both isoforms are trans-membrane anchored and carry either the long (L, 73 aa) or short (S, 10 aa) cytoplasmic domain (27).

CEACAM1 has been controversially discussed as tumor suppressor and driver of invasion (28, 29). Most studies contributing to this discussion were focused on total CEACAM1 or CEACAM1-4L/S. In 2002, Thies and colleagues showed that the CEACAM1 protein expression in primary cutaneous melanoma predicts the development of metastatic disease (30). Furthermore, levels of soluble CEACAM1 in sera from melanoma patients have been shown to inversely correlate with overall survival (31, 32). This spurred discussion of CEACAM1 as a more specific and sensitive biomarker than those currently used, including melan-A, S100β and HMB45, and has implicated CEACAM1 as a potential novel therapeutic target (14). Nevertheless, none of these studies addressed the impact of the four CEACAM1 splice variants.

Furthermore, the clinical impact and the precise mechanism by which the four CEACAM1 variants modulate melanoma progression is completely unknown. Our present study analyzed the biological function and clinical relevance of the individual CEACAM1 splice variants in human melanoma biopsies of increasing disease stages and in cell lines, established from patient’s metastases. We show for the first time that CEACAM1-3S, CEACAM1-3L, CEACAM1-4S and CEACAM1-4L differentially impact cellular function and melanoma progression. Furthermore, we demonstrate that the expression of CEACAM1-3S correlates significantly with the clinical stage and strikingly with a prolonged patient overall survival. Finally, we provide strong evidences that CEACAM1-3S triggers melanoma cells for NK cell-mediated cytolyis by up-regulating cell surface expression of MICA and ULBP2, whereas CEACAM1-4L
CEACAM1 splice variants in melanoma causes the contrary effect due to enhanced shedding of both NKG2D ligands (NKG2DLs).
Material & Methods

Some Materials and Methods are detailed in the Supplementary Data.

Tissues & cell culture

Malignant melanoma cell lines and biopsies were obtained from the Skin Cancer Biobank (SCABIO) of the Dermatology Department, University Hospital Essen, Germany. Informed patient consent and the appropriate institutional review board approval were obtained for all patients. Clinical information including age, gender, stage of disease, tumor load and survival time was documented and retrieved from the electronic database (Achiver Anyware Medical, Achiver Software). Disease staging was performed according to the staging criteria of the American Joint Committee on Cancer (AJCC) (44). Cell lines were established from malignant melanoma as described before (45, 46). RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1% Penicillin/Streptomycin and 1% L-Glutamine (all from PAA Laboratories) was used as culture medium. All cell lines were maintained at 37°C and 5% CO₂ and regularly tested for mycoplasma infection.

Plasmid constructs and transfection

The coding sequences for CEACAM1-3L (NM_001184813.1), CEACAM1-3S (NM_001184816.1), CEACAM1-4L (NM_001712.4) and CEACAM1-4S (NM_001024912.2) were cloned into the pcDNA3.1(-) Neo plasmid (Invitrogen) and verified by sequencing. Constructs were transfected into the Ma-Mel-86a cell line using Metafectene (Biontex) according to manufacturer’s protocol, and single clones selectively grown in medium containing 1mg/ml G418 (Carl Roth).
xCELLigence System
The Real-Time Cell Analyzer System was used to analyze cellular functions (47) and cytotoxicity (48). The experiments were performed as described by manufacturer’s instructions. In short, the half maximum cell index (Cl50) between 0 to 70 hours (Cl50) was used for determination of statistical differences calculated by Student’s t-test. Cytotoxicity results are presented as percentages of cytolysis determined from normalized cell index (nCl): % of specific lysis= [nCl(no effector)-nCl(effector)]/nCl(no effector) x100. Experiments were performed in duplicates.
Please refer to the detailed Supplemental Experimental Procedures.

Immunofluorescence
Melanoma cells on coverslips were grown to 90% confluency. Cells were fixed either with methanol or 4% PFA, blocked with 3% BSA/PBS, then incubated at 4°C overnight with primary antibodies in 1.5% BSA/PBS. Primary antibodies were visualized by fluorescent labeled secondary antibodies. Nuclei were counterstained with DAPI (Carl Roth), and fluorescence microscopy was conducted on a Leica SP8 confocal microscope (Leica), Zeiss AxioObserver.Z1 with Apotome and Zeiss ELYRA PS.1 using SIM technology (Zeiss). Antibody information are provided in Supplementary Material and Methods section.

Flow cytometry
Surface expressions were analyzed using a FACScalibur flow cytometer and the CellQuest Pro software (BD Biosciences), Gallios system (Beckman Coulter) equipped with FlowJo software and Accuri C6 flow cytometer (BD Biosciences). Cells were harvested, incubated with primary antibodies and fluorescence conjugated
CEACAM1 splice variants in melanoma secondary antibodies. Isotype matched control antibody staining served as negative control. Dead cells were excluded from measurements by Propidium Iodide staining. Antibody information are provided in Supplementary Material and Methods section.

**MICA ELISA**

Soluble MICA (sMICA) was quantified, using the DuoSet® ELISA Development System (R&D Systems) according to the to manufacturer’s protocol. Melanoma cells were starved for 24h. Supernatant. was collected and centrifuged to remove cellular debris. Levels of sMICA were normalized to cell number at time of harvesting.

**Enrichment of primary polyclonal NK cells**

CD3⁻CD56⁺ NK cells were enriched from PBMCs of healthy donors using MACS technology (Miltenyi Biotec) according to manufacturer’s protocol. Enrichment of CD3⁻CD56⁺ NK cells was confirmed by flow cytometry and ranged between 90-95%. NK cells were cultured in the presence of 200 IU/ml IL-2 (Chiron) prior to analyses.

**Facs-based cytotoxicity assay**

To determine specific lysis of melanoma cells by NK cells flow cytometric analysis was performed (49). In brief, melanoma cells (5x10⁶ cells/ml) were labeled with 2 nM CFSE for 10 min (Invitrogen). Then antibody pre-treated NK cells were added to 5x10⁶ of CFSE-labeled melanoma cells at various effector to target ratios for 3 h. 7-AAD (Cayman Chemicals) was added to each sample according to manufacturer's protocol. Probes were measured directly in a Gallios flow cytometer (Beckman Coulter). Gating strategy was used as described in Heinemann et al., 2012 (49).

**Statistical analysis**
Quantitative expression values between two groups were compared using Wilcoxon-Mann-Whitney tests. In the case that comparisons were to a group with no observable expression values (stage I/II of CEACAM1-3S), we applied the one-sided t-test (against an expected value of 0). We used Student’s t-test to compare two experimental conditions in functional experiments. Spearman correlations (ρ) were used to assess associations between gene expression levels and tumor stages. Time-to-event data (from melanoma diagnoses till death or the last observation in case of censoring) were displayed using the method of Kaplan-Meier. Given the problems of selecting an appropriate cut-off / cut point value which is often done data-driven, we decided to display our results for the “presence of any transcripts” in contrast to “no transcripts”. Subgroups in Kaplan-Meier plots were compared using log-rank statistics; in addition, we used cox regression to the related estimate hazard ratio. All reported p-values are nominal and two-sided. We applied a significance level of 5% and did not adjust for multiple testing.
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Results

Analysis of CEACAM1 variants in malignant melanoma identified correlation of CEACAM1-3S with patient clinical stage and overall survival

Total CEACAM1 expression has been shown to be significantly higher in melanomas compared to benign nevi (33). To examine the presence of CEACAM1-3S, CEACAM1-3L, CEACAM1-4S and CEACAM1-4L in melanoma, we analyzed 46 cell lines established from human melanoma metastases using RT-PCR. We detected CEACAM1 variant expression in 33 of 46 cell lines (72%, Supplementary Table S1). Remarkably, all CEACAM1-positive cell lines expressed CEACAM1-4L in combination with other variants. We assessed relative variant expression by densitometric quantification. For each case (both CEACAM1-4 and CEACAM1-3), the longer (L)-variant was expressed at a higher level in the melanoma cell lines (Supplementary Table S1).

Next we assessed the impact of CEACAM1 splice variants during melanoma progression, by analyzing variant expression in 51 biopsies from melanoma patients that spanned stages I-IV according to the American Joint Committee on Cancer (AJCC). First, in 45 of 51 (88%) biopsies we observed CEACAM1 variant expression (Table 1, Supplementary Table S2). Surprisingly, both CEACAM1-3 variants were absent in stage I/II melanomas, with only one exception that expressed CEACAM1-3L (Fig. 1A, Supplementary Table S2). CEACAM1-3S and CEACAM1-3L expression was induced during melanoma progression, and significantly positively correlated with clinical stage, reaching the highest expression levels in stage IV tumors (CEACAM1-3S stage III: \( p=0.031 \), stage IV: \( p=0.002 \); CEACAM1-3L stage III: \( p=0.002 \), stage IV: \( p<0.0001 \) versus stage I/II; Fig. 1A). Furthermore, expression of both CEACAM1-3 isoforms was even higher in late stage melanoma (stage IV).
CEACAM1 splice variants in melanoma compared to stage III biopsies (CEACAM1-3S p=0.004; CEACAM1-3L p=0.015, Fig. 1A). CEACAM1-4S expression was detected in 40 of 51 (78%) biopsies but did not vary according to stage (Fig. 1A, Supplementary Table S2). CEACAM1-4L expression was detected in all CEACAM1-positive patient biopsies (88%) (Supplementary Table S2) and was significantly induced during early disease progression from stages I/II to stage III (p=0.030, Fig. 1A). Moreover, CEACAM1-4L was either expressed alone or in combination with other isoforms, while no other isoform was expressed without CEACAM1-4L (Supplementary Table S2). In correspondence to our in vitro observations, CEACAM1-3S exhibit the lowest expression intensities while CEACAM1-4L was predominantly expressed (Fig. 1A, Supplementary Table S2).

Then we applied the CEACAM1 isoforms data to analyze the prognostic power. Surprisingly, patients with advanced melanomas expressing CEACAM1-3S showed significantly (p=0.039) prolonged overall survival compared to patients with melanomas lacking CEACAM1-3S expression (hazard ratio: 0.43; 95% confidence interval [0.19…0.98], Fig. 1B), whereas expression of CEACAM1-3L, CEACAM1-4S and CEACAM1-4L did not affected the clinical outcome (data not shown).

**CEACAM1 variants localize to different intracellular compartments in melanoma cells**

To explore CEACAM1 isoform functionality, we stably transfected CEACAM1-3S, CEACAM1-3L, CEACAM1-4S or CEACAM1-4L, respectively, into the CEACAM negative cell line Ma-Mel-86a, established from a stage III metastases (Fig. 2). Empty vector Ma-Mel-86a-transfection served as a negative control. Expression of
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Exogenous CEACAM1 isoforms were identified by RT-PCR (Fig. 2A), western blot (Fig. 2B) and flow cytometry (Fig. 2C).

Next we investigated the subcellular localization of each CEACAM1 isoform using immunohistochemistry. Beside their surface expression, the CEACAM1-3S and CEACAM1-3L isoforms were primarily detected in vesicle-like structures that accumulated around the nucleus (Fig. 3A, I and II), while the CEACAM1-4S and CEACAM1-4L isoforms were predominantly localized to sites of cell-cell contact on the cell surface (Fig. 3A, III and IV, arrow heads). No CEACAM1 staining was detectable in control cells transfected with empty vector (data not shown). Interestingly, CEACAM1-3S- and CEACAM1-3L-positive vesicle-like structures were arranged like a “string of pearls” orientated towards sites of cell-cell contact (Fig. 3A, I and II, arrows), pointing to a recruitment of both variants to areas of cellular interactions. To analyze how this linear orientation is achieved, CEACAM1-3L and CEACAM1-3S transfectants were immunostained for total CEACAM1 and F-actin. Co-staining of CEACAM1-3S transfectants revealed that CEACAM1-positive vesicular structures associated with F-actin fibers in cell protrusions extending towards contact points with adjacent cells (Fig. 3B). Similar observations were made in CEACAM1-3L transfectants (data not shown). Taken together, all CEACAM1 isoforms were predominantly expressed at cell-cell contacts, but cellular localization of the CEACAM1 isoforms varied and was dependent on the presence of the extracellular A2 domain.

CEACAM1 isoforms differently impact migration and invasion of melanoma cells
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CEACAM1-4L expression has been shown to enhance the migratory capacity and invasive potential of melanoma cells without affecting cell proliferation (13). We found that expression of the different CEACAM1 isoforms transfected into Ma-Mel-86a did also not alter proliferation (data not shown). To investigate the specific contributions of each CEACAM1 isoform to cellular motility, we monitored the CEACAM1 transfectants in real time by using xCELLigence impedance measurement. Expression of CEACAM1-4S (p=0.009) and CEACAM1-4L (p=0.025) had the strongest migration enhancing influence on melanoma cells, compared to control cells (Fig. 4A and B). Also CEACAM1-3L significantly increased migratory capacity, but to a lesser extent (p=0.023, Fig. 4A and B). In accordance to this finding expression of CEACAM1-4S (p=0.006) and CEACAM1-4L (p=0.005) resulted in enhanced invasive behavior, which was only slightly increased by CEACAM1-3L (Fig. 4C and D). Interestingly, CEACAM1-3S expression significantly decreased both cell migration (p=0.031, Fig. 4A and B) and invasion (p=0.020, Fig. 4C and D) compared to control cells. Analyses of a second set of independently generated CEACAM1 transfectants confirmed these results (data not shown).

Now we hypothesized that activity of matrix metalloproteinases (MMPs), enzymes known to be key regulators of invasive potential in melanoma cells (34, 35) could be involved in modulating the cellular function of the individual CEACAM1 isoforms. Consequently, we treated our CEACAM1 transfectants with the MMP inhibitor Marimastat (36-38), and re-assessed invasive capacity after inhibition of MMP activity. In accordance to our hypothesis, Marimastat treatment impaired the invasive promoting effect of CEACAM1-4S or CEACAM1-4L (Fig. 5).
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**CEACAM1-3S and CEACAM1-4L direct tumor immunogenicity by deregulating MICA and ULBP2 expression**

It has been reported that the CEACAM1 expression influences the immunogenicity of cancer cells by modulating the surface expression of NKG2D ligands. These data prompted us to analyze the impact of the four CEACAM1 isoforms on the expression of NKG2D ligand MICA and ULBP2 by flow cytometry. Whereas expression of CEACAM1-3L or CEACAM1-4S did not modulate MICA and ULBP2 expression, significant upregulation of MICA (p=0.006) and ULBP2 (p=0.011) on CEACAM1-3S transfectants was detected compared to control cells (Fig. 6A and B). Remarkably, expression of CEACAM1-4L resulted in damped cell surface expression of MICA (p=0.020) without affecting ULBP2 levels (Fig. 6A and B).

To verify if CEACAM1-3S indeed affected cell surface expression of NKG2D ligands, MICA and ULBP2 expression was determined by confocal microscopy. Expression of CEACAM1-3S resulted in enhanced recruitment of MICA and ULBP2 to the Ma-Mel-86a-cell surface (Fig. 6C). Parallel we analyzed ligand expression levels in the CEACAM1 transfectants using western blot. MICA and ULBP2 expression in whole-cell lysates of CEACAM1-3L, CEACAM1-3S and CEACAM1-4S transfectants was consistent with the flow cytometric data (Fig. 6D). Furthermore, we confirmed that CEACAM1-3S transfected cells upregulated MICA and ULBP2 expression (Fig. 6D). Unexpectedly, CEACAM1-4L expression did not alter the total protein expression of MICA and enhanced ULBP2 protein expression compared to control cells (Fig. 6D). These data did not correlate with our flow cytometric findings, unless the reduced MICA and ULBP2 surface levels in CEACAM1-4L positive cells would appear due to shedding. Accordingly, we examined the levels of soluble MICA (sMICA) and ULBP2 (sULBP2) in media conditioned by the CEACAM1 transfectants using western blot,
CEACAM1 splice variants in melanoma and exclusively detected enhanced amounts of sMICA and sULBP2 in conditioned medium obtained from CEACAM1-4L-expressing cells (Fig. 6E). Quantification of sMICA by ELISA revealed an approximately 2-fold elevation (p=0.033) in conditioned medium from CEACAM1-4L-expressing cells compared to control cells (Fig. 6F). Noteworthy, Marimastat treatment reduced the level of soluble MICA (p=0.0034) to the control level (Fig. 6G).

Next we asked, whether the ~2-fold induction of MICA and ULBP2 on the surface of CEACAM1-3S-expressing cells would impact melanoma cell sensitivity to NK cell-mediated recognition and cytolysis. Thus, the cytolytic activity of NK cells was measured by a CFSE/7-AAD cytotoxicity assay. As shown in Fig. 6H and 6I the sensitivity of CEACAM1-3S-expressing melanoma cells to NK cell-mediated cytolysis was proportional to MICA and ULBP2 cell surface expression. Cytolysis of CEACAM1-3S cells were significantly enhanced compared to control cells at an effector to target ratio of 10:1 (p=0.031, Fig. 6I) with a corresponding outcome using a 5:1 ratio (data not shown). Blocking the NKG2D receptor by antibody significantly reduced NK cell-mediated cytolysis (control, p=0.0113; CEACAM1-3S, p=0.0085) compared to cultures to which isotype control antibody was added (Fig. 6I).

To analyse whether the reduced MICA-surface level on CEACAM1-4L transfectants could dampen the NK cell-mediated killing we performed xCELLigence based cytotoxicity assays. Expression of CEACAM1-3S resulted in significant up-regulation of NK cell-mediated cytolysis compared to control transfectants (Supplementary Fig. S1A, p=0.0016). The specific lysis of control (p=0.0257) and CEACAM1-3S transfectants (p=0.0137) was reduced by blockage of NKG2D (Supplementary Fig. S1A). The expression of CEACAM1-4L revealed no significant modulatory effect although blockage of NKG2D significantly reduced cytolysis (p=0.0141,
CEACAM1 splice variants in melanoma Supplementary Fig. S1A). Nevertheless, data presented in Supplementary Fig. S1A are averaged values of 5 independent experiments. Therefore NK cells of 5 different donors have been used. Notably, two experiments showed reduced killing, two enhanced killing and one no alteration of the NK cell-mediated killing if CEACAM1-4L was expressed.

Furthermore, we validated the expression of the NKG2D ligand ULBP1 and ULBP3, the DNAM-1 ligands CD112, CD155 and ligands for Nkp30 and Nkp46 receptors on CEACAM1 transfectants and control cells by flow cytometry (Supplementary Fig. S2). We found enhanced expression for CD155 in Ma-Mel-86a-CEACAM1-3S (p=0.0124), whereas expression of CEACAM1-4S (p=0.0442) and CEACAM1-4L (p=0.0117) resulted in decreased expression (Supplementary Fig. S2A, I). Also CD112 was found to be reduced in CEACAM1-4S (p=0.0169) and CEACAM1-4L (p=0.0424) transfectants (Supplementary Fig. S2A, II). Nkp30 and Nkp46 did not show altered expression (Supplementary Fig. S2A, III and IV). Based on these findings we focused the cytotoxicity assays on CD155. Blocking the NKG2D receptor, the DNAM-1 receptor and both on NK cells before co-culture with the CEACAM1-3S transfectant and control cells, respectively (Supplementary Fig. S2B). First, 2h after NK cell addition (E/T=1:1) anti-NKG2D decreased the lysis of CEACAM1-3S transfectant (6.6%), and to lesser extend the vector control (1.7%). This confirmed our prior observation (Fig.6 H). We observed similar effects utilizing anti-DNAM-1, resulting in reduced cytolysis of CEACAM1-3S (15%) and control (5.1%) transfectants under corresponding conditions. Nevertheless, simultaneous blockage of NKG2D and DNAM-1 resulted in synergistic effects and reduced the NK cell-mediated lysis of both target cell lines to a comparable level (CEACAM1-3S: 26%; control: 21.6%, Supplementary Fig. S2B, 2h after NK cell addition, E/T=1:1).
Discussion

Tumor progression is a complex multistep process orchestrated by a variety of cellular factors, such as cell adhesion molecules, but also influenced by host-derived microenvironmental cell populations including cells of the immune system. Most recent studies discussed CEACAM1 as a novel promising target for immunotherapy of malignant melanoma patients (3, 39).

Here we report CEACAM1-3S, CEACAM1-3L, CEACAM1-4S and CEACAM1-4L expression in melanoma cell lines and biopsies. The isoform expression pattern differed during malignant progression. Early in tumor establishment (stage I/II), CEACAM1-4L and CEACAM1-4S were exclusively expressed in 50% of melanoma biopsies, whereas CEACAM1-4L was expressed to a higher extend in metastatic melanoma compared to primary tumors. Interestingly, CEACAM1-3L was predominately and CEACAM1-3S was solely expressed in progressed melanoma (stage III/IV). Previously, Gambichler and colleagues reported elevated CEACAM1 expression in melanoma compared to benign nevi (33). The data we present suggest, that the enhanced CEACAM1 expression levels observed by Gambichler et al. in primary melanomas were due to changes in the expression of CEACAM1-4S/L. Thus they seemed to have underestimated the impact of individual biological functions of the four CEACAM1 isoforms on melanoma progression and metastasis whose importance has been implicated by our study. The lack of CEACAM1-3S expression in early melanoma (stage I/II) followed by the dramatic increase in de novo expression for both CEACAM1-3 variants (86%) in later stages (stage III/IV) identified CEACAM1-3 and, in particular, CEACAM1-3S as potential novel biomarker for disease progression. This finding, however, requires a prospectively planned longitudinal evaluation.
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Cellular functions of CEACAM1 require its localization at the cell surface and CEACAM1 has been shown to be recruited to sites of cell-cell contacts (40). Interestingly, by selective expression of individual CEACAM1 isoforms in melanoma cells, we identified variant-specific cellular localizations that are determined by the extracellular domain. However, CEACAM1-4 variants were primarily membrane-associated, whereas CEACAM1-3 isoforms were predominantly localized in to vesicular-like structures that accumulated around the nucleus. Schumann et al. (41) has previously reported that CEACAM1-S binds to F-actin. Together with our data, this leads to the speculation that CEACAM1-3L- and CEACAM1-3S-positive vesicular structures move towards sites of cell-cell contact via association with actin fibers. No evidence currently exists in the literature to identify which specific motor proteins would mediate this process. Sadekova et al. described localization of CEACAM1 in lamellipodia, suggesting a potential role in cell motility (42). However, it is still not understood if and how the extracellular domains of CEACAM1 affect downstream effectors, which modulate subsequently the localization of CEACAM1.

Early studies reported enhanced cell migration and invasion after transfection of CEACAM1-L into human melanoma cells, whereas, overexpression of the CEACAM1-S variant had no effect (13). Contrary to this report, our data precisely show that CEACAM1-4L and CEACAM1-4S trigger the migratory capacity and invasive potential of melanoma cells, whereas CEACAM1-3S diminishes these cellular properties. In this context, Ebrahimnejad et al. has proposed that Tyr-488 within the ITIM domain of the CEACAM1 long cytoplasmic domain is essential for the invasive and migratory effect in CEACAM1-transfected melanoma cells (13). The fact that our study show that the expression of CEACAM1-3L (also containing Tyr-488) influenced migratory and invasive potential less strongly than CEACAM1-4L and CEACAM1-4S, argues against the hypothesis. Moreover, the here shown
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contradictory effects of CEACAM1-4S and CEACAM1-3S on cell motility, implicate that the modification of function occurs independent of the long cytoplasmic domain. To our knowledge, this is the first report showing that the extracellular domain of CEACAM1 has the potential to modulate cellular functions. The migratory behavior and invasive potential of cancer cells is, of course, multifaceted and strongly influenced by the tumor microenvironment. In this context, our in vitro experiments implicated the involvement of MMPs in regulating CEACAM1-4L- and CEACAM1-4S-mediated invasion.

Recently, it was shown that CEACAM1-3L and CEACAM1-3S dampen antitumor immunity by down-regulating the surface expression of ligands for the activating NK cell receptor NKG2D in colon cancer cells (22). Contrastingly, our data clearly point to a differential isoform-specific function of CEACAM1 in melanoma cells. In our experiments, CEACAM1-3S expression triggered enhanced expression of the NKG2D ligands MICA, ULBP2 and DNAM-1 ligand CD155 on the melanoma cell surface, which drove these cells to NK cell-mediated cytolysis. In contrast, expression of CEACAM1-4L resulted in reduced cell surface expression of MICA, ULBP2, CD155 and CD112 whereas the lower surface expression of NKG2DLs was mediated by enhanced shedding of these ligands.

Our differing data on the impact of CEACAM1-4L for NK-cell mediated tumor cell cytolysis could be caused by donor specific variations. In line with this idea, Markel et al. presented data that melanoma patient-derived NK cells show an irregular phenotype and lower levels of NKp46, NKp30 and CD16 while the expression NKG2D was not altered (31). Furthermore, they showed that NK cells from healthy donors behaved differently than patient-derived NK cells. Thus, utilizing melanoma patient-derived NK cells expressing increased amounts of CEACAM1 on their cell
CEACAM1 splice variants in melanoma surface, could result in stronger interaction with CEACAM1-4L on melanoma cells (Fig. 3 A) and consequently show higher susceptibility to CEACAM1-mediated inhibition of NKG2D-triggered lysis.

Interestingly, treatment with Marimastat reverted the soluble level of MICA in medium conditioned by CEACAM1-4L-expressing melanoma cells almost back to the level of control cells. These data implicate a possible involvement of MMPs in the regulation of NKG2D ligand shedding by CEACAM1-4L in melanoma cells. Data from Liu et al. strengthen our hypothesis. They could show that matrix metalloproteinases mediates MICA shedding independent of disintegrin; other MMPs and ADAMs (43). Our data implicate the possibility that CEACAM1-4L expressing melanoma cells reduce the tumor-immune response by MMP-mediated shedding of NKG2DL but further studies are clearly needed to unravel the involvement of all the players in this process. Taken together our results provide for the first time evidence that the expression of CEACAM1-3S, CEACAM1-3L, CEACAM1-4S and CEACAM1-4L differentially impact disease progression in malignant melanoma. Importantly, we clearly demonstrated that CEACAM1 variants differentially modulate attenuation of immune surveillance by regulating ligand expression for the NKG2D receptor on tumor cells in malignant melanoma. These findings are of major interest, since CEACAM1 is discussed for use in clinical applications of immunotherapy. Caused by the fact that CEACAM1-3S potentially inhibits melanoma cell migration and invasion, and drives these cells through NK cell-mediated cytolysis, whereas CEACAM1-4L is acting in the opposite direction by supporting tumor progression, it is indispensable to define the impact of different CEACAM1 variants to secure therapeutic efficiency. Hence, determination of all four CEACAM1 isoforms opens up new possibilities for diagnosis and prognosis of melanoma patients.
Acknowledgements

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CEACAM1 splice variants in melanoma


CEACAM1 splice variants in melanoma


Table 1

Characteristics of melanoma patients

<table>
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<th>Biopsies positive for specific CEACAM1 splice forms</th>
<th>Total (N=51)</th>
<th>CEACAM1-3S (N=26)</th>
<th>CEACAM1-4S (N=40)</th>
<th>CEACAM1-4L (N=45)</th>
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<td>CEACAM1-4L</td>
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* according to AJCC criteria 2010

Abbreviations: y, years; mm, millimeter; N, number of biopsies
Figure legends

Figure 1

CEACAM1-3S expression correlates with clinical stage and overall survival in melanoma patients. (A) Scatter plot for expression of indicated CEACAM1 splice variant relative to total CEACAM1. Patients are grouped according to their clinical stages, based on the AJCC system (stage I/II, N=8; stage III, N=21; stage IV, N=22). Mann-Whitney Test; (°) one-sampled t-test to hypothetical value = 0; *p<0.05, **p<0.01, *** p<0.001; Error bars, mean ± SEM. (B) Kaplan-Meier curve of patients (N=51) with (N=26, solid line) or without (N=25, dotted line) CEACAM1-3S expression. Short vertical lines represents censored observations. Statistical significance was calculated by log-rank test.

Figure 2

Transfectants of the human melanoma cell line Ma-Mel-86a show specific expression of CEACAM1 splice variants. (A) Expression of CEACAM1-4L, CEACAM1-4S, CEACAM1-3L and CEACAM1-3S transfected into Ma-Mel-86a was analyzed by RT-PCR. Multiple PCR products result from the lack of the A2 domain and the different length of the cytoplasmic domains (CEACAM1-4L=779 bp; CEACAM1-4S=726 bp; CEACAM1-3L=491 bp; CEACAM1-3S=438 bp). Cells transfected with empty vector served as controls. (B) Western blot analyses of the CEACAM1 transfectants. Beta-actin served as loading control. (C) Flow cytometric analyses of CEACAM1 cell surface expression. Cells were stained for CEACAM1 (thick line) and for isotype-
CEACAM1 splice variants in melanoma matched control (thin line). Numbers indicate MFI values. For A-C one representative experiment out of three are shown.

**Figure 3**

CEACAM1 splice variants localize to different cellular compartments. (A) Immunofluorescence-based assessment for cellular localization of CEACAM1 (green) in the indicated CEACAM1 isoform transfectants. Nuclei were counterstained using DAPI (blue). White arrow heads, CEACAM1 staining at cell-cell contact sites; white arrow, CEACAM1 positive vesicles arranged in a line towards cell-cell contacts. (B) Double staining for total CEACAM1 (mAb 4/3/17) (green) and F-actin (Phalloidin, red) in CEACAM1-3S transfectants, nuclei were counterstained using DAPI (blue). Similar results were observed in CEACAM1-3L cells (data not shown). Representative images show maximum z-projection of confocal optical sections. Analyses were repeated three times by using different passages of each transfectants. Scale bar, 10µm.

**Figure 4**

CEACAM1 splice variants differentially affects cell motility. (A-D) Functional analysis of CEACAM1 transfectants using the real-time cell analyzer system (xCELLigence). Verification of migration (A+B) and invasiveness (C+D) of each CEACAM1 variant. xCELLigence analyzes were repeated three times, each variant performed in triplicates per assay with corresponding results. A representative experiment is shown. (B+D) Statistical analyses were performed by comparing the half maximum CI values (CI_{50}) in the range from 0-70h of each transfectant with control cells by
CEACAM1 splice variants in melanoma

Student’s *t*-test. The inverted mean values of three independent experiments are shown. *p<0.05, **p<0.01; Error bars, mean ± SEM.

**Figure 5**

Enhanced invasiveness of CEACAM1-4S and CEACAM1-4L is mediated by MMPs. The xCELLigence system was used to analyze invasive potential of CEACAM1-4L, CEACAM1-4S transfectants in the presence or absence of the MMP-inhibitor Marimastat compared to CEACAM1 negative control cells in real time. Monitoring was conducted in triplicates and experiments were repeated three times. One representative experiment out of three is shown.

**Figure 6**

CEACAM1-3S enhances the immunogenicity of melanoma cells by up-regulating cell surface expression of NKG2D ligands. (A) MICA and ULBP2 cell surface expression of control cells compared to CEACAM1 transfectants by flow cytometry. Representative histograms are shown: background fluorescence of control cells (thin black line) or of indicated CEACAM1 transfectants (thin grey line), expression for NKG2D ligands of control cells (thick black line) or of CEACAM1 isoform transfectants (dashed black line). (B) Normalized mean MFI values for MICA and ULBP2 in the indicated transfectants (N=5). (C) IHC for NKG2DL (green) in control cells and CEACAM1-3S transfectants. Nuclei were counterstained using Dapi (blue). White arrow heads indicate cell surface expression of MICA/ULBP2. Scale bar, 10µm. (D) Immunoblot analysis for MICA and ULBP2 expression in CEACAM1 transfectants. One representative blot is shown (N=4). (E) Soluble MICA and ULBP2
CEACAM1 splice variants in melanoma expression in conditioned media of indicated CEACAM1 transfectants analyzed by Western Blot and (F) MICA-specific sandwich ELISA (N=6). Sample loading was normalized to cell number. One representative blot is shown. (G) MICA specific ELISA of supernatants collected from CEACAM1-3S, CEACAM1-4L transfectants and controls after treatment with Marimastat or DMSO. Sample loading was normalized to cell number. Mean values of three independent experiments are presented. (H) NK cell-mediated cytotoxicity at various E/T ratios was measured by CSFE/7AAD cytotoxicity assay. To block NKG2D dependent recognition of melanoma cells, NK cells were incubated with anti-NKG2D mAb. Controls were incubated with IgG antibody. One representative experiment is shown. (I) Cytolysis measured at an effector to target ratio of 10:1 (N=4, *p<0.05); All statistical tests were run on unnormalized data. Means ± SEMs are shown.
Figure 1

(A) Expression relative to total CEACAM1 [%] for CEACAM1-3S, CEACAM1-3L, CEACAM1-4S, and CEACAM1-4L across different stages: Stage III and Stage IV.

(B) Kaplan-Meier survival analysis showing overall survival probability [%] with different expressions (expressed vs. not expressed) and the p-value (0.039) for CEACAM1 expression.
**Fig. 2**

**A**

<table>
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<tr>
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<th>CEACAM1-3S</th>
<th>CEACAM1-3L</th>
<th>CEACAM1-4S</th>
<th>CEACAM1-4L</th>
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**B**

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**C**

- **Control**
- **CEACAM1-3S**
- **CEACAM1-3L**
- **CEACAM1-4S**
- **CEACAM1-4L**

Histograms showing events against CEACAM1.
Phalloidin CEACAM1

DAPI Merge

I II

III IV

CEACAM1-3S CEACAM1-3L

CEACAM1-4S CEACAM1-4L

A

B

Phalloidin

CEACAM1

DAPI

Merge

Scale bars for all images are 5 μm.
Ullrich et al., Fig. 6

A

CEACAM1-3S

CEACAM1-3L

CEACAM1-4S

CEACAM1-4L

B

MICA ULBP2

Events

MFI MICA

MFI ULBP2

C

MICA

ULBP2

D

Control

CEACAM1-3L

CEACAM1-3S

CEACAM1-4L

CEACAM1-4S

MICA

ULBP2

β-Actin

E

70kDa

55kDa

35kDa

40kDa

sMICA

sULBP2

F

CEACAM1-3S

CEACAM1-4L

Control

Relative sMICA

G

DMSO

Marimastat

Relative sMICA

H

CEACAM1-3S + IgG

CEACAM1-3S + aNKG2D

Control + IgG

Control + aNKG2D

% of specific lysis

E:T

I

CEACAM1-3S + aNKG2D

CEACAM1-3S + IgG

Control + aNKG2D

Control + IgG

Specific lysis (normalized to control)
CEACAM1-3S drives melanoma cells into NK cell-mediated cytolysis and enhances patient survival

Nico Ullrich, Anja Heinemann, Elena Nilewski, et al.

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