Research Paper

Silencing of Lactotransferrin Expression by Methylation in Prostate Cancer Progression

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Original manuscript submitted: 02/01/07
Manuscript accepted: 04/28/07

This manuscript has been published online, prior to printing for Cancer Biology & Therapy, Volume 6, Issue 7. Definitive page numbers have not been assigned. This manuscript has been published online, prior to printing for Cancer Biology & Therapy, Volume 6, Issue 7. Definitive page numbers have not been assigned.

ABSTRACT

Background: Cancer cells gain selection advantages by the coordinated silencing of protective and by the activation of cell proliferation/cell survival genes. Evaluations of epithelial cell transcriptome of benign and malignant prostate glands by laser capture microdissection (LCM) identified Lactotransferrin (LTF) as the most significantly downregulated gene in prostate cancer (CaP) cells (p < 10−6). Frequent downregulation, significant association of LTF with PSA recurrence-free survival in CaP patients and the established antitumorigenic effects of LTF in experimental cancer models have provided impetus to evaluate LTF expression features and mechanisms in CaP specimens.

Methods: LTF mRNA expression analysis was performed in LCM derived benign and malignant prostate epithelial cells by using Affymetrix GeneChip and QRT-PCR. LTF protein expression was assessed in tissue specimens by immunohistochemistry and in serum samples from CaP patients compared to healthy male control by using ELISA. Mechanism of LTF downregulation was analyzed in 5-azadeoxycytidine treated LNCaP and LAPC4 cells using MALDI-TOF MS. Proliferation and cell cycle analysis of CaP cells by FACS flow cytometry was assessed in LNCaP cell cultures.

Results: Quantitative analysis of LTF mRNA expression in tumor cells revealed marked downregulation of LTF with significant associations to decreased PSA recurrence-free survival of CaP patients (n = 100, p ≤ 0.0322). Moreover, low levels of LTF protein expression was observed in tumor tissues as well as in sera from CaP patients (p ≤ 0.0001). LTF promoter downstream CpG island methylation was found in LNCaP and LAPC4 cells. Furthermore, replenishing of LTF by supplementing growth media with LTF protein resulted in the reduced cell growth. Cell cycle analysis revealed robust increases in apoptosis in response to LTF treatment.

Conclusion: This study highlights the potential for LTF in chemoprevention and to become a biologically relevant prognostic marker of CaP, suggesting that silencing of the LTF gene may be causally linked to CaP progression.

INTRODUCTION

While recent trends in stabilizing incidence and decreasing mortality rates are encouraging, prostate cancer (CaP) remains a major health burden for American men.1 Similar to other cancers, prostate cancer develops through a multi-step process of genetic changes, each providing some type of growth advantage for cells, leading to their progressive conversion into aggressive cancer cells. Intensive investigations on CaP-specific genetic alterations are beginning to define common genetic changes in CaP.2,3 Chromosomal rearrangements/translocations leading to activation of ETS transcription factors (ERG, ETV1 and ETV4) or methylation mediated silencing of protective genes such as GSTP1 appear to be most common potentially causal gene alterations in CaP.2,5 Expression alterations of the GSTP1,6 DD3,7 AMACR,8 and proto-oncogene, ERG,9 represent the most frequent CaP associated gene expression changes.

LTF, a non-heme iron binding glycoprotein, belongs to the transferrin gene family and arose from an ancient intragenic duplication.10 LTF was first discovered in milk and was also found in a variety of secretions derived from glandular epithelium cells, such as prostate and salivary glands as well as in many biological secretions including tears and semen. LTF plays an immunomodulatory role and participates in inflammatory response. A number of reports suggested that LTF has an antitumorigenic role11 utilizing a variety of mechanisms including regulation of NK (natural Killer) cell activity,12 modulation of expression of G1 proteins,13 and enhancement of apoptosis.14 LTF has anti-tumor function and could inhibit development of tumors in animal models.10,15

KEY WORDS

LTF, downregulation, methylation, PSA doubling time, chemoprevention
Bovine lactotransferrin (bLTF) has been found to significantly inhibit colon, esophagus, lung, bladder and liver cancers in rats.\textsuperscript{16-20} Moreover, bLTF was shown to be chemopreventive in liver carcinogenesis induced by diethyl nitrosamine (DEN) alone or by DEN plus 2-amino-3, 8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), or in MeIQx-induced colon carcinogenesis in rat animal models.\textsuperscript{21}

However, the role of LTF in human prostate cancer remains to be defined. In this study, we evaluated the expression of LTF gene in CaP specimens and assessed the prognostic value of decreased or lost LTF expression in CaP progression. Furthermore, we investigated the mechanism of LTF downregulation and the cell growth inhibitory effects of LTF in prostate cancer cell models systems.

**MATERIALS AND METHODS**

**GeneChip\textsuperscript{®} analyses.** Total RNA was isolated from LCM derived tumor and benign epithelial cells according to protocol number WRAMC WU# 04-2871-98k. The RNA was quantitated, amplified, biotinylated, and hybridized to high-density oligonucleotide human genome array HG U133A (Affymetrix, Santa Clara, CA).\textsuperscript{9} The gene expression data from paired benign and malignant prostate epithelial cells from 20 patients (40 GeneChips\textsuperscript{®}) were subjected to multidimensional scaling (MDS) analysis as described previously.\textsuperscript{9}

**Validation of LTF expression by QRT-PCR.** Total RNA isolated from the LCM derived tumor and benign epithelial cells, was converted to cDNA (Sensiscrypt, Qiagen, Valencia, CA). Quantitative gene expression analysis was performed by TaqMan-based QRT-PCR as described.\textsuperscript{22} LTF copy number was determined in tumor and benign samples (n = 100) and normalized to GAPDH copy numbers.\textsuperscript{22} Statistical analyses were performed using the SAS software package (version 9.0, SAS Institute Inc., Cary, NC). PSA recurrence free survival was correlated with LTF expression ratio.\textsuperscript{23}

**Northern blot analysis.** Twenty micrograms of total RNA from LNCaP and LAPC4 cells and RNA Millennium\textsuperscript{™} Markers (Ambion Inc., TX, USA) were electrophoresed in denaturing formaldehyde 1% agarose gels. Following overnight capillary transfer to Protran Pure Nitrocellulose Transfer and Immobilization Membranes (Schleicher & Schuell Keene, N.H., USA). Blots were probed with LTF cDNA of 2.9 Kb in size (OriGene Technologies, Inc., Rockville, MD USA), GAPDH-Mouse DECAprobe template and Millennium Marker\textsuperscript{™} Probe Template (Ambion Inc., TX, USA), which were radioactively labeled with \( \alpha \)-32p-dCTP (GE Healthcare, Buckinghamshire, UK) using a random-primed DNA labeling kit named, Amersham Rediprime\textsuperscript{™} II Random Prime Labeling Systems (GE Healthcare, Buckinghamshire, UK). Hybridization was carried out for approximately 18 h in NorthernMax Prehybridization/ Hybridization buffer (Ambion Inc., Tex., and USA). The membrane was washed twice with 1x SSC and 0.1% SDS for 15 min each and then washed twice with 0.25 x SSC and 0.1% SDS for 15 min each and autoradiographed. Sizes of the target genes were determined from RNA Millennium\textsuperscript{™} Markers on the autoradiograph.

**Analysis of LTF protein expression by immunohistochemistry (IHC).** All tissues were fixed in 10% neutral buffered formalin and were paraffin embedded. Prostate tissue specimens were selected with the criteria of having sufficient tissue within the paraffin block to perform the immunohistochemistry (IHC). Tissue sections (100 \( \mu \)m) of the radical prostatectomy derived whole-mount prostate specimens from 30 CaP patients were stained with H&E and anti-LTF by IHC.\textsuperscript{24}

**Determination of LTF protein concentration in CaP patient Serum.** Serum LTF protein concentration of biopsy-verified CaP patients who underwent radical prostatectomy at Walter Reed Army Medical Center (WRAMC) was compared to CaP-free control group with normal digital rectal examination (DRE) and a low serum PSA (<2.5 ng/ml). Serum LTF levels were measured in a cohort of 34 CaP patients and 35 healthy male controls using Bioxytech Lacto-f-EIA (OxisResearch, Portland, OR) and a Multiskan Ascent ELISA plate reader according to the manufacturer’s recommendation.

**Activation of LTF expression in LNCaP cells by 5-azacytidine treatment.** LNCaP and LAPC4 cells were treated with 5 \( \mu \)M of 5-azacytidine DNA methyl transferase inhibitor (Sigma-Aldrich Co., St. Louis, MO) for 14 days at concentration of 5 \( \mu \)M with the change of cell culture media every two days (concentration was optimized prior to the actual experiment to avoid toxicity). Quantitative methylation analysis of genomic DNA from treated and untreated LNCaP cells were performed by SequEnom\textsuperscript{®} (San Diego, CA) using EpiTYPER, a bisulfite-treatment-based MALDI-TOF MS method for detection and quantitation of methylation.\textsuperscript{25} To amplify the bisulfite modified +200 to +600 CpG-rich region of the LTF gene, a primer pair was designed as follows: forward primer, 5′-GGGTTAAGTTTTGAATAGGGG-3′ and reverse primer 5′-TAAAACCCAAAAACTTATTCC-3′. The GSTPI gene -300; +100 region was amplified by using the following primer set: forward 5′ TGGGAAAGGGAAAGTTTTT-3′ and reverse 5′-CCCATAAAACTCTAAACCCCATC-3′. For gene expression analysis total RNA was extracted from untreated and 5-azacytidine treated LNCaP and LAPC4 cells using RNeasy Mini kit (QIAGEN\textsuperscript{®}, Valencia, CA). For gene expression analysis total RNA was extracted from untreated and 5-azacytidine treated LNCaP and LAPC4 cells using RNeasy Mini kit (QIAGEN\textsuperscript{®}, Valencia, CA). The RNA was transferred on nitrocellulose membrane to assess the expression of LTF by Northern Blot analysis in both LNCaP and LAPC4 cells as described above. GAPDH expression was assessed as the quality control of RNA samples.

**Effects of LTF on LNCaP cell proliferation and cell cycle.** LNCaP cells (5 \( \times \) 10\(^4\) cells/well, passage 28) in RPMI medium were plated into 12-well plates and allowed to grow in a 5% CO\(_2\) incubator at 37°C for 24 hours. LTF dose response was assayed by supplementing the media with various concentrations of LTF protein (Sigma-Aldrich, St. Louis, MO) from a 1000 \( \mu \)M stock solution to final concentrations of 0, 10, 30 and 50 \( \mu \)M. Cell growth was determined at 24, 48, 72 and 96 hours. The experiment was performed in triplicate (n = 3) and repeated twice. Cell morphology was monitored and photographed using an inverted microscope (DMIRE2, Leica Microsystems, Bannockburn, IL). Statistical analysis was performed with SPSS version 12 software package. p < 0.05 values were considered to be statistically significant. For cell cycle analysis Propidium Iodide (PI) staining and FACS analysis was performed. The fluorescent dye Propidium Iodide preferentially binds to double-stranded nucleic acids and allows fluorescent intensity to be used as an indicator of the cellular DNA content. LNCaP cells were grown to ~70% confluence in 25-cm\(^2\) flasks and were treated with 10 \( \mu \)M of human lactoferrin for 24 h and 48 h at 37°C. Following treatments, the adherent cells were trypsinized and washed twice 1x PBS and then fixed with 70% cold ethanol at -20°C for 30 min. Cells were washed twice with cold PBS following ethanol fixation. Cells were resuspended in PBS containing 10 \( \mu \)g/ml RNase (Boehringer Mannheim, Indianapolis, IN) and incubated at 37°C for 30 min to remove double stranded RNA. Subsequently, cells were stained with PI (Boehringer Mannheim, Indianapolis, IN) at a concentration of 50 \( \mu \)g/ml and stored at 4°C in the dark until flow cytometry was performed.

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performed. PI stained LNCaP cells were analyzed using an EPICS ELITE ESP (Beckman Coulter, Miami, FL) flow cytometer. Cell cycle analysis of DNA histogram is performed with ModFit LT software (Verity Software House, Topsham, ME). Experiments were carried out in triplicates.

RESULTS

LTF mRNA and protein levels are downregulated in prostate cancer. Evaluations of prostate epithelial cell (PEC) transcriptome using laser capture microdissection (LCM) derived benign and tumor epithelial cells and Affymetrix GeneChipO revealed LTF (downregulated in tumor cells) and AMACR (upregulated in tumor cells) as the most significant (p < 10−6) malignant cell-specific differential expression (Fig. 1A). CaP associated LTF expression alteration was validated at protein level. Significant downregulation of LTF protein was noted in all 30 whole-mount prostate specimens analyzed by IHC. As expected, AMACR overexpression (95%) and decreased GSTP1 expression (100%) was observed in virtually all tumor specimens (Supplementary Fig. 1). Low LTF “Tumor (T) / Benign (B)” expression (copy number) showed a significant correlation with shorter PSAR-free survival by Kaplan-Meier unadjusted analysis (p value ≤0.0322; Fig. 2B). Using chi-square analysis, a significant association

Figure 1. LTF is downregulated in prostate cancer. (A) Gene expression signature representing LTF downregulation in prostate tumor (T) cells compared to matched benign (B) epithelium. The heat map displays tumor and benign expression of LTF in 20 CaP patients (9 with High Risk and 11 with Moderate Risk form of CaP) based on signals obtained by probe set 202018_s_at (Gene Bank ID: NM_002343) on the Affymetrix GeneChip HG U133A. The color scale from 1/86 fold to 86-fold ratio is shown under the heat map. Gleason scores are shown in parenthesis at the top of the heat map. Expression data was analyzed by multidimensional scaling (MDS) using the MATLAB package (http://arrayanalysis.nih.gov/marray.html) from NHGRI. Genes with the most significant expression differences were selected by t-test (by lowest p-Values). (B) LTF immuno-staining of tissue sections (4 μm) of 30 whole-mount prostate specimens revealed overall high levels of LTF protein in benign glands as compared to malignant glands. Panel B shows LTF protein expression in four prostate tissue sections of CaP patients by IHC using anti-LTF goat polyclonal antibody. Black arrows indicate strongly stained benign cells. In contrast, white arrows indicate negative staining of tumor cells (focal staining in less than 20% of CaP). In 30 of 30 (100%) cases, benign glands (black arrows) adjacent to cancer areas were highly positive for LTF. Magnification, 126. C, decreased LTF protein level in serum of CaP Patients. Serum was drawn from CaP patients [n = 34] undergoing their first diagnostic prostate biopsy, and from healthy males [n = 35]. A significant two-fold decrease in mean LTF levels was observed in sera from CaP patients compared to controls [p < 0.0001] (EUSA). Whisker-box plot technique was used to illustrate serum LTF concentration by patient groups [CaP vs control]. Students’ t test confirmed a significant difference in LTF concentration between cancer and control groups (cancer vs control: 775.71 vs 1381.6, p = 0.0001).
was noted between “T/B” LTF expression (copy number) and PSAR after RP such that men with low T/B ratios had a greater proportion of PSAR (p = 0.02; Fig. 2C). Similarly, multivariate Cox proportional hazards regression demonstrated significantly greater odds of PSAR-free survival for men with higher LTF T/B ratios (p = 0.04) adjusting for relevant covariates (Supplementary Table 1). These data suggest that low “T/B” LTF expression is a potential biomarker of poor prognosis of CaP. These data suggest that low “T/B” LTF expression is a potential biomarker of poor prognosis of CaP.

Mechanism of LTF downregulation in LNCaP cells. To elucidate the possible mechanism of LTF gene silencing we analyzed the LTF promoter upstream and downstream sequences for CpG islands. The analysis revealed a CpG island downstream to the transcription initiation site. To assess whether or not DNA methylation played a role in LTF gene silencing LNCaP and LAPC4 cells harboring mutant or wild type AR respectively, were incubated in the absence or presence of 5 μM 5-aza-deoxycytidine (decitabine) DNA methyl transferase inhibitor for 15 days. Genomic DNA was isolated from the cells and quantitative methylation analyses was carried out by EpiTYPER MALDI-TOF MS, a bisulfite-treatment-based method for the detection and quantitation of DNA methylation.25 In cells without decitabine treatment the CpG-rich region spanning from exon 1 to the downstream intronic sequences (+200 to +600 relative to the transcription initiation site of LTF promoter), an average of 80% methylation was found in mutant AR expressing LNCaP cells (Fig. 3A). In contrast, an average of 35% methylation was observed in the same region in wild type AR expressing LAPC4 cells. (Fig. 3C). Proportional demethylation was observed in response to 5-aza-deoxycytidine treatments at CpG sites throughout the assessed region in both cell lines. Consistent decreases in GSTP1 methylation in response to 5-aza-deoxycytidine treatments confirmed that decitabine concentration was in the subtoxic concentration range facilitating the demethylation of CpG islands (Fig. 3, B and D). Assessment of LTF mRNA levels by Northern blot analyses indicated robust increases in wild type AR harboring LAPC4 cells and in a lesser extent in LNCaP in response to decitabine (Fig. 3E). These observations were consistent with the finding of high levels of DNA methylation in genome of mutant AR harboring LNCaP cells (Fig. 3A).26

LTF protein inhibits the growth of prostate cancer cells. Numerous studies have demonstrated the negative effect of LTF protein supplementation on the growth of various cancer cells. Therefore, we evaluated the effect of LTF on prostate cancer cell
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The expression of LTF mRNA was determined in untreated LNCaP and LAPC4 cells and in LNCaP and LAPC4 cells treated with 5-azadeoxycytidine (Fig. 4A and B). We noted that cell number was not reduced within the first 24 hours of LTF treatment (data not shown). After 48 hours the number of LNCaP cells was significantly reduced in response to 10 μM LTF treatment (Fig. 4A). While 10 μM LTF concentration was cytostatic (p < 0.01 for each pair), ≥30 μM LTF appeared to be cytotoxic (Fig. 4B). Both cytostatic and cytotoxic effects of LTF on LNCaP cells were evident by the observed changes in cell morphology.

**DISCUSSION**

Detection of the frequently downregulated LTF in CaP may provide a valuable tool for monitoring disease progression. Association of LTF downregulation with increased PSA recurrence-free survival of CaP patients suggests that LTF may be suitable for disease prognosis. Consistent with the LTF mRNA expression data, LTF protein downregulation was observed both in tumor tissue and in serum (p < 0.0001). In quantitative gene expression evaluation of LCM-selected cells, downregulation of LTF mRNA expression was observed in 74% of the samples. IHC results of whole-mount prostates from 30 patients suggested absence of LTF expression in approximately 80% of tumors. Interestingly, specimens with increased LTF expression showed better prognosis (longer PSA recurrence free survival) of CaP that may reflect the preventive function of LTF in these patient group.

**Figure 3.** Detection and quantitation of LTF gene methylation in LNCaP and LAPC4 cells: mechanism of LTF downregulation. Methylation analysis of genomic DNA from 5-azadeoxycytidine treated and untreated LNCaP and LAPC4 cells were performed at SEQUENOM using Epityper MALDI-TOF MS, which is a bisulfite-treatment-based method for the detection and quantitation of DNA methylation. Blue bars indicate % methylation. Red bars mark % of methylation after 5-azadeoxycytidine treatment. (A) In the analyzed CpG island of LTF gene, +200; +600 relative to the transcription initiation site, 74 to 97% methylation was detected in LNCaP cells. (B) 71 to 100% methylation of the analyzed CpG sites was detected in the -300; +100 region of GSTP1 gene in LNCaP cells. (C) In LAPC4 cells modest methylation of the LTF gene, +200; +600 region was further decreased in response to 5-azadeoxycytidine treatment. (D), an approximately 40% of methylation of the GSTP1 gene -300; +100 region in LAPC4 showed further decreases in 5-azadeoxycytidine treated cells. (E) 5-azadeoxycytidine dose dependent expression of LTF mRNA is pronounced in LAPC4 as opposed to LNCaP cells as shown in Northern blot assays. GAPDH mRNA blots are shown as controls.
we previously shown that CaP cells harboring wild type or mutant alleles of AR are different in their genomic DNA methylation levels and in their response to inhibition of DNA methyl transferases. Therefore, we addressed the question of whether or not silencing of LTF promoter is affected by the allelic status of AR in CaP cell culture models. Indeed, in mutant AR harboring LNCaP cells the LTF promoter downstream CpG island was heavily methylated, and LTF expression was undetectable. In contrast, analysis of wild type AR expressing LAPC4 cells indicated modest methylation within the promoter downstream CpG island and detectable levels of LTF expression. Inhibition of DNA methyl transferases by decitabine resulted in robust activation of LTF expression in LAPC4 and in to a lesser extent activation of LTF in LNCaP cells. These data together indicate that methylation silencing of LTF may mirror the functional status of androgen receptor. Although, methylation silencing of LTF gene closely resembles the silencing of the protective GSTP1 gene that is an early event in CaP, LTF methylation may be intimately linked to the chromatin changes associated with AR functions in prostate cancer.

Early recurrence of CaP after RP is detectable by a rise in serum PSA level and PSA recurrence develops in a significant number of patients who undergo RP, which is commonly considered as an early indication of progressive disease. Prognostic molecular markers of CaP progression predicting PSA recurrence and disease-specific survival have been extensively evaluated in CaP tissues. Expression features of genes such as p53, BCL2, EZH2, AMACR8 and ERG19 appear to be associated with PSA recurrence after RP. Significant association of LTF downregulation with PSA recurrence also suggests that LTF may play protective roles in the prostate epithelium.

Use of natural compounds such as lactoferrin in chemoprevention and chemotherapy may become a useful strategy in suppressing and inhibiting tumor growth and carcinogenesis. Interestingly, growth inhibition of LNCaP cells by LTF protein suggests potential anti-tumorigenic activity of LTF in CaP. We evaluated changes in the cell cycle of prostate cancer cells in response to lactoferrin treatment to assess underlying mechanisms of the lactoferrin-mediated anti-tumorigenic activity. Robust apoptotic response (G1), growth arrest at G1 and reduced S phase was observed in response to lactoferrin treatment suggesting a role for specific cell cycle regulatory mechanisms in LTF-mediated cell growth inhibition. Similar observations were reported in Head and Neck cancer cell, hepatocyte and breast cancer cell models. Numerous reports suggested that LTF has antitumorigenic activity in animal models in various cancer types. These studies have shown that LTF can inhibit development of tumors in experimental models. Our data warrants similar investigations for LTF in prostate cancer.

Taken together, this study has established LTF as a frequently downregulated gene in CaP. As the magnitude of LTF mRNA downregulation in prostate tumor cells is significantly associated with PSA recurrence after RP, quantitative features of LTF expression have prognostic utility in disease progression. Reduced levels of LTF protein in tumor specimens and blood samples hold the promise for providing alternative strategies for assessing the prognostic value of LTF in the serum of CaP patients. Finally, the observed cell growth inhibitory effects of this natural milk product, warrants future experiments evaluating the anti-tumorigenic effects of LTF in prostate cancer in the context of chemoprevention.
Acknowledgements

The authors would like to thank Dr. Katsuaki Masuda for providing 5-azadeoxycytidine treated LNCaP cells. The authors also thank Mr. Stephen Doyle, Graphics Designer, for his assistance in preparing the figures. The opinions and assertions contained herein are the private views of the authors and are not to be construed as reflecting the official views of the US Army or the Department of Defense.

Note

Financial disclosure. This work was funded by the CPDR through an ongoing grant from the US Army Medical Research and Materiel Command, and by NIH Grant RO1 DK065977.

Ethics statement. Prostate tissue specimens used in this study were obtained under an IRB-approved protocol at Walter Reed Army Medical Center. Informed consent was obtained from each subject.

Competing financial interests statement. The authors declare that they have no competing financial interests. Mathias Ehrich is an employee of the Sequenom Inc., San Diego, California.

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