Cancer-Associated Mutations Reveal a Novel Role for EpCAM as an Inhibitor of Cathepsin-L and Tumor Cell Invasion

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Abstract

Background

Epithelial cell adhesion molecule (EpCAM) is a 40-kD type-I transmembrane protein that is frequently overexpressed in human epithelial cancers. Recent evidence implicates EpCAM in the regulation of oncogenic signaling pathways and epithelial-mesenchymal transition. Of note, multiple proteins with thyroglobulin-type-1 (TY-1) domains are known to inhibit cathepsin-L (CTSL), a cysteine protease that promotes tumor invasion and metastasis.

Methods

Human cancer sequencing studies reveal that somatic EpCAM mutations are present in up to 5.1% of tested tumors from public database search. To determine how EpCAM mutations affect cancer biology we studied C66Y, a damaging TY-1 domain mutation identified in liver cancer, as well as 13 other cancer-associated EpCAM mutations. Using *in-vitro* and *in-vivo* models, immunoprecipitations and localizations we demonstrate EpCAM inhibits CTSL activity based mutations and thereby its localization.

Results

We demonstrate that wild type (WT) EpCAM, but not C66Y EpCAM, inhibits CTSL activity *in vitro*, and the TY-1 domain of EpCAM is responsible for this inhibition. WT EpCAM, but not C66Y EpCAM, inhibits tumor cell invasion *in vitro* and lung metastasis *in vivo*. In an extended panel of human cancer cell lines, EpCAM expression is inversely correlated with CTSL activity. Previous studies have demonstrated that EpCAM germline mutations can prevent EpCAM from being expressed at the cell surface. We demonstrate that C66Y and multiple other EpCAM cancer-associated mutations prevent surface expression of EpCAM. Cancer-associated mutations that prevent EpCAM cell surface expression abrogate the ability of EpCAM to inhibit CTSL activity and tumor cell invasion.

Conclusions

These studies reveal a novel role for EpCAM as a CTSL inhibitor, confirm the functional relevance of multiple cancer-associated EpCAM mutations, and suggest a therapeutic vulnerability in cancers harboring EpCAM mutations.

Background

Epithelial cell adhesion molecule (EpCAM) is a 40-kD type-I transmembrane protein that is expressed at the basolateral membrane in the majority of epithelial tissues. EpCAM is overexpressed in the majority of human epithelial cancers including colorectal, breast, gastric, prostate, ovarian, and lung cancer [1, 2]. EpCAM was the first human tumor-associated antigen to be identified using monoclonal antibodies [3], and was the first target of monoclonal antibody therapy in humans [4]. Although initial attempts to target EpCAM were disappointing, a number of second-generation molecular therapies are currently under

development. Recently, we and others have demonstrated that overexpressed EpCAM modulates oncogenic signaling pathways including ERK [5], AP-1 [6], NF- κ B [7] and Wnt [8, 9] pathways. EpCAM expression can differentially regulate oncogenic signaling pathways and invasion depending on the cancer type. These studies suggest that the precise role of EpCAM in cancer biology remains to be elucidated.

At the structural level, EpCAM contains a well characterized thyroglobulin-type-1 (TY-1) domain [10]. The EpCAM TY-1 domain is located between amino acids 63 and 135 in the extracellular region (**Fig. 1**). There are 17 proteins with TY-1 domains in the human genome (**Fig. S1**). TY-1 domains are characterized by a unique sequence motif of 60–80 residues containing six conserved cysteine residues, forming three disulfide bonds [11]. TY-1 domains are conserved in a number of species, and multiple proteins with TY-1 domains function as protease inhibitors [12], (**Table S1**). Proteins with TY-1 domains have been shown to inhibit cathepsin-L (CTSL), a protease implicated in tumor invasion [13, 14].

CTSL is frequently overexpressed in many cancers, and CTSL expression has been associated with higher histologic tumor grade and metastatic potential [15]. CTSL is secreted by tumor cells [16, 17] and is capable of degrading extracellular matrix proteins and promoting tumor cellular invasion [18–20]. Because of its frequent overexpression and prominent role in metastasis, CTSL inhibitors have been the focus of multiple preclinical investigations. CTSL inhibition has demonstrated promising results [15]. Interestingly, both EpCAM [21, 22] and CTSL [23, 24] are known to be secreted in the tumor microenvironment and are present in the serum of human cancer patients. To date, the potential impact of EpCAM on CTSL activity has not been studied.

The Cancer Genome Atlas (TCGA) and other high throughput cancer sequencing studies have dramatically improved our understanding of the genetic basis of cancer. Functional analyses of specific cancer-associated mutations have provided critical insights into how mutated proteins contribute to the biology of cancer, and how to best target these proteins. To date, over 130 coding, cancer-associated mutations in the EpCAM gene have been identified (**Fig. 1**). Studies reveal that somatic mutations in EpCAM are present in up to 5.1% of some tested cancer cohorts, including squamous and melanoma skin cancers. EpCAM mutations have also been observed in metastatic lesions and other cancers at a frequency of 1–2% (**Fig. S2**). No reports to date have investigated the potential role of EpCAM mutations in human cancer, although germline mutations in EpCAM impact cellular localization and are associated with congenital tufting enteropathy and Lynch syndrome [25, 26]. In the current report we demonstrate for the first time that secreted wild type (WT) EpCAM inhibits extracellular CTSL activity. More importantly, we demonstrate that EpCAM cancer-associated mutations alter EpCAM cellular function and localization, abrogate the ability of EpCAM to inhibit CTSL activity, and impact CTSL-driven cancer cell invasion.

Methods

EpCAM mutation analysis

To determine the position, amino acid change, and occurrences of somatic EpCAM coding mutations in cancer, we queried the Catalogue of Somatic Mutations in Cancer (COSMIC) database [27] and tabulated reported mutations. To determine the frequency of EpCAM mutations in tested cancer cohorts, we analyzed 178 non-redundant datasets including 47,005 samples in the cBioPortal for Cancer Genomics [28]. The potential impact of mutations of interest on EpCAM protein structure and function was analyzed *in silico* using PolyPhen-2 [29].

Cell lines

All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and used at a low passage number with the exception of WHIM-3. WHIM-3 is a breast cancer cell line isolated from a patient-derived xenograft. WHIM-3 was provided by the patient derived xenograft (PDX) core at Washington University of School of Medicine (WUSM). All cell lines were maintained in RPMI or DMEM supplemented with 10% FBS and antibiotics (GIBCO BRL, Grand Island, NY). All cell lines were tested for mycoplasma and maintained in ciprofloxacin.

Recombinant Proteins

Recombinant EpCAM-Fc (Gln24-Lys265-IgG1Pro100-Lys300, carrier free) and recombinant CTSL (Glu113-Val333) were purchased from R&D Systems (Minneapolis, MN).

RNA interference

Lentiviral-mediated RNA interference was performed as previously described [5]. shRNA sequences targeting murine cathepsin-L were provided by the McDonnell Genome Institute at Washington University in St Louis. Specific sequences used for RNA interference are detailed in **Table S2**.

Plasmid constructs and site directed mutagenesis

The full-length open reading frame of EpCAM was amplified from the MCF-10A mammary epithelial cell line and subcloned into both pcDNA3 and the retroviral vector pBABE. EpCAM deletion mutants were generated by PCR amplification as shown in **Fig. 3E**. C66Y EpCAM and other EpCAM cancer-associated mutation constructs were generated using synthetic gene fragments from Integrated DNA Technologies (IDT, Coralville, IA). For example, C66Y EpCAM was generated as a G-block fragment (197G > A; substitution position 197, G→A). Other mutations were generated based on tumor DNA mutation information available at COSMIC [27] or cBioPortal [28] databases. For EpCAM-GFP fusion constructs generation, EGFP cDNA was amplified from pLL3.7 vector and subcloned at the c-terminal of EpCAM in pCDNA3.1 and pBabe-Puro vectors.

Retroviral transduction

Phoenix-ECO packaging cells were transfected when nearly confluent with 2.5 µg of pBABE-hygro-EpCAM using FuGENE HD (Promega, Madison, WI). Forty eight hours after transfection viral supernatants were collected, filtered through 0.45 micron filters, and then added to B16-F10 and other cells in media

containing 8 µg/mL protamine sulfate. After two successive retroviral infections, cells were grown for 48 hours and selected in puromycin for two weeks.

Flow cytometry

EpCAM expression levels were measured by flow cytometry using PE-labeled EpCAM antibody. Expression was quantified as mean fluorescence intensity (MFI) using a FACScan flow cytometer and FlowJo (BD Biosciences, San Jose, CA).

Cathepsin-L activity assay

Cells were suspended in ice-cold lysis buffer (400 mM sodium phosphate buffer, pH 6.0, 75 mM NaCl, 4 mM EDTA, and 0.25 % Triton X-100) and incubated on ice for 30 minutes. Lysates were centrifuged at 15,000 rpm for 20 minutes at 4 °C. Protein concentration was determined by the BCA protein assay kit (Pierce, Rockford, IL). CTSL activity was measured by fluorometric assay [30]. Briefly, 10 µg of total cell lysate was diluted in 100 µL of 0.34 M sodium acetate buffer, pH 5.5, containing 2 mM EDTA, and 4 mM dithiothreitol (DTT). To discriminate between CTSL and cathepsin-B activities, a selective cathepsin-B inhibitor, CA074 (Sigma), was added at a final concentration of 5 µM and pre-incubated for 15 minutes at 37 °C. Fluorogenic substrate Z-Phe-Arg-AMC (Sigma) was added to a final concentration of 5 µM and samples were incubated for an additional 30–60 minutes at 37 °C. Fluorescence of the degradation product, 7-amino-4-methylcoumarin (7-AMC), was measured at an excitation wavelength of 370 nm and an emission wavelength of 460 nm, using a spectrometer (BioTek). Cysteine protease inhibitor l-trans-epoxy-succinyl-leucylamido-(4-guanidino)-butane (E64, Sigma) was used as a CTSL inhibitor at 50 µM concentration.

Protein immunoblots

Cells were washed with ice-cold PBS and lysed in cell lysis buffer with a protease inhibitor cocktail (Cell Signaling Technology, Danvers, MA). Protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL). Approximately 20–30 µg of protein was subjected to SDS-PAGE (NuPAGE, Life Technologies), and transferred by electrophoresis to a PVDF membrane. EpCAM (C-10) and actin-HRP antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). CTSL antibodies were obtained from AbCAM (Cambridge, MA). Signal detection was performed using the SuperSignal West Pico chemiluminescent immunodetection system (Thermo Scientific, Rockford, IL). To quantify band density, immunoblots were developed on film, scanned, and pixels in each band were measured using Image J software.

Immunoprecipitation

For protein extraction, cells were scraped in PBS supplemented with 1 mM Na₃VO₄, centrifuged and re-suspended in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.25% NP-40, 0.2% Triton-X, 1 mM Na₃VO₄, and 1 mM PMSF). Five hundred µg of protein was precleared with Protein A/G-Sepharose. Precleared lysates were then incubated for 1 hour using 2 µg of specific antibodies or control IgG at 4 °C with 25 µl

of Protein A (or Protein G)-Sepharose. The immunobeads were washed 3 times in lysis buffer and then eluted in 50 μ L of 2 $\times$ reducing sample buffer, boiled, and proceeded for immunoblotting.

Immunofluorescence and confocal microscopy

Retrovirus transduced MDCK or transiently transfected HEK-293T cells with EpCAM-GFP constructs were lightly trypsinized after 48 hours and plated on a glass bottom 35 mm dish with serum-free Opti-MEM media (ThermoFisher Scientific, Grand Island, New York). Cells were visualized and captured with a fluorescence microscope (EVOS digital inverted microscope at 20X or 40X magnifications). Duplicate cell culture was used for data measurements on a Zeiss LSM 880 microscope equipped with the AiryScan detector, an Argon laser (Melles-Griot) for 488 nm excitation and a Zeiss Plan-Apochromat 63 $\times$ /1.4 NA DIC M27 Oil objective. The microscope is equipped with temperature and CO₂ controls that were kept at 37 °C and 5%, respectively.

EpCAM ELISA

HEK-293T transiently transfected cells or tumor cell lines were plated in serum free Opti-MEM media. After 48 hours, conditioned media was used as a source of secreted/soluble EpCAM. Human EpCAM DuoSet ELISA kit (DY960) was used from R&D Systems Inc. (Minneapolis, MN) as recommended.

Invasion assays

Cells (4×10^4) were added to Matrigel transwell invasion chambers or control transwell chambers (BD Biosciences, San Jose, CA) and incubated for 24–72 hours with chemoattractant media (Clonetics, Walkersville, MD) supplemented with growth factors. Cells invading through the Matrigel or control membranes were fixed using 70% ethanol, stained with 0.1% crystal violet, and photographed in four fields to cover the entire area. Cells were counted from all fields by a scientist blinded to the experimental conditions.

Animal experiments:

Tumor cell expansion for cathepsin-L activity assays were done by xenografting stable lines in 4–6 weeks old female NOD-SCID gamma (NSG) IL2Rgamma^{null} mice (The Jackson Laboratory, Bar Harbor, Maine). Tumor cells were transduced with pBABE-puro retroviruses to express EpCAM or empty vector as described earlier. Cells were re-suspended in DMEM, 1×10^7 per 100 μ L. Before tumor cell injection, the mice were anesthetized with 2.5-4% isoflurane under continuous infusion via a nose. Cells were injected subcutaneously then allowed to expand in mice for 2–3 weeks. Two mice were used per cell line to establish cell lines. When the tumor growth reached 0.5-1 cm³, tumors were removed aseptically, minced, filtered and plated in appropriate growth media. After 2 days, tumor cells were selected in puromycin containing media for one week. These lines were used within 5–8 passages for CTSL activity assay experiments.

Tumor challenge/lung metastasis assay

Six to ten weeks old female C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) were used for all lung metastasis experiments. Mice were housed at institutional animal facility. B16-F10 cells (5×10^4) were re-suspended in 200 μ L PBS. For tail vein injections, mice were immobilized in a rodent holder and kept under heating lamp for one minute to dilate blood vessels. 200 μ L cell suspensions were injected via tail vein into mice using hypodermic syringes. Animals were monitored weekly. Five mice per group were used in individual studies, and each study was repeated at least three times. Three weeks following tumor challenge, the mice were euthanized by CO₂ asphyxiation or cervical dislocation according to the approved IACUC protocol. Lung nodules were photographed and counted using a dissecting microscope.

Statistical analysis

Numerical data are presented as the mean values $\pm$ the standard deviation. Statistical significance was evaluated using the Student's *t* test. A *p*-value < 0.05 was considered to be statistically significant. GraphPad PRISM (GraphPad Software Inc., La Jolla, CA) was used for statistical analysis of all experiments.

Results

Somatic EpCAM mutations are present in a significant number of human cancers. To analyze somatic EpCAM mutations in human cancers, we queried the COSMIC database as well as 178 non-redundant datasets including 47,005 samples in the cBioPortal for Cancer Genomics [27, 28]. We identified 115 unique somatic/missense coding EpCAM mutations (**Fig. 1**). Depending on the dataset and cancer type, EpCAM mutations are present at a frequency between 0 to 5.13% in human cancers (**Fig. S2**). We identified multiple cancer-associated mutations which may affect the overall structure and function of EpCAM. C66Y EpCAM is an exemplary TY-1 domain EpCAM cancer-associated mutation identified in a liver cancer specimen. The cysteine residue is part of a critical disulfide bond, and the C66Y mutation is likely to extensively perturb EpCAM structure [10] and thereby disrupt function of the EpCAM TY-1 domain (**Fig. 1**). Analysis by PolyPhen-2 [29] predicts the C66Y mutation to have a highly damaging effect (data not shown) on protein structure and function. We demonstrate below that the C66Y mutation also impacts EpCAM cellular localization as well.

WT, but not C66Y EpCAM, inhibits tumor cell invasion *in vitro* and *in vivo*.

EpCAM has been implicated in the regulation of cancer invasion. To investigate the role of cancer-associated EpCAM mutations, we initially focused on C66Y. We expressed WT, or C66Y EpCAM, in the human WHIM-3 breast and murine PyMT BO-1 mammary cancer cell lines. Both cell lines have minimal endogenous EpCAM expression and an invasive, mesenchymal phenotype with high CTSL activity. Cells were transduced with retroviruses expressing either GFP (control), WT EpCAM, or C66Y EpCAM. EpCAM expression was confirmed by flow cytometry (data not shown). Expression of WT EpCAM decreased invasion *in vitro* in both cell lines approximately 70% compared to cells expressing C66Y EpCAM or GFP (**Fig. 2A-B**). To extend these findings, we expressed WT or C66Y EpCAM in B16-F10 cells and performed

both *in vitro* and *in vivo* studies. B16-F10 has minimal EpCAM expression, is known to be highly invasive, and is dependent on CTSL activity for migration and invasion [31]. Specific ablation of CTSL decreased B16-F10 invasion *in vitro*, confirming that this cell line is dependent on CTSL for invasion (**Fig. S3A-C**). We transduced B16-F10 cells with WT or C66Y EpCAM and selected stable cell lines. We confirmed that expression of WT or C66Y EpCAM was comparable in these cell lines by protein immunoblot (**Fig. 2B and S4, left panel**). Expression of WT, but not C66Y EpCAM, significantly decreased B16-F10 tumor cell invasion *in vitro* (**Fig. 2B, right panel**), and the number of lung cancer metastases following tumor challenge *in vivo* (**Fig. 2C, Fig. S3D**). CTSL promotes tumor cell invasion and metastasis by degradation of the interstitial matrix and basement membranes. C66Y EpCAM failed to suppress CTSL-mediated lung metastasis *in vivo* (**Fig. 2C**). These results suggest that EpCAM expression has the potential to regulate cancer invasion in the B16-F10 cell line.

WT, but not C66Y EpCAM, inhibits CTSL activity.

Based on the known role of TY-1 domains in the regulation of CTSL (**Fig. S1, Table S1**), we tested the hypothesis that EpCAM can inhibit CTSL. First, we measured EpCAM expression and CTSL activity in a panel of cell lines. EpCAM expression was assessed by flow cytometry, and CTSL activity was assessed using a fluorescent substrate. We observed a striking inverse correlation between EpCAM expression and CTSL activity (**Fig. 3A & 3B**). As previously discussed, EpCAM [21, 22] and CTSL [23, 24] are both secreted into the extracellular space where they likely come in contact in the tumor microenvironment. To determine if soluble EpCAM can inhibit CTSL activity, we incubated recombinant EpCAM-Fc with SKOV3 cell lysates as a source of CTSL (SKOV3 cells have the highest CTSL activity [**Fig. 3A**] in the panel of tested cell lines). Recombinant EpCAM-Fc decreased CTSL activity in a dose dependent manner, and at 10 ng/mL, CTSL activity was suppressed approximately 60% (**Fig. 3C**). We also tested the ability of cancer cell lines transduced with EpCAM to inhibit CTSL. In cell line A549, which has minimal endogenous EpCAM expression (**Fig. 3A**), WT, but not C66Y EpCAM was able to significantly inhibit CTSL activity (**Fig. 3D**). To evaluate the potential role of the EpCAM TY-1 domain in the inhibition of CTSL, we generated EpCAM deletion mutants (**Fig. 3E**). Stably transduced A549 cell lines with EpCAM deletion mutants were cultured in serum-free media for 24 hours and assayed for CTSL activity. Only EpCAM deletion mutants with intact TY-1 domains were capable of inhibiting CTSL activity (**Fig. 3F**). As previously reported [10] and shown here, the cysteine at residue 66 forms a critical disulfide bond in the EpCAM TY-1 domain. The C66Y mutation likely impacts protein structure and CTSL inhibition. Taken together, these studies confirm that EpCAM is capable of inhibiting CTSL activity, presumably via the TY-1 domain.

WT EpCAM physically interacts with CTSL

The ability of TY-1 domain proteins to inhibit CTSL activity typically depends on a physical interaction between the TY-1 domain protein and CTSL. To determine if EpCAM can physically interact with CTSL, we performed immunoprecipitation and protein immunoblot assays. We used the MDA-MB-468 breast cancer cell line, which expresses moderate amounts of both EpCAM and CTSL. MDA-MB-468 cell lysates

were incubated with IgG, anti-EpCAM, or anti-CTSL antibodies, and immunoprecipitated proteins were immunoblotted for EpCAM or CTSL. EpCAM immunoprecipitation pulls down CTSL (**Fig. 4A and S4**), and CTSL immunoprecipitation pulls down EpCAM (**Fig. 4B and S4**), demonstrating a physical interaction between these proteins.

Many cancer-associated mutations prevent EpCAM cell surface expression and secretion and abrogate the ability to inhibit CTSL

EpCAM is a type-I transmembrane glycoprotein, which is predominantly localized on the cell surface. The extracellular domain of EpCAM is secreted (and/or cleaved) and is detectable in cell culture media and the serum and ascites of cancer patients. Soluble/secreted EpCAM is a 242 amino acid (aa) fragment lacking the signal peptide (23 aa), transmembrane domain (23 aa), and cytoplasmic tail (26 aa) [32]. CTSL, while typically expressed in the endosome, has also been shown to be secreted from tumor cells and potentiates invasion in multiple cancer types [33, 34]. A study of congenital tufting enteropathy patients demonstrated that EpCAM mutations can alter cellular trafficking and localization of EpCAM protein to the cell surface [25]. In that study, multiple germline EpCAM mutations prevented EpCAM expression at the cell surface.

To investigate whether cancer-associated EpCAM mutations affect its cellular localization, and/or its potential to inhibit CTSL, we cloned multiple cancer-associated EpCAM mutations in expression vectors alone, or fused to GFP at the C-terminal, and tracked EpCAM localization *in vivo*. As shown in **Fig. 5A**, flow cytometry and confocal microscopy demonstrate that WT EpCAM and some mutants (EpCAM-M115T) localize to the cell surface of epithelial MDCK cells, whereas other mutants (EpCAM-C66Y and EpCAM-L240A) localize in the cytosolic compartments. To verify that soluble EpCAM can inhibit CTSL activity, HEK-293T cells were then transfected with GFP-tagged EpCAM mutants and after 48 hours, conditioned media was collected to measure soluble EpCAM levels by ELISA and/or to test the ability of conditioned media to inhibit CTSL activity. As expected, EpCAM mutants that are not expressed at the cell surface were not detected in culture media by ELISA (**Fig. 5B, right**), and these conditioned medias could not inhibit CTSL activity from SKOV3 media (**Fig. 5C, right**). In contrast, WT EpCAM and EpCAM mutants expressed at the cell surface were readily detected in culture media (**Fig. 5B, left**), and conditioned media robustly inhibited CTSL activity (**Fig. 5C, left**). Together, these results demonstrate that secreted EpCAM inhibits secreted CTSL activity, while cancer-associated EpCAM mutations that prevent cell surface expression also prevent the ability to inhibit CTSL activity.

To confirm and extend these findings, we tested A549 lung cancer cells under multiple conditions. CTSL activity is required for A549 invasion [35] and at baseline A549 cells are less invasive compared to other CTSL-secreting cells. Addition of CTSL-rich SKOV3 conditioned media enhanced A549 invasion 3-fold, and this was abrogated by the CTSL inhibitor E64 (**Fig. 5D, last 2 bar graphs**). This confirms previous reports that CTSL contributes to A549 invasion. To assess the effect of cancer-associated EpCAM mutations on A549 invasion, A549 cells were plated on Matrigel chambers for 2 hours followed by the addition of conditioned media from transfected HEK-293T cells expressing WT or mutant EpCAM. After

30 minutes, CTSL-rich SKOV3 conditioned media was added and invasion was monitored over 24 hours. As expected, WT and cancer-associated EpCAM mutants expressed at the cell surface and secreted suppressed invasion, while cancer-associated EpCAM mutants with no expression at the cell surface did not affect invasion. Together, our findings suggest that soluble/secreted EpCAM can suppress extracellular CTSL protease activity via its TY-1 domain, while cancer-associated EpCAM mutants that are not expressed on the cell surface do not retain this function.

Discussion

EpCAM is overexpressed in many epithelial cancers, and plays a complex role in cancer biology, stimulating or inhibiting diverse cancer signaling pathways depending on the cancer type [5–7]. There are 17 proteins with TY-1 domains in the human genome (**Fig. S1**). TY-1 domains are dependent on three conserved disulfide bonds, creating a structure that can function as a cathepsin family protease inhibitor [36, 37]. In studies performed here, we demonstrate that WT EpCAM has the ability to inhibit CTSL, and that this inhibition is dependent on the TY-1 domain of EpCAM. C66Y is a cancer-associated EpCAM mutation that disrupts an important disulfide bond in the EpCAM TY-1 domain. The findings reported here provide important insights into the biology of this and other cancer-associated EpCAM mutations.

Cathepsin proteases play a key role in tissue remodeling, tumor invasion, and metastasis. CTSL promotes tumor cell invasion and metastasis by degradation of the interstitial matrix and basement membranes [38]. Cathepsin protease activity is frequently dysregulated during neoplastic transformation, and increased activity within the tumor microenvironment leads to cancer progression, proliferation, metastasis, and drug resistance. CTSL is a powerful lysosomal cysteine proteinase, and CTSL expression and activity is markedly increased in advanced cancers [39]. Forced expression of CTSL is associated with increased migration, invasion, and chemotherapy resistance [31, 40, 41], and inhibition of CTSL activity decreased tumor growth and invasion [42]. As such, a role for WT EpCAM in the regulation of CTSL in normal epithelia and/or cancer (as demonstrated here) is consistent with the known roles for EpCAM in the regulation of both epithelial-to-mesenchymal transition (EMT) and tumor cell invasion.

EpCAM mutations have been identified in many human epithelial cancer types. In the present study we focused on C66Y EpCAM, a mutation resulting in a profound structural and functional alteration in the EpCAM TY-1 domain. We expect other cysteine mutations such as C135G in the TY-1 domain will disrupt EpCAM structure and interaction with CTSL. We also studied 13 other cancer-associated EpCAM mutations and discovered that approximately 50% of these mutations prevented EpCAM cell surface expression. In addition to these nonsynonymous mutations, EpCAM expression is often silenced in cancer cells by promoter methylation, histone modification, and/or aberrant transcription factor signaling [43]. EpCAM is silenced in various cancer types [44, 45], and silencing may exceed the observed rate of mutations. Our data suggest that EpCAM gene silencing is likely associated with increased CTSL activity. Therefore, EpCAM gene silencing may represent an additional therapeutic opportunity to target CTSL, although further studies would be needed.

Based on a detailed crystal structure analysis, the first loop of the EpCAM TY-1 domain interacts with the C-terminal domain of adjacent EpCAM to form EpCAM *cis*-dimers [10]. This suggests that the EpCAM TY-1 domain may not be available to inhibit CTSL activity when dimerized on the cell surface due to steric hindrances. However, EpCAM is frequently present as a monomer under different experimental conditions [10, 46–48], and EpCAM is known to be cleaved and secreted by cancer cells as shown by us (**Fig. 5**) and by others [32]. We demonstrate here that soluble EpCAM inhibits CTSL activity in a dose dependent manner (**Fig. 3C**) and that this inhibitory effect is abrogated by deletion of the TY-1 domain of EpCAM (**Fig. 3E**). These findings are consistent with other studies demonstrating that thyroglobulin domains in other proteins can inhibit cathepsins (**Table S1**). Together, these results suggest that EpCAM likely inhibits CTSL in a soluble and/or monomeric state.

These studies reveal a novel role for EpCAM in the regulation of CTSL, but also have potential therapeutic implications as well. Cancers harboring cancer-associated EpCAM mutations and/or EpCAM silencing may have increased CTSL activity. While many cancers demonstrate overexpression of CTSL, it is unclear if this directly corresponds to increased CTSL and metastatic activity. Laboratory techniques have been developed that measure cathepsin activity in tissue specimens, but greater specificity is needed for CTSL [49], and these are not routinely performed. EpCAM mutations could potentially serve as a biomarker of increased CTSL activity in the tumor microenvironment and identify patients that would benefit from protease inhibitors targeting CTSL. Initial clinical trials of protease inhibitors (predominantly matrix metalloprotease inhibitors) showed limited efficacy due to off target activity causing significant drug toxicity [15]. However, more recent preclinical studies have identified and tested multiple different types of cathepsin inhibitors that have been shown to specifically target cathepsin subtypes with less toxicity [50]. Taken together, our findings and these recent preclinical studies suggest that CTSL inhibition in cancers harboring EpCAM mutations may prove to be an effective strategy.

Conclusions

Our study demonstrates molecular mechanism of tumor progression in EpCAM mutated tumor cells. Cathepsin-L is often overexpressed or activated in advanced epithelial cancers. To date, over 130 coding, somatic EpCAM mutations have been reported and deposited in public databases [27, 28]. Our study demonstrates that some of these mutations, drives EpCAM accumulation in cytosol compartments instead transported on membrane. TY-1 domain mutations also disrupted CTSL binding. In an era where genomic testing is being increasingly used to drive biomarker-driven therapeutic decisions in patient care, additional study is required to understand the role of EpCAM mutations in cancer biology, and the potential to target these mutations with existing and/or novel therapies.

Abbreviations

CTSL: Cathepsin-L, EpCAM: Epithelial Cell Adhesion Molecule; TY-1: thyroglobulin-type-1 domain; PDX: patient derived xenograft;

Declarations

All manuscripts must contain the following sections under the heading 'Declarations':

Ethics approval and consent to participate

Animal experiments were followed the NIH guidelines (NIH Pub. No. 85-23, revised 1996) and have been approved by the Animal Protection and Use Committee of Washington University School of Medicine.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Author contributions

NVS and WEG were responsible for study conception, design, and supervision. NVS, AAA, ANL performed the *in vitro* experiments. NVS, JMH performed animal experiments. NVS, TCB, TPF, and WEG wrote the manuscript. All authors have read and approved the manuscript.

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Figures

Figure 1

Cancer-associated EpCAM mutations. Histogram (top) shows location and frequency of EpCAM cancer-associated mutations as reported in COSMIC (<https://cancer.sanger.ac.uk/cosmic>). Protein schematic (bottom) indicates EpCAM protein structure. EpCAM is a type-1 transmembrane protein with a signal peptide (SP, green), an N-terminal domain (ND, blue), a thyroglobulin type-1 domain (TY-1, red), a C domain (CD, tan), a transmembrane domain (TM, gray), and an intracellular domain (EpIC, purple). Domain positions noted as reported by Pavsic et al [10]. The EpCAM C66Y mutation in the TY-1 domain is predicted to be highly damaging by Polyphen2 (<http://genetics.bwh.harvard.edu/pph2/>).

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Figure 2

Wild type, but not C66Y EpCAM, inhibits tumor cell invasion in vitro and in vivo. A, WHIM-3 and PyMT BO-1 breast cancer cell lines were transduced with GFP, wild type EpCAM, or C66Y EpCAM. Invasion was assessed in vitro using Matrigel chambers. B, B16-F10 cells were transduced with GFP, wild type EpCAM, or C66Y (EpCAM colony 1 and 2). Protein expression is shown by immunoblot (left). Invasion (right) was assessed in vitro using Matrigel chambers. C, B16-F10 cells were transduced with GFP, wild type EpCAM, or C66Y EpCAM. Transduced cells were injected into animals by tail vein, and the lungs were harvested 2 weeks later. Representative lungs samples are shown (left). Lung metastases were counted using a dissecting microscope by two independent researchers (right). All experiments were performed at least three times. Full-length blots are presented in Supplementary Figure 4.

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Wild type, but not C66Y EpCAM, can inhibit CTSL activity. A-B, EpCAM expression negatively correlates with CTSL activity in a panel of cancer cell lines. EpCAM expression was measured by flow cytometry, and CTSL activity was measured using a fluorescent substrate. C, Recombinant EpCAM inhibits CTSL activity. Increasing doses of recombinant EpCAM were added to conditioned media from SKOV-3 cells (source of CTSL). CTSL activity was measured after 30 minutes. D, Wild type, but not C66Y EpCAM, inhibits CTSL activity. A549 cells were stably transduced with GFP, wild type EpCAM or C66Y EpCAM. Endogenous CTSL activity was measured. E-F, Wild type EpCAM and EpCAM deletion mutants containing

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Wild type EpCAM physically interacts with CTSL. A, MDA-MB-468 cell lysates were immunoprecipitated with EpCAM (clone C-10) or control (IgG) antibody as described in methods section. EpCAM immunoprecipitation pulls down CTSL. B, Cell lysates were immunoprecipitated with CTSL or control (IgG) antibody. CTSL immunoprecipitation pulls down EpCAM. Full-length blots are presented in Supplementary Figure 4

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A. EpCAM localization

B. Soluble EpCAM

C. Soluble CTSL activity

D. Invasion

Figure 5

Cancer associated EpCAM mutations prevents EpCAM-CTSL localization, interaction and Cathepsin L mediated invasion. A, MDCK cells were transduced with WT, M115T, C66Y, and L240A EpCAM and surface expression was analyzed by flow cytometry or confocal microscopy, respectively. B, GFP-tagged EpCAM mutants were transfected in 12 well culture plates into HEK-293T cells. 500uL of Opti-mem media was added to harvest secreted EpCAM in conditioned media after 48 hours and assayed by ELISA to measure soluble EpCAM. C, After 48 hours of culturing, 1-mL of conditioned media was collected from 6-well SKOV3 culture as a source of soluble CTSL. Conditioned media from transformed HEK-293T cells was used as a source of soluble EpCAM. CTSL activity was determined as discussed in methods. A5-CM (A549 conditioned media serves as a control). D, A549 cells were plated on Matrigel for 6 hours. Conditioned media and soluble EpCAM were added, followed by soluble CTSL. After 24 hours cells were tabulated to measure invasion.

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