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Novel Anti-CRR9/CLPTM1L Antibodies with Antitumorigenic Activity Inhibit Cell Surface Accumulation, PI3K Interaction, and Survival Signaling

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Abstract

We and others have recently shown Cisplatin Resistance-Related Protein 9 (CRR9)/Cleft Lip and Palate Transmembrane 1-Like (CLPTM1L) to affect survival and proliferation in lung and pancreatic tumor cells. Our research has indicated that CLPTM1L affects multiple survival signaling pathways in tumor cells under oncogenic, genotoxic, and microenvironmental stress. We have confirmed the association of CLPTM1L with pancreatic cancer by demonstrating overexpression of CLPTM1L in pancreatic tumors and poor survival in patients with high tumor expression of CLPTM1L. Predicting a transmembrane structure, we determined that CLPTM1L could be targeted at the plasma membrane. Herein, we describe the development of monoclonal antibodies targeting CLPTM1L. Lead antibodies inhibited surface accumulation of CLPTM1L, Akt phosphorylation, anchorage-independent growth, and chemotherapeutic resistance in lung and pancreatic tumor cells. Gemcitabine promoted a physical interaction between CLPTM1L and p110α in pancreatic tumor cells, which was inhibited by anti-CLPTM1L. In-vivo treatment with anti-CLPTM1L robustly inhibited the growth of both lung and pancreatic adenocarcinoma xenografts. The efficacy of anti-CLPTM1L correlated with specific epitopes representing important targets in human cancers, particularly those driven by KRas, for which effective targeted therapies have been elusive. This study is the first to report cell-surface exposure of the tumor survival protein CLPTM1L and inhibition of the function of surface CLPTM1L with novel, systematically developed inhibitory monoclonal antibodies establishing proof of concept of clinically practical agents inhibiting this compelling new tumor survival target in cancer.

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Keywords

Pancreatic cancer; Lung cancer; PI3K; Akt; chemoresistance; CRR9; CLPTM1L; targeted therapy; monoclonal antibody therapy

Introduction

The CRR9/CLPTM1L gene resides within a locus at chromosome 5p15.33 that is associated with risk of non-small cell lung cancer, pancreatic cancer, and a variety of other cancers (1–11). CLPTM1L is commonly overexpressed in multiple tumor types (12–14) (OncomineTM Compendia Bioscience, Ann Arbor, MI.). There is increasing evidence for tumor specific CLPTM1L expression. In fact, increased CLPTM1L transcript expression was found to be a highly specific marker of laryngeal carcinoma using microarray analysis (13). In addition, an in-frame fusion of the 297 amino acid N-terminus of CLPTM1L with the GLIS3 in a rare pediatric case of hepatocellular carcinoma and was found to be oncogenic (15). Little has been reported regarding the expression of CLPTM1L in normal tissues. However, we have shown that CLPTM1L expression in normal lung epithelium is low relative to that in tumor tissues (16). CLPTM1L expression was characterized as weak in 30–40% of normal pancreatic ductal epithelial tissues, with expression being undetectable in the remainder of normal tissues in one study (14). In addition to tumor-specific expression, CLPTM1L functions in several tumor-specific pathological processes. In particular, we previously found CLPTM1L to confer resistance to chemotherapy and survival of anoikis in the context of anchorage independent tumor growth (12). We found that the role of CLPTM1L in chemoresistance was not limited to cisplatin, as resistance to Topoisomerase I inhibition was also dependent on CLPTM1L. Our laboratory was the first to demonstrate a role for CLPTM1L in tumorigenesis. Our previous results demonstrated that CLPTM1L was necessary for Ras-induced Akt phosphorylation in association with an interaction between PI3K and CLPTM1L in mouse fibroblasts. CLPTM1L blockade also caused decreased expression of Bcl-xL, which was independent of Akt status (16). Anchorage independent growth inhibition by CLPTM1L blockade in these cells was dependent on inhibition of both Akt phosphorylation and Bcl-xL expression.

CLPTM1L is predicted to have 6 hydrophobic helical domains as indicated by Uniprot annotation, indicating membrane localization. It was previously suggested that CLPTM1L may exert its survival function in the mitochondria (17). Glycosylation of CLPTM1L has previously been overlooked, and given its role in the intrinsic apoptosis pathway, a focus on the mitochondria has resulted in a failure to recognize the presence of CLPTM1L on the cell surface by previous studies. However, recent studies in pancreatic tumor cells and the current study provide strong evidence that CLPTM1L resides predominantly in the endoplasmic reticulum and on the cell surface (14). It is unknown whether cell surface-associated CLPTM1L plays a specific and critical role in survival signaling or if it can be targeted therapeutically. We have shown CLPTM1L to be critical for Ras transformation and tumorigenesis, making this signaling pathway a novel potential way to target Ras-driven cancers. Therefore, this study focuses on lung and pancreatic cancers, of which approximately 35 and 90% are driven by K-Ras, respectively.

Here, we present the first evidence for cell-surface exposure of CLPTM1L in lung and pancreatic tumor cells and demonstrate an interaction of CLPTM1L with p110 α in tumor cells. We demonstrate the first proof of concept of clinically practical anti-cancer agents inhibiting the function of surface CLPTM1L with systematically developed inhibitory monoclonal antibodies. These novel agents inhibit p110 α -CLPTM1L interaction and Akt phosphorylation, inhibit tumor cell growth and anchorage independence, and sensitize lung and pancreatic tumor cells to killing by cisplatin and gemcitabine, respectively. Furthermore, we demonstrate effective inhibition of the growth of established adenocarcinomas in *in-vivo* models. Our findings provide strong justification for investigation of CLPTM1L-targeting antibodies as therapeutics and chemosensitizers for human cancers.

Materials and Methods

Cell culture and reagents

Panc1, MiaPaCa, A549, H838, HeLa, U251, GBM4, and Beas-2B cells were obtained from ATCC or authenticated by DNA typing of STR and microsatellite loci and comparison to ATCC reference profiles within 6 months of experiments. Primary human pancreatic adenocarcinoma cell lines MCW462 and MCW670 were established at the Surgical Oncology Biorepository at MCW and maintained in DMEM/F12 with 6% FBS and supplements. Cell lines were generated from heterotopic murine xenografts established from primary and metastatic human pancreatic cancer (PC) specimens. Cell lines were established after enzymatic digestion of the xenografts. Mouse CD326- MHC Class I+(H-2Kd) cells were eliminated from the cell lines by flow cytometric FACS sorting using human-specific CD326 (EpCAM) and murine-specific MHC Class I (H-2Kd) antibodies (eBioscience, San Diego, CA). Short tandem repeat (STR) profiling was performed using seventeen STR loci plus the gender determining locus using the commercially available PowerPlex 18 D Genetic Analyzer. Data were analyzed using GeneMapper ID-X v1.2 (Applied Biosystems). Samples did not match any cell line in either the American Type Culture Collection database. Cell lines were characterized by immunohistochemistry (IHC) for epithelial (CK19) and pancreatic (PDX-1) markers, doubling time, colony forming efficiency, and *in vivo* tumorigenicity. Mutations in KRAS and TP53 were assessed using Sanger sequencing. Both cell lines harbor KRAS G12A mutations. Human lung adenocarcinoma cell lines (A549 and H838) were cultured in RPMI1640 plus 10% FBS (Life Technologies, Carlsbad, CA). Beas-2B cells were cultured in LHC-8 media plus epinephrine (Life Technologies, Carlsbad, CA). Panc1 cells were grown in DMEM/F12 media with 10% FBS. Cisplatin and gemcitabine were purchased from Sigma-Aldrich (St. Louis, MO) and prepared immediately before use in 5 mM and 50 mM aqueous stock solutions, respectively. Polyclonal anti-CLPTM1L (ab155119, Abcam, Cambridge, MA) was used in polyclonal anti-CLPTM1L inhibition studies. Antibody diluent as described by Abcam was used as a vehicle control for polyclonal antibody treatment where indicated to account for any effect of diluent constituents. Normal mouse IgG was used as a non-specific antibody control for monoclonal antibody treatment where indicated. Rabbit α -HA (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a non-specific antibody control for polyclonal antibody treatment where indicated. Mouse α -HA (Cell Signaling, Boston, MA) was used as a non-specific antibody control for experiments with purified monoclonal antibodies, and mouse α -human Von

Willibrand Factor (hVWF) ascites was used for experiments with monoclonal ascites. Monoclonal antibody production was contracted to Biomatik Corporation, Cambridge, Ontario, Canada. Polyclonal antibodies provided preliminary results and the use of polyclonal, monoclonal ascites, and purified monoclonal antibody was dictated by appropriate characterization of each antibody and secondarily by availability. The shRNA depletion of CLPTM1L with shCLP, as well as the specificity and efficacy of knockdown with this vector were previously validated and described (12, 16).

Immunohistochemistry

IHC staining was performed on the Dako Autostainer Plus using the Dako EnVision™ FLEX High pH Detection Kit (K8010) (Dako, Carpinteria, CA). Slides were deparaffinized to DI water. Antigen Retrieval was performed on Dako PT Link water bath. The antigen retrieval was done at 97°C for 20 minutes. The slides were cooled until they reached 65°C. All slides for all antibodies were placed in Tris/EDTA pH 9 (Dako TRS High pH). Slides were washed in Dako wash buffer for 5 minutes. Slides were subjected to a peroxidase Block for 5 minutes. Slides were rinsed twice with wash buffer. Slides were incubated with primary antibody CLPTM1L (rabbit polyclonal, Sigma Aldrich cat# HPA014791, lot A57952) diluted to 1:400 for 30 minutes. Slides were rinsed with wash buffer. Slides were incubated with secondary antibody for 20 minutes and rinsed twice with wash buffer. Slides were incubated with DAB substrate for 10 minutes and rinsed with wash buffer. Slides were stained with hematoxylin for 7 minutes and rinsed with DI water. Slides were dehydrated and coverslipped for viewing. Staining intensity in tumor tissue was scored by three pathologists and averaged. Tumors were scored as negative – 0, weak – 1, intermediate – 2, or strong – 3. Independent scores were averaged. Antibodies for IHC on xenograft tumors were Ki-67 (CRM 325 A,B,C) and Cleaved Caspase-3 (Asp175, Cell Signaling, Boston MA) 1:300.

Survival Studies

Twenty-five pancreatic cancer patients with IHC CLPTM1L expression data on treatment naïve tumors were divided into tertiles according to CLPTM1L staining intensity in tumor tissues, which resulted in division into groups with average staining intensities of <1.15, 1.15–1.75, and >1.75 in low, intermediate, and high expression groups, respectively. Differences in survival and clinicopathologic variables between expression groups were analyzed using STATA software (StataCorp, College Station, Texas,) Chi Square tests.

Cell-surface protein isolation

Proteins exposed on the cell surface were biotinylated and isolated using the Pierce Cell Surface Protein Isolation Kit according to manufacturer's instructions (Pierce Biotechnology, Rockford, IL). Briefly, subconfluent cells in T75 flasks were washed with cold PBS and treated with sulfo-NHS-SS-Biotin in PBS for 30 minutes at 4°C. The reaction was stopped with quenching buffer and cells were scraped into solution in a 50 mL conical tube. Cells were pelleted, resuspended and washed in TBS, and re-pelleted. Cells were lysed in the presence of protease inhibitors and sonicated. Lysates were incubated on ice with periodic vortexing for 30 minutes and clarified by centrifugation. Biotinylated proteins were captured and washed in a NeutrAvidin Agarose loaded column. Proteins were eluted with

500 µL of SDS PAGE Sample Buffer (62.5mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol) with 50mM DTT for 60 minutes at room temperature. Lysates were analyzed by western blotting as described. CLPTM1L antibody precipitated Panc1 cell lysate (CLP +Ct) and a whole cell Panc1 lysate (Lysate +Ct) were used as positive controls. Surface associated EGFR and nucleus associated Cyclin D1 were blotted for as loading and surface specificity controls, respectively.

Immunofluorescence

Cells were grown on tissue culture treated slides, washed with PBS, and fixed with 4% formaldehyde at room temperature for 30 minutes. Cells were gently washed twice with PBS for 2 minutes. Cells were blocked with 10% FBS in PBS for 1 hour at room temperature. Slides were incubated with the indicated primary antibodies (1:100 – 1:250) in PBS with 10% FBS overnight at 4°C. Slides were washed three times for 5 minutes with PBS with gentle shaking. Slides were incubated with secondary Alexa Fluor 594 goat anti-mouse, Alexa Fluor 488 goat anti-rabbit (Molecular Probes, Inc., Eugene, OR), or anti-mouse Cy2 antibodies (1:500) at room temperature for 1 hour protected from light. Slides were washed three times for 5 minutes with PBS. For plasma membrane staining, wheat germ agglutinin (WGA) conjugated with Alexa Fluor 594 (ThermoFisher Scientific, Carlsbad, CA) was added to the slides for 10 min. and slides were washed with PBS per manufacturer's instructions. Thirty microliters of SlowFade Gold with DAPI anti-fading agent (Molecular Probes, Inc., Eugene, OR) was added to the slide, over which a coverslip was laid. Signal was observed using fluorescence and confocal microscopy on an Olympus Fluoview FV1000 confocal laser scanning microscope (Olympus Life Science Europa GmbH, Hamburg, Germany) with a UPLSAPO 20× (dry, NA:0.75) objective.

Flow cytometry

Panc-1 cells were harvested, centrifuged, and resuspended in cold FACS buffer (2% FCS in PBS) at a density of 1×10^6 /mL. Samples (200,000 cells) were incubated for 2 hours with 1:50 anti-CLPTM1L (5–2) ascites at 4°C. Cells were washed with 1 mL FACS buffer three times and incubated with 1:100 anti-mouse-PE (R&D Systems, Minneapolis, MN) for 1 hour at 4°C. After washing 3 times with 1 mL FACS buffer, analysis was carried out on FACS Calibur cytofluorimeter using CellQuest software (Beckton Dickinson, Franklin Lakes, NJ).

Western blotting

Cells were lysed with 100 µL of 1X NP40 lysis buffer containing proteinase inhibitors, sheared 10 times with a 28 gauge needle, spun at $16,000 \times g$ for 30 min, normalized by protein concentration as determined by the Bradford method, and the supernatant boiled for 5 minutes. Twenty microliters of normalized lysate was resolved by SDS-PAGE and immunoblotting analyzed with indicated antibodies. The following antibodies were used: rabbit anti-CLPTM1L (Sigma-Aldrich, St. Louis, MO), mouse anti-Actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bcl-xL – rabbit Bcl2L1 (AbCam, Cambridge, MA), anti-AKT, anti-pAKT (Thr308), anti-EGFR, anti-Cyclin D1 (Cell Signaling, Boston, MA). Quantitation of Western analyses of three independent cultures was done using Image J software (NIH).

Viability assay

Sub-confluent cells plated in triplicate at equal densities on 12-well tissue culture plates were treated with antibodies and/or cisplatin at the indicated concentrations for 24 or 48 hours. One-hundred microliters of CellTiter 96 Aqueous One-Solution Cell Proliferation Assay reagent (MTS, Promega, Madison, WI) was added to 0.5 mL of media on the growing cells. The assay was incubated for 3 hours at 37°C and 5% CO₂. The media and MTS solution was mixed with a pipette and 200 µL was transferred to a 96-well assay plate for measurement of absorbance at 490 nm.

Co-immunoprecipitation assay

AminoLink Co-immunoprecipitation Kit (Pierce Biotechnology, Rockford, IL) was used according to the manufacturer's instructions for co-precipitation of proteins from tumor cell lysates using the indicated antibodies. Polyclonal antibodies α-p110α (Cell Signaling, Boston, MA), α-CLPTM1L (Sigma Aldrich, St. Louis, MO) were covalently linked to resin for immunoprecipitation. Eluted proteins were analyzed by western blotting as described.

Cytotoxicity Assay

Cells were plate at equal density on 24 well tissue culture plates and treated as indicated. Cells were treated with purified 5 µM monoclonal anti-CLPTM1L (5–2) or control antibody for 24 hours followed by 72 hours of treatment with 200 µM gemcitabine or vehicle control (sterile distilled water). Dead cells were fluorescently stained with Yoyo-1 (Life Technologies, Carlsbad, CA) and enumerated on an Incucyte FLR live cell imager (Essen Bioscience, Ann Arbor, MI). Total cell numbers were then enumerated on the imager by staining with Vybrant Dye Cycle Green (Life Technologies, Carlsbad, CA). A killing index was calculated for each well by dividing the number of dead cells by the number of total cells. The killing indices of triplicate groups were averaged.

Soft agar assay

For anchorage independent growth, cells were suspended in 0.4% agarose in complete growth media and plated over 0.8% bottom agar at 10,000 cells per well of a six-well tissue culture dish in triplicate. Cells were fed twice a week over 4 weeks in culture, and colonies were stained using cell staining reagent and protocol from Millipore's Cell Transformation Detection Assay. Images of wells were captured and analyzed by Image J software to count colonies. Average counts per well were determined. P-values were determined using a two-tailed Student's t-Test.

Colony formation assay

Panc1 cells were plated at a density of 1000 per well on 6-well tissue culture plates in triplicate for each treatment group. Cells were treated with the indicated antibodies (5 µg/mL). Colonies were methanol fixed and stained after 7 days in culture with 0.5% crystal violet and counted using ImageJ software using a particle (colony) size cutoff of 4 square pixels (14, 18). Average counts per well were determined.

Tumor spheroid assay

Cells were counted and plated (2,500 cells/well) on ultra-low attachment round-bottom tissue culture plates (Corning Life Sciences, Inc., Tewksbury, MA). Plates were centrifuged at 1000 g for 5 minutes and placed in an Incucyte FLR live cell imager (Essen Biosciences, Ann Arbor, MI) for monitoring of tumor spheroid size using confluence as a measure.

A549 xenograft assay

Tumor cells were cultured, counted, and resuspended in sterile PBS at a concentration of 1×10^7 cells/mL. A volume of 200 μ L (2×10^6 cells respectively) was injected subcutaneously into the flanks of athymic nude mice at an age of 6 weeks. Mice were kept in individual ventilated cages to avoid infection. All mice were fed a commercial sterile diet and water ad libitum and were housed in an animal facility under a 12 h light/dark cycle at constant temperature and humidity. Five mice per treatment group were treated with 1 mL/kg of the indicated ascites intraperitoneally in 200 μ L of sterile PBS on days 0, 5, and 12 after growth of tumors to an average of 30 mm³ and randomization into groups. The health of these mice was monitored 3 times weekly and tumor sizes were measured periodically using digital calipers until sacrifice. Tumor volumes were calculated by the formula for an ellipsoid, $4/3\pi r^1 r^2 r^3$. P-values were determined by one-way ANOVA with Tukey HSD post test where $K > 2$. Animal weights were measured throughout the study to monitor toxicity. Significance of differences between groups was determined by two-tailed Student's t-test. Mice were euthanized and tumors excised and frozen for further analysis. Protein was isolated from 30 mg of tumor tissue using a TissueLyser LT (Qiagen, Venlo, Limburg, Netherlands) and 300 μ L of RIPA buffer with HALT protease/phosphatase inhibitor (Life Technologies, Carlsbad, CA).

Panc1/MiaPaCa xenograft assay

Twelve week old female NOD CB17-Prkdc^{scid}/NcrCrl mice (Charles River, Innovo Kft., Hungary) were kept in individual ventilated cages to avoid infection. All mice were fed a commercial sterile diet and water ad libitum and were housed in an animal facility under a 12 h light/dark cycle at constant temperature and humidity. For the induction of rodent model of human pancreas tumors, 5×10^6 Panc1 or MiaPaCa human pancreatic ductal adenocarcinoma cells were injected in a 100 μ L volume of PBS subcutaneously into the right flank of the mice. Fifteen days after cancer cell inoculation, mice were randomized and treated once weekly intravenously through the tail vein for 6 weeks. Tumors were measured by calipers every three days. Two different groups, each containing 9 mice, were studied: (1) ascites control (2.5 mL/kg) and (2) ascites containing antibody 5-2 (2.5 mL/kg). For MiaPaCa studies, 2.5 mg/kg purified control or indicated antibody was administered intravenously as described. The study was performed according to the Institutional and National Animal Experimentation and Ethics Guidelines under ethical clearance (XXIX./3610/2012). Animal weights were measured throughout the study to monitor toxicity. Significance of differences between groups was determined by two-tailed Student's t-test. Mice were euthanized and tumors excised and frozen for further analysis. Protein was isolated from 30 mg of tumor tissue using a TissueLyser LT (Qiagen, Venlo, Limburg, Netherlands) and 300 μ L of RIPA buffer with HALT protease/phosphatase inhibitor (Life

Technologies, Carlsbad, CA). Panc1 tumor samples (collected 8 days after last anti-CLPTM1L treatment) were paraffin-embedded. HE and IHC staining was carried out.

Results

CLPTM1L is overexpressed in lung and pancreatic tumors and associated with poor clinical outcome

Having previously demonstrated overexpression of CLPTM1L in lung adenocarcinomas compared to that in adjacent normal lung tissue (12), we employed immunohistochemical analysis to evaluate its expression in human pancreatic ductal adenocarcinoma and confirm its expression in human lung tumors. Staining was uniformly negative in normal ductal epithelia (n=16), while positive in 90% (28/31) of tumor cells ($r=0.78$, $p<5\times10^{-10}$) (Figure 1A). Staining intensity, while variable between patients, was highly consistent within the tumor tissue of individual patients. Therefore, distribution of staining was invariably 100% and was not a factor in calculating positivity. Negative tissues surrounding the positively stained tumor cells in these sections are tumor stroma. Tumors had an average staining score of 1.46 while that of normal ductal epithelium was 0. Tumors expressing low levels of CLPTM1L as defined by an average score of 1.15 or less were associated with increased overall survival, while patients with higher expression had shorter median overall survival times. Median overall survival among all patients was 18.5 months. At that time point, the survival rate of patients with low expression in tumor tissues was significantly higher ($p=0.0294$) at 86%, compared to 44 and 33% in intermediate and low expressing groups. Patients with low CLPTM1L expression had a median overall survival time of 31 months, while patients with intermediate and high expression had median survival times of 17 and 14 months, respectively (Table S1). Chi-square testing verified that clinicopathological features of age, race, gender, and disease stage did not affect expression. Survival studies excluded 6 patients who had received neoadjuvant therapy. Representative tissues stained with CLPTM1L antibody are shown in Figure 1B, showing positivity in tumor over normal tissues. Similarly, human lung adenocarcinoma tissues were positive for CLPTM1L expression by IHC (Figure 1C). Demographic information and clinical characteristics of the patients in this study are detailed in Table S1.

Cell-surface localization of CLPTM1L in Lung and Pancreatic Tumor Cells

Immunofluorescence staining of non-permeabilized A549 cells for CLPTM1L revealed a punctate cell-surface staining pattern in both pancreatic adenocarcinoma cells and in lung adenocarcinoma cells (Figure 1D). To confirm the localization of CLPTM1L at the surface of tumor cells, we isolated cell-surface proteins from A549 human lung tumor cells by biotinylation and extraction with a streptavidin resin. Western analysis of whole cell and cell-surface lysates demonstrated a prevalence of CLPTM1L at the cell-surface (Figure 1E). EGFR, as a cell surface-localized control protein, was detected at the cell-surface. The ratio of signal for cell surface CLPTM1L to that for total protein was greater than that of EGFR, suggesting that a larger fraction of CLPTM1L was localized to the cell surface in these cells. Cyclin D1, used as a nuclear protein control, was not detected at the cell surface. We confirmed surface exposure of CLPTM1L in Panc1 cells by labeling of live cells with purified anti-CLPTM1L monoclonal antibody and a phycoerythrin conjugated secondary

antibody. Cells labeled with anti-CLPTM1L were positive for fluorescence with mean fluorescence intensity of 341 units compared to 149 in control labeled cells (Figure 1F) confirming the presence of surface-exposed CLPTM1L in live pancreatic tumor cells.

Transmembrane structure prediction of CLPTM1L

CLPTM1L is predicted to be a serpentine transmembrane protein with 6 transmembrane domains by analysis using a hidden Markov model (TMHMM - <http://www.cbs.dtu.dk/services/TMHMM/>) (Supplementary Figure S1A). Protein disorder prediction using IUPred (<http://iupred.enzim.hu>) supports this model and indicates a disordered region within the large predicted extracellular region near the N-terminus of the protein (Supplementary Figure S1B). Areas of high order are predicted to contain helical transmembrane regions, and those of the highest disorder are predicted to be without secondary structure. Areas on either side of the disordered region (residues 141–162) are predicted extracellular globular domains. We have designated these domains extracellular region 1 (ECR1) and extracellular region 2 (ECR2). Figure 2A represents the overall structural prediction for CLPTM1L including transmembrane domains, extracellular globular domains, the disordered region, and N-glycosylated residues.

Design and development of anti-CLPTM1L monoclonal antibodies

To effectively target CLPTM1L we developed a panel of monoclonal antibodies against exposed epitopes of the protein. A target region of the CLPTM1L protein was defined as the hydrophilic loop region that is likely to be exposed on the cell surface in the protein's native form (residues 32–264), excludes the identified disordered region (residues 141–162), and excludes N-glycosylation sites (residues 91, 101, and 229). The hydrophilic loop region has been found to be necessary for CLPTM1L function (14). A proprietary algorithm was utilized to select epitopes with minimal similarity to other proteins, hydrophilicity, surface-orientation, and flexibility (Supplementary Figure S2). Monoclonal antibodies were raised in balb/c mice by immunization with peptides representing these epitopes, generation of hybridoma clones, collection of ascites, and Protein A affinity purification. The resulting ten antibodies targeted epitopes designated as epitopes 1 through 5. Epitopes 1 and 2 reside within ECR1, while epitopes 3, 4, and 5 reside within ECR2. These epitopes are represented in Supplementary Table S2. Aliquots of these monoclonal antibodies were used for *in-vitro* and *in-vivo* screening. The three best performing candidates (1–1, 5–2, and 5–3) in terms of inhibition of survival signaling, cisplatin resistance, anchorage independent growth, and tumorigenesis were selected for propagation of hybridoma cell lines and purification of antibodies.

Our panel of 10 monoclonal antibodies targeting the extracellular globular domains of CLPTM1L was tested for detection of CLPTM1L and immunofluorescent labeling of surface CLPTM1L. Monoclonal antibodies labeled plasma membrane-localized CLPTM1L in non-permeabilized Panc1 cells (Figure 2B). All antibodies resulted in a cell-surface staining pattern when used for immunofluorescence on non-permeabilized A549 cells, with antibodies targeting ECR2 exhibiting a more distinct punctate staining in cisplatin-treated A549 cells (Figure 2C). Monoclonal antibodies strongly detected a single 62-kilodalton CLPTM1L protein band by western blot corresponding to the major band detected with

polyclonal anti-CLPTM1L (Figure 2D left panel). The antibody clone 1-1 blot included two faint higher molecular weight bands. No additional bands were detected. In addition, the single band detected by monoclonal antibodies was diminished upon specific depletion of CLPTM1L with shRNA, demonstrating the specificity of the antibodies for CLPTM1L (Figure 2D center panel). Specificity of the polyclonal antibody and lead monoclonal antibody 5-2 was confirmed by western blotting in cells expressing endogenous CLPTM1L and those also expressing a FLAG-tagged exogenous CLPTM1L (Figure 2D right panel).

Anti-CLPTM1L inhibits surface accumulation and chemotherapy-induced interaction of CLPTM1L with p110 α

With our previous evidence that CLPTM1L interacts with the catalytic subunit of PI3-kinases in fibroblasts, we sought to determine if CLPTM1L interacts with p110 α in tumor cells, and whether that interaction is affected by anti-CLPTM1L treatment. Given the role of CLPTM1L in chemoresistance, we also sought to determine if gemcitabine treatment affected this interaction in human pancreatic adenocarcinoma cells. Co-immunoprecipitation assays were conducted in Panc1 cells. Gemcitabine caused a modest increase in the amount of CLPTM1L that co-precipitated with p110 α in these cells, suggesting that the interaction between these proteins may be enhanced upon treatment (Figure 3A). The increase in co-precipitation of CLPTM1L and p110 α was not observed upon treatment with monoclonal α -CLPTM1L (5-2) or (1-1). To determine if cell surface accumulation of CLPTM1L was diminished by treatment with anti-CLPTM1L, which occurs through internalization with other antagonistic immunoglobulin drugs, we conducted biotinylation of surface proteins followed by isolation and western blotting. Cells were treated with anti-CLPTM1L (5-2) ascites or control ascites (1:1000) for 96 hours. Anti-CLPTM1L treatment resulted in downregulation of surface-associated CLPTM1L (Figure 3B). As evidenced in Figure 3A, levels of CLPTM1L in CLPTM1L immunoprecipitated and whole-cell lysates were not appreciably diminished upon anti-CLPTM1L treatment, suggesting that downregulation was cell surface specific. Masking by the treatment monoclonal antibody is not expected since detection was performed using a polyclonal antibody raised against the larger, residues 75–210 of the protein, which does not include the monoclonal epitope.

Inhibition of AKT phosphorylation and chemotherapeutic resistance with anti-CLPTM1L antibodies

We previously demonstrated that targeting CLPTM1L in tumor cells modulates Bcl-xL and Akt survival signaling and sensitizes cells to killing with cisplatin (12, 16). In the current study, A549 and H838 human lung tumor cells were treated with monoclonal anti-CLPTM1L ascites at 1:5000 and 1:1000 dilutions. After culture for 48 hours with treatment, western blotting for Bcl-xL, total Akt1, and phospho-Akt1 was conducted. Decreased accumulation of Akt1 with polyclonal anti-CLPTM1L treatment was observed in human lung tumor cell lines (Figure 4A). To determine the effect of treatment with polyclonal antibody on sensitivity to cisplatin, cells were treated with control antibody or dilutions of anti-CLPTM1L from 0.01 to 1 μ g/mL for 24 hours. Cells were then exposed to cisplatin at 0, 10, and 20 μ M concentrations for 48 hours. Treatment with anti-CLPTM1L resulted in a decrease in cell viability following cisplatin treatment that was dependent on the dose of antibody (Figure 4B). These results demonstrate sensitization to cisplatin killing using anti-

CLPTM1L. Likewise, treatment of pancreatic adenocarcinoma cells with anti-CLPTM1L 5-2 resulted in dose-dependent inhibition of Akt phosphorylation (Figure 4C). Akt phosphorylation was also inhibited in MCW670 primary human pancreatic tumor cells upon treatment with anti-CLPTM1L (Supplementary Figure S3A). Treatment of pancreatic adenocarcinoma cells with anti-CLPTM1L resulted in significantly increased killing by gemcitabine ($p=0.007$) (Figure 4D). Anti-CLPTM1L treatment resulted in an approximately 2-fold increase in gemcitabine killing (20% vs. 10% with gemcitabine alone).

Treatment of A549 and H838 human lung tumor cells with our panel of monoclonal antibodies resulted in depletion of Bcl-xL and Akt phosphorylation with a range of efficacies. Western analysis revealed that treatment with antibodies targeting epitopes 1 and 5 resulted in dose dependent decreases in Bcl-xL and phospho-Akt1 accumulation (Figure S3B,C). Likewise, sensitization to cisplatin killing was accomplished by treatment of tumor cells with anti-CLPTM1L monoclonal antibodies with a range of efficacies (Supplementary Figure S3D). A summary of these results for each antibody clone is given in Supplementary Table S3.

Inhibition of both KRas and EGF driven anchorage independent growth and colony formation by anti-CLPTM1L

With the knowledge that inhibition of CLPTM1L can inhibit K-Ras driven tumor spheroid formation and tumorigenesis (16), we sought to determine if antibodies targeting of CLPTM1L was inhibitory to the anchorage independent growth of human lung tumor cells. Treatment of A549 and H838 lung tumor cells with 1 $\mu\text{g/mL}$ anti-CLPTM1L significantly decreased the number of anchorage independent colonies by 55% and 65%, respectively compared to solvent control-treated cells ($p<0.05$) (Figure 5A). Treatments with 1 $\mu\text{g/mL}$ antibody or control IgG were administered one day after plating and again one week later. EGF treatment induced anchorage independent growth in Beas2B human bronchial epithelial cells, which was inhibited by 76% upon treatment with 1 $\mu\text{g/mL}$ anti-CLPTM1L antibody (Figure 5B). Colony formation of Panc1 cells in adherent tissue culture was inhibited by anti-CLPTM1L monoclonal antibodies, particularly those targeting epitope 5 ($p<0.005$) (Figure 5C). Anti-CLPTM1L (1-1, 5-2, and 5-3) also robustly inhibited colony formation of A549 lung, GBM4 and U-251 glioblastoma, and HeLa cervical human tumor cells (Supplementary Figure S4). To investigate the role of Akt signaling in colony formation inhibition by anti-CLPTM1L, Panc1 cells were stably transfected with a constitutively active myristoylated Akt (mAkt) or control vector. Expression of mAkt modestly rescued colony formation, with an average of 15 colonies per well with mAkt compared to 6 with vector control (Figure 5D).

Inhibition of anchorage independent growth of A549 cells by monoclonal antibodies is summarized in Supplementary Table S3. Among all monoclonal antibodies raised against the extracellular domains of CLPTM1L, only those raised against epitope 5 were effective in inhibiting anchorage independent tumor spheroid growth of H838 cells (Supplementary Figure S5, Supplementary Table S3).

Inhibition of the growth of established tumors *in-vivo* by anti-CLPTM1L antibodies

To determine if the growth of established human lung tumor xenografts could be inhibited using antibodies targeting CLPTM1L, we utilized an athymic nude mouse model of tumorigenesis. Mice bearing established subcutaneous A549 cell tumors were separated into groups of equal average tumor size and variance. Monoclonal antibodies recognizing epitope 5 in ECR2 were evaluated in xenograft models for inhibition of the growth of established human lung tumor xenografts. A549 cell xenografts were established and mice were separated into groups of equal average tumor size and variance. Mice were treated intraperitoneally with 1 mL/kg of the indicated monoclonal antibody or control ascites on days 0, 5, and 12, post-randomization. Antibodies 5-2 and 5-3 had a robust and significant inhibitory effect on tumor growth (Figure 6A). Monoclonal clone 5-2 inhibited tumor growth by 88% ($p<0.01$). In Panc1 human pancreatic xenograft models, lead candidate monoclonal anti-CLPTM1L 5-2 significantly inhibited tumor growth by 75% over 55 days post treatment initiation ($p<0.01$) (Figure 6B). The difference in average tumor size was significant from day 21 post treatment on. Similar results were obtained with purified monoclonal antibody treatment in MiaPaCa human pancreatic adenocarcinoma xenografts, with significantly decreased growth and regression of tumors as early as 7 days post treatment initiation (Supplementary Fig S6A). Panc1 xenografted mice were treated with 2.5 mL/kg antibodies on days 0, 7, 14, 21, and 28, post-randomization. Both control and treated Panc1 tumors had the same immunophenotype as observed by H&E stain (blind to pathologist) (Figure 6C). Treated tumor tissue was blindly determined by the pathologist to exhibit more widespread cell death and much less vital tumor tissue. Immunohistochemical staining of tumor tissues revealed that positive staining for CLPTM1L was diminished in anti-CLPTM1L-treated mice. As determined by pathologist review, heterogeneity in the staining signal for CLPTM1L was due to technical variation within the sections with regard to thickness of the section, proximity to necrotic tissue, and vacuolation. Again, masking by the treatment monoclonal antibody is not expected since IHC was performed using a polyclonal antibody raised against a different region of the protein. Accumulation of phosphorylated Akt in tumor tissues was ablated by anti-CLPTM1L treatment of both Panc1 ($p<0.005$) and A549 ($p<0.05$) xenografts (Figure 6D). There were no significant differences in average animal weight from that of the control group indicating an absence of acute toxicity (Supplementary Figure S6B).

Discussion

A growing body of evidence has demonstrated a critical role for the tumor survival protein CLPTM1L in tumorigenesis. Our discovery that CLPTM1L resides on the surface of tumor cells reveals the potential targetability of this protein for preventive or therapeutic purposes. As previous publications have overlooked the presence of CLPTM1L at the cell surface, the use of non-permeabilized cells for IF, isolation of surface-exposed proteins, or flow cytometry may be necessary methods for the determination of cell-line and tissue specificity of surface expression.

To our knowledge, we are the first to report an association of CLPTM1L expression with poor outcome in pancreatic cancer patients. While significant, these findings are limited by

the inherently small number of available tumor tissues and will require further validation with larger data sets.

Mutant KRas is the prevalent driver of pancreatic cancer, and KRas and Akt are predominant contributors to chemoresistance in pancreatic tumors (19). K-Ras mutations in lung tumors are associated with poor survival (20). Effective strategies to target K-Ras have been highly sought after. However, little success in targeting this prevalent oncoprotein has been had. Likewise, in lung cancer, targeted therapies exist for EGFR driven tumors, but resistance remains an issue and no effective targeted therapy exists for KRas driven tumors. Our results provide a potential approach for targeting Ras driven cancers as well as cancers driven by other oncogenes. Although our mechanistic findings are not specific to K-Ras, it is apparent that there is a critical interaction between mutant K-Ras signaling, and the CLPTM1L/Akt signaling axis (16). Here we demonstrate the effectiveness of targeting CLPTM1L with specific antibodies in the inhibition of anchorage independent growth of both K-Ras transformed lung and pancreatic tumor cells and EGF transformed human bronchial epithelial cells. A subset of anti-CLPTM1L monoclonal antibodies was also effective in the inhibition of H838 tumor spheroid growth. H838 tumor cells do not harbor activating mutations in K-Ras, nor H-Ras, nor N-Ras, indicating that anti-CLPTM1L antibody treatment is also effective in non-Ras driven tumor cells as well as those driven by Ras oncogenes.

Here we provide a description of the design and development of a panel of monoclonal antibodies targeting specific regions of the CLPTM1L protein, a subset of which affect tumor cell survival signaling and chemotherapy resistance and are highly effective in inhibition of anchorage independent growth and tumorigenesis. Monoclonal inhibitory anti-CLPTM1L antibodies were effective in inhibiting the growth of established tumors *in-vivo*, particularly those targeting epitope 5. Overall, antibodies targeting this epitope were the most inhibitory to CLPTM1L function. Antibodies targeting ECR1 of CLPTM1L (1-1, 1-2, 2-1, and 2-2) were generally less inhibitory and resulted in a more diffuse, less intense staining pattern with immunofluorescence, although clone 1-1 did inhibit Akt phosphorylation. It is possible that antibodies targeting different domains as 1-1 and 5-2 do may bind a differently localized subset of protein, or directly affect localization or staining appearance. Staining appears more punctate and may be more vesicular in Panc1 cells compared to A549 cells. We posit that this may be simply due to differences in cellular morphology. Due to the need to collect endpoint tissues from xenografted mice while treatment remained effective and a finite supply of treatment antibody, the duration of mouse experiments was restricted. This resulted in endpoint tumor volumes that were smaller than is often seen in xenograft experiments that are conducted for longer duration, or with higher cell numbers, but demonstrated striking efficacy against human lung and pancreatic tumors. Clearing of CLPTM1L positive cells from tumor tissues may cause a decrease in the number of positively staining cells by IHC; however, diminished signal intensity upon anti-CLPTM1L treatment as seen in xenograft tumor samples may also be a result of decreased surface-localized CLPTM1L as demonstrated in Figure 3.

Our results also demonstrate that targeting CLPTM1L can sensitize tumor cells to killing by genotoxic agents, including cisplatin and gemcitabine. We have demonstrated

chemosensitization by anti-CLPTM1L antibodies in pancreatic and lung cancer cells. Akt and Bcl-xL are intimately related to survival of tumor cells harboring oncogenic drivers such as K-Ras (21, 22) and chemoresistance in lung and pancreatic cancers, and are key targets for chemosensitization (22–26). Treatment of human pancreatic adenocarcinoma cells with gemcitabine promoted the interaction of CLPTM1L with PI3K p110 α , implicating this interaction and downstream Akt signaling as playing a role in chemoresistance. Further, this promotion of the CLPTM1L-p110 α interaction was inhibited by monoclonal anti-CLPTM1L, providing mechanistic insight into the functional inhibition of CLPTM1L by these immunoglobulin agents. The effect on CLPTM1L-p110 α interaction may have been due to the fact that anti-CLPTM1L caused a general downregulation of CLPTM1L accumulation at the cell surface, potentially because of internalization of the antibody-antigen complex. This mechanism of downregulation has been previously described for antagonistic antibodies, including cetuximab (27). Expression of a myristoylated Akt partially rescued colony formation, providing further evidence for inhibition of the PI3K/Akt signaling pathway as a mechanism of action. Exogenous activation of this pathway only resulted in a modest increase in colony formation, suggesting that other signaling pathways may be involved. While we previously showed constitutive activation of Akt to restore anchorage independent growth, additional CLPTM1L-regulated factors may influence proliferation or survival in adherent culture. Further research, including mechanistic studies and additional *in-vivo* models of combination therapy and chemosensitization will be important for the clinical translation of therapy using CLPTM1L inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Expression of CLPTM1L in tumors and association with clinical outcome

A) Immunohistochemical scoring of staining with antibody specific to CLPTM1L in pancreatic tumor vs. normal ductal epithelial tissues in 31 patients. Intensity scoring – 0=negative, 1=weak, 2=intermediate, 3=strong B). Representative immunohistochemical staining of pancreatic tumor tissues for CLPTM1L. Inset - IHC on non-tumor ductal epithelium within desmoplastic tissue. Scale bars = 50 μ M. C) Representative immunohistochemical staining of human lung adenocarcinoma for CLPTM1L. Scale bars = 100 μ M. D) Immunofluorescent staining of non-permeabilized A549 lung tumor cells and primary pancreatic adenocarcinoma cells (MCW462) using DAPI dye to stain cell nuclei and polyclonal antibody raised against the predicted extracellular hydrophilic loop of human CLPTM1L followed by a secondary FITC conjugated antibody to detect CLPTM1L. A non-specific mouse IgG antibody was used as a control. Staining indicates punctuated plasma membrane localization of the protein in non-permeabilized cells. Scale bars = 10 μ M. E) Western blotting of A549 cell surface and whole cell protein lysates for CLPTM1L,

transmembrane receptor EGFR and nuclear protein Cyclin D1. F) Flow cytometry analysis of live Panc1 cells stained with control or purified anti-CLPTM1L monoclonal antibody 5-2.

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Figure 2. Transmembrane structure prediction and monoclonal antibody targeting of CLPTM1L
 A) Summary of the predicted transmembrane structure, interstitial disordered region (red), and glycosylated residues (green) of full length CLPTM1L. Images were generated using Protter (Omasits, 2013; PMID:24162465) B) Immunofluorescent staining of non-permeabilized Panc1 cells transfected with either vector control or an exogenous CLPTM1L expression construct using monoclonal CLPTM1L (5-2), and co-stained with a red fluorescent cell membrane stain (WGA). Scale bars = 50 μM. C) Immunofluorescent staining of non-permeabilized, A549 cells (treated with 20μM cisplatin for 48 hours) with monoclonal CLPTM1L antibodies 1-1 (1000X) and 5-2 (400X). D) Western blotting for CLPTM1L in A549 cells in duplicate with monoclonal and polyclonal antibodies. Blotting of lysates from A549 cells with vector control or shRNA depletion of CLPTM1L (center

panel), and of lysates from Panc1 cells transfected with vector control or a Flag-tagged exogenous CLPTM1L expression construct (right panel) with monoclonal antibodies.

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Figure 3. Anti-CLPTM1L depletes cell surface CLPTM1L and inhibits chemotherapy-induced interaction of CLPTM1L with p110α

A) Western blotting of Panc1 cell lysates precipitated with a resin covalently linked with anti-p110α or anti-CLPTM1L rabbit polyclonal antibody. Cells were treated with non-relevant control antibody (αHA), monoclonal anti-CLPTM1L (5-2), or monoclonal anti-CLPTM1L (1-1) for 48 hours and 200 μM gemcitabine for 24 hours at the time of lysis as indicated. Input blots represent lysates without antibody precipitation as loading controls. B) Western blotting in duplicate of cell surface proteins isolated from Panc1 cells treated with 1:1000 control ascites (VWF) or anti-CLPTM1L ascites (5-2) for 96 hours. EGFR serves as a surface protein loading control and Cyclin D1 as a non-surface protein control.

Figure 4. Inhibition of AKT phosphorylation and cisplatin or gemcitabine resistance by anti-CLPTM1L antibodies

A) Western blotting for phospho-Akt in A549 cells treated with polyclonal anti-CLPTM1L 48 hours. B) Relative cell viability of A549 cells after treatment with polyclonal anti-CLPTM1L for 24 hours followed by 48 hours of treatment with cisplatin (CDDP). * $p < 0.05$. C) Western blotting demonstrating loss of Akt phosphorylation upon 48 hrs anti-CLPTM1L 5-2 treatment of pancreatic tumor cells and quantification of phospho- to total Akt signal ratio (bottom panel). D) Relative viability of pancreatic tumor cells treated with 5 μg/mL anti-CLPTM1L (5-2) and/or 100 μM gemcitabine for 72 hrs.

Figure 5. Inhibition of both KRas and EGF driven anchorage independent growth and colony formation of human lung and pancreatic tumor cells by anti-CLPTM1L

A) Soft agar colony formation assays of A549 and H838 human lung tumor cells treated with control or anti-CLPTM1L polyclonal antibodies. * $p < 0.05$ B) Anchorage independent colony formation of Beas2B human airway epithelial cells upon EGF stimulation, with control or polyclonal anti-CLPTM1L treatment. * $p < 0.05$. C) Colony formation of Panc1 PDAC cells treated for 5 days with control or anti-CLPTM1L monoclonal antibodies (1-1, 5-2, or 5-3). Average colony counts per well (below). Error bars represent standard deviations. *** $p < 0.0005$, * $p < 0.05$. D) Colony formation of Panc1 cells stably expressing myristoylated Akt (mAkt) or vector control and treated with control or anti-CLPTM1L monoclonal antibody (5-2). Average colony counts per well (Below). Error bars represent standard deviations. Western blotting confirmed Akt phosphorylation in mAkt cells (below, right).

Figure 6. Inhibition of the growth of established tumors *in-vivo* by anti-CLPTM1L antibodies
 A) Mean tumor volume in athymic nude mice injected subcutaneously with A549 tumor cells and treated with 1 mL/kg control ascites or monoclonal anti-CLPTM1L ascites (5-2, or 5-3) on days 0, 5, and 12 after tumor establishment. Error bars represent standard error of the mean. ** $p < 0.01$ as calculated for the final time point. B) Mean tumor volume in athymic nude mice injected subcutaneously with Panc1 tumor cells and treated with intravenous injection of 2.5 mL/kg ascites control or ascites containing antibody 5-2. Error bars represent standard error of the mean. ** $p < 0.01$ Arrows indicate antibody treatments. C) Representative H&E staining and immunohistochemistry for CLPTM1L, Caspase 3, and Ki67 on control and anti-CLPTM1L 5-2-treated Panc1 xenograft tumors. Scale bars represent 50 μ m. D) Western blotting for phospho- and total Akt in control and anti-CLPTM1L 5-2-treated Panc1 xenograft tumor lysates. Three individual tumors are

represented per group. Blot quantitation (right panel). Values are expressed as pAkt:total Akt ratios relative to average control values. **p<0.01, *p<0.05.

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