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LL-37 as a therapeutic target for late stage prostate cancer

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Abstract

BACKGROUND—The antimicrobial peptide, LL-37 (leucine-leucine-37), stimulates proliferation, angiogenesis and cellular migration, inhibits apoptosis and is associated with inflammation. Since these functional processes are often exaggerated in cancer, the aim of the present study was to investigate the expression and role of LL-37 in prostate cancer (PCa) and establish its value as a therapeutic target.

METHODS—We evaluated the expression of LL-37 and the murine orthologue, Cathelicidin Related Anti-Microbial Peptide (CRAMP) in human and murine prostate tumors, respectively. Compared to normal/benign prostate tissue, both LL-37 and CRAMP were increasingly over-expressed with advancing grades of primary prostate cancer and its metastasis in human tissues and in the Transgenic Adenocarcinoma Mouse Prostate (TRAMP) model, correspondingly. We subsequently knocked down CRAMP in the highly tumorigenic TRAMP-C1 cell line via a RNA interference (RNAi) strategy to examine the importance of CRAMP on cellular proliferation, angiogenesis, invasion, apoptosis, activation of signaling pathways and tumor kinetics.

RESULTS—Abrogation of CRAMP expression led to decreased proliferation, invasion, type IV collagenase, and the amount of phosphorylated Erk1/2 and Akt signaling *in vitro*. These results were paralleled *in vivo*. Syngenic implantation of TRAMP-C1 cells subjected to CRAMP knock-down resulted in a decreased tumor incidence and size, and the down regulation of pro-tumorigenic mechanisms.

CONCLUSIONS—CRAMP knockdown in a murine prostate cancer model analogously demonstrated the tumorigenic contributions of LL-37 in PCa and its potential as a novel therapeutic target for the treatment of PCa and potentially, other cancers over-expressing the peptide.

Keywords

CRAMP; androgen independent; proliferation; angiogenesis; metastasis

INTRODUCTION

LL-37 is named for the first 2 amino acids (leucine-leucine) in its 37 amino acid sequence and is the only cathelicidin derived antimicrobial peptide in humans. (1–6). In a non-pathological system, beyond its anti-microbial properties, LL-37 has been shown to augment proliferation, angiogenesis, protection from apoptosis and epithelial-mesenchymal-transition (7–12). These mechanisms, when taken out of their normal physiological context all serve as hallmarks of cancer and are utilized by transformed/malignant cells to promote tumor growth and metastasis (13). Interestingly, clinical findings have shown LL-37 to be up-

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regulated in several aggressive solid tumor types including carcinomas of the breast, ovary and lung; however, studies invoking the causative effects of LL-37 on tumorigenesis and metastasis have not been comprehensively performed (14–17). Although LL-37 is normally cleaved from its precursor, human cathelicidin antimicrobial protein-18 (hCAP-18), by neutrophil protease 3 to become activated, evidence suggests cancer cells also produce an enzyme to proteolytically cleave their secreted hCAP-18 independent of neutrophils (16,18). To date, no study has reported over-expression of LL-37 in prostate cancer or analyzed mechanisms of LL-37 expression in genitourinary tract tumor progression for potential use as a therapeutic target.

The present study demonstrates that LL-37 is over-expressed progressively in human prostate tumors as the Gleason score increases and in bone metastasis. The murine orthologue, CRAMP, is also over-expressed progressively in murine prostate tumors spontaneously developing in the TRAMP model and was additionally found in areas of lung metastasis. Since CRAMP exhibits similar structure, source and function as LL-37, this allowed for a comparable, pre-clinical evaluation using CRAMP within an immunocompetent mouse model to further elucidate the role of LL-37 in prostate cancer and its potential as a therapeutic target (2,19–25). Using the TRAMP model of prostate cancer, the present study examined tumorigenic mechanisms affected by CRAMP modulation and analogously demonstrated the value of LL-37 as a therapeutic target in human prostate cancer. Since CRAMP is produced by leukocytes in addition to cancer cells, the study considered it important to employ an immunocompetent mouse prostate cancer model while non-systemically targeting the cancer's endogenous source of CRAMP in order to pre-clinically assess the efficacy of specifically targeting the peptide in the prostate cancer tissue itself.

By genetically altering CRAMP expression in a highly tumorigenic TRAMP prostate cancer cell line with high levels of endogenous CRAMP expression, the present study suggests that CRAMP has an important role in cancer cell proliferation, invasiveness, and tumor angiogenesis. Further, down-regulation of CRAMP led to inhibition of subcutaneously implanted tumor growth in the recipient host. In correlation with other studies, Erk 1/2 was found to be a central signaling pathway regulated by CRAMP. These findings illustrate the potential of LL-37 to be a therapeutic target in PCa as well as in other cancers also over-expressing this peptide.

MATERIALS AND METHODS

Tissue samples

Human prostate tissue and xenograft tumor samples were obtained from the NCI sponsored tissue procurement facility at UAB in accordance with approved institutional IRB protocol (W. Grizzle, director). Tissues obtained from this consortium were processed by a whole mount technique after fixation in 10% neutral buffer. All tissue samples were reviewed by board certified pathologists and malignant lesions characterized using the Gleason Score according to the International Society of Urological Pathology criteria (26). Tissues from the TRAMP model were obtained from the study's TRAMP breeding colony in a pure C57BL/6 background, heterozygous for the probasin-Tag transgene and histopathic scoring of tissue samples were based on assessments used in characterizing the TRAMP model (27). Animal care and treatments were conducted in accordance with established guidelines and protocols approved by the UAB Institutional Animal Care Committee.

Cell lines and Reagents

The mouse prostate cancer cell lines TRAMP-C1 and TRAMP-C3 (CRL-2730 and CRL-2732 respectively, American Type Culture Collection, Manassas, VA) were cultured in DMEM high glucose w/L-glutamine, without sodium pyruvate media (DMEM-D5796, Sigma-Aldrich Inc., St. Louis, MO) supplemented with 5% Nu-serum IV (BD Nu-Serum, Franklin Lakes, NJ), 5% fetal bovine serum (Sigma-Aldrich, Inc.), 0.005mg/mL insulin (Sigma-Aldrich, Inc.), 1% penicillin-streptomycin (Invitrogen, Grand island, NY) and 10 nM dihydrotestosterone (Sigma-Aldrich, Inc.). The culture medium was changed every 2–3 days and maintained at 37°C in a humidified, 5% CO₂ incubator. Antibodies used were: CRAMP for immunocytochemistry (cat.# sc-34169, Santa Cruz Biotechnology Inc., Santa Cruz, CA); CRAMP for immunohistochemistry (cat. # PA-CRPL-100, Innovagen, Lund, Sweden), LL-37 (cat. # PA-LL37-100, Innovagen); Akt (cat. #9272), Phospho-Akt (clone # 193H12), Beta-Actin (clone # 13E5), Erk1/2 (clone # 137FS), Phospho-Erk1/2 (cat. # 9101), Phospho-Erk 1/2 for IHC (clone # D13.14.4E), Ras (cat. # 3965), RhoA (clone # 137FS), Cleaved Caspase-3 (cat. # 9661) and MMP9 (clone # G657), all from Cell Signaling Technologies, Inc. Danvers, MA); Ki67 (cat. # ab15580) and CD31 (cat. # ab28364) from Abcam, Cambridge, MA.

Construction of a shRNA vector and development of stable clones with CRAMP knockdown

Nineteen-base-pair targets were selected from unique regions of the CRAMP coding sequence using T. Tuschl siRNA design guidelines (28). Among the tested shRNA sequences, the one that showed maximum efficacy, and was used in the present study consisted of 19-mer sense (GAACCGGACAAAGACCTAA) and anti-sense strands separated by a 9-bp region and contained BamHI or HindIII restriction sites respectively, for directional cloning (Integrated DNA Technologies, Coralville, IA). The oligos were annealed and directly ligated into the pRNAT.U6/Neo expression plasmid (Genscript, Piscataway, NJ). TRAMP-C1 cells were cultured in 6-well plates to approximately 90% confluency in antibiotic-free medium. The cells were then transfected with shRNA expressing plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions and incubated at 37°C in the presence of 5% CO₂. After 24 hrs, the transfection medium was replaced with normal medium and after another 24 hrs, the cells were trypsinized and split 1:4 and cultured in selection medium containing 800 µg/mL neomycin.

RNA Isolation and Analysis

Total RNA was isolated from TRAMP-C1, TRAMP-C1^{CRAMP^{sh}}, TRAMP-C1^{scrambled} and TRAMP-C3 cells using Trizol Reagent (Invitrogen) and concentrations measured colorimetrically with an Eppendorf BioPhotometer (Eppendorf NA, Hauppauge, NY). The samples were further checked on a formaldehyde gel to verify the purity of RNA and lack of degradation. Single-stranded cDNAs were synthesized from the total RNA using a Biorad cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) containing RNase H⁺ MMLV reverse transcriptase and oligo (dT) primers. The cDNA was then used for RT-PCR analysis using SYBR green (Sigma-Aldrich) to detect expression levels of CRAMP mRNA. GAPDH was used as the internal control for the RT-PCR analysis. The primer sequences used in PCR reactions were:

CRAMP-forward	5'-AACCAGCAGTCCCTAGACACCAAT-3'
CRAMP-reverse	5'-TTCCTTGAAGGCACATTGCTCAGG-3'
MMP-2-forward	5'-ATGACCACCGCCCTGCAGGTCCA-3'
MMP-2-reverse	5'-TCCGACCAGTCACCGAACCCCATAGG-3'
MMP-9-forward	5'-GCCCTGGAAGTCTAGACGACA-3'

MMP-9-reverse 5'-TTGGAACTCACACGCCAGAAG-3'
 GAPDH-forward 5'-TCAACAGCAACTCCCACTCTTCCA-3'
 GAPDH-reverse 5'-ACCCTGTTGCTGTAGCCGTATTCA-3'

Immunocytochemistry Analysis

Cells were grown to 90% confluency on slide chambers, washed with PBS and fixed with 10% formalin containing 0.1% Triton X-100 for 20 minutes, followed with PBS (containing Ca^{+2} and Mg^{+2}) and blocked with 2% BSA. Cells were then stained with an anti-CRAMP antibody (Santa Cruz Bio-Technology) overnight at 4°C and washed with PBS containing 0.05% Tween-20 and stained with a FITC-conjugated secondary antibody (Invitrogen) for 1 hr and washed with PBS (3 × 10 minutes). The cells were imaged at ×400 original magnification on a Leica DMI4000B fluorescent microscope (Leica Microsystems Inc, Bannockburn, IL) to qualitatively determine CRAMP levels.

MTS Proliferation Assay

Cells were seeded in multiple 96 well plates at 1×10^4 cells/well in replicate cultures. Twenty-four hours after plating, 20 μL /well of CellTiter 96 AQueous One Solution Reagent (Promega, Madison, WI) was added to a plate and incubated for 1 hr at 37°C in a humidified, 5% CO_2 incubator, then removed and assayed on a microplate reader at 490 nm to establish baseline readings. Successive readings were conducted on remaining plates every 24 hrs up to 96 hrs post seeding to establish growth curves.

Matrigel Invasion Assay

Insert chambers for 24 well plates containing a Matrigel Basement Membrane-like Matrix over an 8 μm pore PET membrane (BD Biosciences) were re-hydrated with serum-free medium for 2 hrs at 37°C in a humidified, 5% CO_2 incubator. The hydration medium was removed from chambers and 1.5×10^4 cells, suspended in 0.5 mL of fresh serum-free medium, were plated into the hydrated chambers and placed into wells containing 0.75 mL/well of medium plus 5% FBS and were incubated at 37°C in a humidified, 5% CO_2 incubator for 22 hrs. Cells not invading through the Matrigel to the underside of the membrane were removed by scrubbing the interior side of membrane with cotton swabs and the invading cells, undisturbed on the underside, were fixed and stained using a Diff-Quik fixing/staining kit (Dade Behring Inc, Newark, DE). After allowing the membranes to dry, they were cut from insert chambers, mounted on slides and photographed so that counting of stained, invasive cells could be done.

Zymography Analysis

Serum-free media in which the cells were cultured was collected after 48 hrs incubation. Media was concentrated and washed with PBS using a 100 KDa cut-off Americon Ultra centrifugal filter device (Millipore, Billerica, CA). Equal amounts of protein (50 μg) from each cell group were run on 10% zymogram gels (Biorad) for 2 hrs, then incubated for an additional 2 hrs in renaturation buffer (Biorad) and developed overnight in development buffer (Biorad) at 37°C and stained with coomassie blue for 2 hrs followed by de-staining and photographing.

Western Blot Analysis

Unmodified TRAMP-C1 and TRAMP-C1 cells stably transfected with shRNA plasmids targeting CRAMP or non-targeting scrambled sequence, were harvested for protein isolation using a lysis buffer containing protease (Roche Diagnostics, Indianapolis, IN) and phosphatase (Thermo Scientific, Rockford, IL) inhibitors. Following denaturation, the

samples were separated on a 10% polyacrylamide gel and transferred overnight to a nitrocellulose membrane (Millipore, Bedford, MA) followed by blocking using 2% non-fat milk and incubated with primary antibodies (Cell Signaling Technology). Beta-actin antibody was used as a loading control (Cell Signaling Technology). Following overnight incubation with primary antibody at 4 degrees Celsius and subsequent washes (3×10 minutes) with $1 \times$ Tris-Buffered Saline with Tween-20 (TBST), a secondary antibody, conjugated to horseradish peroxidase was applied for 1 hour at room temperature (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), then washed with TBST (3×10 minutes) and blots were then incubated with enhanced chemiluminescence reagent (GE Healthcare Bio-Sciences Corp.) according to manufacturer's directions and developed on a Fuji LAS-3000 chemiluminescence developer. Densitometric scanning was performed to quantify band intensities.

Tumorigenicity Study

TRAMP cells (4×10^5) were implanted subcutaneously into 12–13 week old C57BL/6 male mice ($n=8$) and measured for allograft tumor development starting one week post-implantation and thereafter every three to four days via external measurement in two dimensions using a digital caliper. The average radius values from these measurements were used in the formula for spherical volume to calculate tumor volumes. At time of sacrifice, tumor samples were collected and formalin-fixed for immunohistochemistry analyses.

Immunohistochemistry

Immunohistochemistry was performed on 5 μ m sections of paraffin-embedded tumor tissues to determine levels of LL-37 or CRAMP, proliferation (Ki67), apoptosis (cleaved caspase-3), angiogenesis (CD31), MMP9 and Phospho-Erk1/2 using the specific antibodies mentioned above. After rehydration, slides were incubated with citrate buffer for 20 minutes in a steamer for antigen retrieval and endogenous peroxidase quenched by incubation with a 3% H_2O_2 aqueous solution for 20 minutes at room temperature. Primary antibody incubation was overnight at 4°C. Secondary antibodies used were linked with HRP and incubated with sample for 1 hour at room temperature. Visualization of the bound complex was performed using diaminobenzidine tetrahydrochloride. The slides were counterstained minimally with hematoxylin. All murine IHC was performed on tumors of similar size to insure comparable assessment of tumorigenic factors. To accomplish this, TRAMP-C1^{CRAMP^{sh}} tumors used for IHC were permitted to grow in the host for longer periods of time in order to attain a tumor volume analogous to control-sized tumors.

Statistical Analysis

Data were analyzed by Student's *t* test. Values provided are the Mean $\pm$ SEM and the differences were considered significant if $p < 0.05$.

RESULTS

Human prostate tumor tissues over-express LL-37 in direct proportion to Gleason score

To establish the clinical relevance and importance of LL-37 to PCa, immunohistochemistry for LL-37 expression was performed on human prostate tissue arrays containing benign and malignant prostate tumor tissue samples. A total of 30 cases of PCa and 5 normal/benign prostate tissue samples were analyzed. The samples of PCa ranged from low to high Gleason scores and included metastasis to bone. Staining with LL-37 antibody demonstrated that prostatic adenocarcinomas expressed higher levels of the peptide compared to normal/benign tissues (Fig. 1A). Additionally, there was a concomitant increase of LL-37 as the Gleason score of tumor increased, including high expression in bone metastasis (Fig. 1A).

Interestingly, we noticed that the LL-37 over-expression was found at or near the luminal surface or within the cytoplasm of cells in primary tumor tissues that were well- to moderately-differentiated (Fig. 1A, black arrows), but in poorly-differentiated primary and metastatic bone lesions, expression of LL-37 was predominantly located in the nuclei (Fig. 1A, black arrows).

Androgen independent PCa is commonly more aggressive and difficult to treat. Xenograft tumors grown from the CWR22 cell line typically respond very well to androgen depletion (29). To examine the correlation of LL-37 with androgen dependence, the LL-37 expression in androgen dependent CWR22 tumors was compared to that of CWR22 tumors that had developed androgen independence. The androgen independent xenografts had higher expression of LL-37 compared to those that remained androgen dependent (Fig 1B). Again, the cellular localization of LL-37 was primarily nuclear.

TRAMP prostate tumor tissues over-express CRAMP in a severity of grade dependence

Prostate tissues obtained at different time points from TRAMP mice and age-matched, non-transgenic, littermate controls were analyzed by immunohistochemistry. Results indicated that by week 13, TRAMP prostate tissues showed a slightly higher level of CRAMP than negative controls (Fig. 2A). By week 32, there were substantial differences between age-matched samples, with relatively low expression in controls versus high expression in well-developed and poorly differentiated prostate adenocarcinomas of the TRAMP model (Fig. 2A). Additionally, metastatic lesions in lung tissue had high CRAMP expression compared to surrounding normal lung and stromal tissue (Fig. 2B).

TRAMP cell lines with phenotypical polarities differentially express CRAMP and genetic transfer of shRNA established stable cell lines for studying the pro-tumorigenic importance of CRAMP

The subsequent study compared CRAMP expression in TRAMP-C1 and TRAMP-C3 cells that were derived from the same prostate tumor of a 32-week old TRAMP mouse but, are opposed in terms of proliferation rates and tumorigenicity. TRAMP-C1 cells have a higher rate of proliferation than TRAMP-C3 cells and are also tumorigenic when syngeneically transplanted into C57BL/6 mice, whereas TRAMP-C3 cells never establish tumors in hosts (30). TRAMP-C1 cells were found to express CRAMP nearly 4-fold higher than TRAMP-C3 cells (Fig. 3A). To examine the role of CRAMP in pro-tumorigenic mechanisms, we developed a stable cell line having down-regulated CRAMP expression using CRAMP shRNA. CRAMP knocked-down TRAMP-C1 (TRAMP-C1^{CRAMPsh}) cells had a greater than 5-fold reduction of CRAMP RNA expression than the parental TRAMP-C1 cells (Fig. 3A). As an additional control, a cell line stably transfected with a scrambled 19-mer shRNA sequence with no homology to the mouse genome (TRAMP-C1^{scrambled}) was developed that had no significant alteration of CRAMP RNA expression as compared to the parental TRAMP-C1 cells (Fig. 3A). CRAMP protein levels of TRAMP-C1 and TRAMP-C1^{scrambled} controls, and TRAMP-C1^{CRAMPsh} were qualitatively compared using immunocytochemistry. Results correlated with the RT-PCR data to further demonstrate successful knockdown of CRAMP in the TRAMP-C1^{CRAMPsh} cells (Fig. 3B). These established cell lines were subsequently used to determine the role and significance of CRAMP for tumorigenicity and other functional characteristics in prostate cancer.

CRAMP knock-down inhibits proliferation

The *in vitro* MTS assays of cells demonstrated similar proliferation rates amongst the TRAMP-C1 and TRAMP-C1^{scrambled} cell lines. In contrast, the TRAMP-C1^{CRAMPsh} cell line proliferated at a significantly lower rate than parental or scrambled control cell lines ($p < 0.001$; Fig. 4).

CRAMP knock-down results in decreased invasion and matrix metalloproteinase levels

The results of the Matrigel Invasion assay indicated no significant change in migration/invasion between TRAMP-C1 and TRAMP-C1^{scrambled} cells, suggesting that the scrambled sequence had no significant off target effects on the cell's ability to invade. However, knock-down of CRAMP expression in the TRAMP-C1^{CRAMP^{sh}} cells resulted in a significant reduction of invasion as compared to parental TRAMP-C1 cells and TRAMP-C1^{scrambled} cells ($p < 0.001$ and $p < 0.05$ respectively; Fig 5A). Based on the results of the Matrigel Invasion assay which demonstrated CRAMP's ability to promote cellular invasion, we reasoned that CRAMP expression would promote cells to produce type IV collagenase(s) and therefore, break down extracellular matrix. Semi-quantitative RT-PCR was performed to examine relative gene expression levels of two type IV collagenases (MMP2 and MMP9) associated with increased metastasis. The RT-PCR analysis indicated a 45 percent reduction of MMP2 among the TRAMP-C1^{CRAMP^{sh}} vs. control cells, but this did not reach statistical significance (Fig. 5B). However, comparison of MMP9 expression between the cell lines demonstrated that CRAMP knockdown resulted in almost complete abolishment of MMP9 expression ($p < 0.05$; Fig 5B). To corroborate the RT-PCR data, zymograms were performed to measure the MMP2 and MMP9 protein levels. Qualitative results of the zymograms indicated that protein levels of both collagenases were decreased to levels approximating the relative gene expression levels shown by semi-quantitative RT-PCR (Fig. 5C). Taken together, these assays demonstrated that CRAMP knock-down resulted in decreased prostate cancer cell invasion and decreased levels of MMP2 and MMP9.

CRAMP knock-down results in inhibition of Erk1/2 and Akt signaling

Following the above results, which demonstrated that CRAMP augments proliferation and invasion of TRAMP cells *in vitro*, key signaling pathways were analyzed to clarify the findings at a molecular level. Signaling pathways were analyzed based on their established roles in tumorigenesis. Among these, the study demonstrated that CRAMP knock-down resulted in down-regulation of both phosphorylated Erk1/2 and phosphorylated Akt (Fig. 6).

Down-regulation of CRAMP expression by shRNA results in decreased tumor incidence, tumor volume, proliferation, angiogenesis, MMP9 and phosphorylated Erk1/2 levels *in vivo*

Mice receiving TRAMP-C1^{CRAMP^{sh}} cells had a 12-fold decrease in average tumor volume as compared to the mice implanted with TRAMP-C1^{scrambled} cells ($p < 0.01$; Figure 7A & B). Furthermore, knock-down of CRAMP in TRAMP-C1^{CRAMP^{sh}} cells resulted in a 37.5 percent reduction in tumor incidence as compared to TRAMP-C1^{scrambled} cells. Immunohistochemical staining of tumors with CRAMP antibody confirmed TRAMP-C1^{CRAMP^{sh}} tumors had maintained a decreased CRAMP expression *in situ* compared to the TRAMP-C1^{scrambled} control tumors (Fig. 7C). Additional IHC of tumor tissues was performed to illustrate the *in vivo* effects of CRAMP knock-down on proliferation, angiogenesis, apoptosis, MMP9 and phosphorylated Erk1/2. To ensure comparable assessment of these factors, the TRAMP-C1^{CRAMP^{sh}} tumors used for IHC were permitted to grow in the host for longer periods of time in order to attain a tumor volume analogous in size to TRAMP-C1^{scrambled} control tumors before being harvested. Comparisons of markers for proliferation (Ki67), angiogenesis (CD31), MMP9, and phospho-Erk1/2 within tumors derived from these two cell lines demonstrated that the knock-down of CRAMP in TRAMP-C1 cells led to an *in vivo* inhibition of these properties/molecules (Fig. 7C). Additionally, TRAMP-C1^{CRAMP^{sh}} tumors demonstrated more necrotic areas as would be expected with decreased angiogenesis. There was no difference between the two groups in the induction of apoptosis, observed with an antibody directed against cleaved caspase 3 (Fig. 7C).

DISCUSSION

The study's clinically relevant finding that LL-37 is up-regulated in human prostate cancer along with previous findings by others documenting the peptide's over-expression in other cancers suggest its importance as a collective pro-tumorigenic constituent in many types of cancer. This issue will be elucidated as more cancers are examined for LL-37 over-expression. Over-expression of LL-37 in prostate tissue suggest a possible, but as yet, unexplored therapeutic approach to the treatment of PCa; the second leading cancer in terms of mortality rates among men in the United States (31). Because LL-37 expression was found to be greater with increasing Gleason score and in metastatic lesions suggest its importance during neoplastic progression and in the metastatic phase as well as its potential therapeutic value for targeting advanced primary and metastatic prostate cancer. Additionally, the finding that LL-37 is highly over-expressed in androgen independent CWR22 xenograft tumors suggest that targeted LL-37 therapy may be useful in treating patients having recurrence following androgen ablation. The usefulness of the TRAMP model for study of LL-37 in human prostate cancer and the value of LL-37 as a therapeutic target was demonstrated by the model having CRAMP expression in prostate tissues similar to that of LL-37 in human prostate tissues, including a relationship to normal prostate tissue as well as the noted increase in expression with advancing histopathic score (mice) and Gleason score (humans), and additionally in metastasis.

Previously unreported in cancer was our observation that over-expressed LL-37 was on or near the luminal surface of cells within well differentiated tumors, however as the tumors progressed and became more poorly differentiated or metastasized, there were increased levels of expression as well as a change in localization of LL-37 to the nuclei. This observation had been made with bacterial infections, in which LL-37 was shown to bind exogenous DNA via its cationic charge and translocate with the DNA to the nucleus (32). The advantage or function of this process in cancer requires future study.

Both our *in vitro* and *in vivo* studies demonstrated that proliferation and invasive potential were decreased as a result of targeted down-regulation of CRAMP, indicating the targeting of LL-37 in human prostate cancer may serve both to inhibit primary tumor growth and to decrease the metastatic potential of PCa. In addition to the knockdown of CRAMP in the tumorigenic TRAMP-C1 cell line, the study also examined the effects of up-regulating CRAMP expression in the non-tumorigenic TRAMP-C3 cell line via cDNA. This resulted in a significant increase in proliferation and invasion (data not shown). This further strengthens the hypothesized importance that LL-37 may have in human prostate cancer in terms of neoplastic progression.

The decrease in both tumor incidence and size in mice transplanted with the TRAMP-C1^{CRAMP^{sh}} cell line again demonstrates that LL-37 in humans may play an important role in prostate tumorigenesis and may serve as a valuable therapeutic target. Ki67 staining for proliferation showed a modest decrease in proliferation within CRAMP knockdown tumors and was comparable to data on *in vitro* proliferation. High endogenous levels of CRAMP in tumors formed from the TRAMP-C1^{scrambled} cells also correlated with a rich tumor vasculature. The decreased angiogenesis and subsequent increase of necrotic lesions in TRAMP-C1^{CRAMP^{sh}} tumors suggests angiogenesis may be very important with respect to the pro-tumorigenic effects of CRAMP and that down-regulation of LL-37 might have parallel results in human prostate tumors. It has been proposed that LL-37 works independent of VEGF (10). Because many anti-angiogenic therapies for PCa cancer are aimed at inhibiting VEGF from binding to its receptors, targeted down-regulation of LL-37 could be used as an adjuvant to anti-VEGF therapies or be efficacious for patients refractory to anti-VEGF therapies (33). Immunohistochemical analysis of MMP9 demonstrated a

reduction in tumor tissue levels of the collagenase, which again confirmed the *in vitro* data. Since metastasis is usually the cause of mortality rather than the primary tumor, targeted down-regulation of LL-37 may prove efficacious in decreasing metastasis and mortality among patients with prostate cancer. Because evasion of apoptosis is another hallmark of cancer, and studies have demonstrated the ability of LL-37 to protect cells from programmed death, we sought to determine if targeted down-regulation of CRAMP would result in increased apoptosis. However, immunohistochemical analysis for cleaved caspase 3 did not indicate CRAMP knock-down has any discernable effect on apoptosis as there was no clear advantage regarding increased apoptosis among tumor tissues with inhibited levels of CRAMP. It is certainly possible that other molecules along the complex apoptosis intrinsic and extrinsic pathways are affected and this requires further investigation.

Results on key signaling pathways conducive for tumor growth in the present study demonstrated that both phosphorylated Erk1/2 and phosphorylated Akt are modulated by CRAMP. These results confirm other studies demonstrating the involvement of Erk1/2 in LL-37 and CRAMP signaling (17,34,35). Additionally, activation of Erk1/2 and Akt pathways facilitate angiogenesis and Erk1/2 also stimulates prostate cancer cellular proliferation (36–39). Furthermore, MMP2 and MMP9 secretion in prostate cancer cells, and their migration/invasion are regulated in part through Erk1/2 activation (40–41). Taken together with this study's findings that activation levels of Erk1/2 and Akt are downregulated following CRAMP knock-down, this provides a likely explanation of how targeted knock-down of CRAMP affected the observed decreases in angiogenesis, proliferation, invasion and MMP levels.

CONCLUSIONS

The present study defined CRAMP as having a significant role in the growth of prostate cancer. More importantly, based on overexpression of LL-37 in human prostate cancer tissues, this study analogously established a proof-of-principle that targeting of LL-37 might serve as an efficacious therapy for prostate cancer. The standout therapeutic advantages were clearly those of decreased angiogenesis and decreased collagenase levels. Therefore, targeted down-regulation of endogenous LL-37 in prostate tumors by approaches such as small molecular inhibitors, or targeted disruption by gene transfer could prove to be efficacious in therapies based upon angiogenesis and metastasis suppression. Furthermore, though this study addresses this point in a model of prostate cancer, it is reasonable to postulate that the proposed use of targeted LL-37 therapy will be applicable to other cancers overexpressing this small yet potent peptide.

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Figure 1A **Figure 1B** **Figure 1. LL-37 is upregulated in human prostate cancer**

Immunohistochemistry demonstrated that LL-37 is up-regulated in primary and metastatic lesions of prostate cancer. Tumors with moderate differentiation had LL-37 located primarily on the luminal surface of the cells (black arrows), while more poorly differentiated and metastatic tumors had LL-37 located primarily in the nuclei (black arrows) (original magnification $\times 400$) (A). Immunohistochemistry also demonstrated androgen independent xenograft tumors of the human prostate cancer cell line CWR22 grown in nude mice have over-expression of LL-37 compared to androgen dependent CWR22 xenograft tumors (original magnification $\times 200$) (B).

Figure 2A

Figure 2B

Figure 2. CRAMP is upregulated in TRAMP prostate cancer

Immunohistochemistry demonstrated CRAMP is highly expressed in primary prostate tumors from the TRAMP model as compared to age-matched controls and increases with severity of histopathic grade (original magnification $\times 400$) (A). Metastatic lung lesions also over-expressed CRAMP (* denotes bronchiole) (original magnification $\times 600$) (B).

Figure 3A

Figure 3B

Figure 3. TRAMP-C1 has lower CRAMP expression than TRAMP-C3 and TRAMP-C1^{CRAMPsh} clones have knocked-down CRAMP levels

RT-PCR indicated TRAMP-C1 cells have significantly higher CRAMP expression than non-tumorigenic TRAMP-C3 cells ($p < 0.001$). TRAMP-C1^{scrambled} clones showed no off target effect on CRAMP expression, whereas CRAMP expression in the TRAMP-C1^{CRAMPsh} clone was significantly decreased greater than 5-fold ($p < 0.001$) (A). *In situ* fluorescent staining of TRAMP-C1, TRAMP-C1^{scrambled} and TRAMP-C1^{CRAMPsh} cells demonstrated a decrease in CRAMP protein following shRNA targeting of CRAMP compared to the parental and scrambled controls (B).

Figure 4. CRAMPsh inhibits proliferation

A MTS proliferation assay illustrated a significant decrease in proliferation of TRAMP-C1^{CRAMPsh} cells compared to TRAMP-C1 and TRAMP-C1^{scrambled} ($p < 0.001$).

Figure 5A

Figure 5B

Figure 5C

Figure 5. CRAMPsh decreases invasion and matrix metalloproteinase levels

A Matrigel Invasion assay demonstrated the invasion of TRAMP-C1^{CRAMPsh} cells were reduced to nearly half that of the TRAMP-C1 cell line (original magnification $\times 200$, $p < 0.001$) (A). RT-PCR analysis illustrated near abolishment of MMP9 expression following CRAMP knockdown ($p < 0.05$) and a 45% decrease of MMP2, though not significant (B). Zymography analysis corroborated this observation with a similar reduction in the collagenase protein levels (C).

Figure 6. Knockdown of CRAMP expression results in down-regulation of Akt and Erk1/2 signaling pathways

Western blotting comparing TRAMP-C1, TRAMP-C1^{scrambled} and TRAMP-C1^{CRAMPsh} cells for key signaling proteins involved in tumorigenesis demonstrated that phosphorylated Akt and Erk1/2 were decreased in the TRAMP-C1^{CRAMPsh} cells compared to TRAMP-C1 and TRAMP-C1^{scrambled} cells.

Figure 7A

Figure 7B

Figure 7C

Figure 7. Targeted downregulation of CRAMP on tumor growth and pro-tumorigenic proteins *in vivo*

Mice implanted with TRAMP-C1^{CRAMPsh} had a significant reduction in tumor growth compared to mice implanted with TRAMP-C1^{scrambled} group (**A**), and in volume on day 34 between the two groups ($p < 0.01$) (**B**). Immunohistochemistry of tumor tissues with indicated antibodies demonstrated CRAMP knock-down resulted in decreased proliferation (Ki67), angiogenesis (CD31), MMP9, and phosphorylated-Erk1/2 in tumor tissues, but had no observable effect on apoptosis (cleaved caspase-3), (original magnification $\times 400$) (**C**).