Cysteine-rich Secretory Protein-3: A Potential Biomarker for Prostate Cancer

Farhad Kosari,1 Yan W. Asmann,1 John C. Cheville, and George Vasmatzis2
Division of Experimental Pathology [F. K., Y. W. A., G. V.] and Division of Anatomic Pathology [J. C. C.], Department of Laboratory Medicine and Pathology, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Abstract

Electronic profiling of publicly available expressed sequence tag databases identified a gene, cysteine-rich secretory protein-3 (CRISP-3), that is up-regulated in prostate cancer, and of which the expression is relatively prostate-specific. The objective of this study was to examine the potential of CRISP-3 as a biomarker for prostate cancer. In transient transfection studies, CRISP-3 was found to be a secretory protein. Using a multiple tissue dot blot experiment, CRISP-3 transcript was identified in a limited number of human tissues including the prostate. In situ hybridization experiments indicated that CRISP-3 mRNA is epithelial-specific and is up-regulated in prostate adenocarcinoma compared with benign prostate tissue. CRISP-3 mRNA overexpression in cancer was confirmed using quantitative real-time reverse-transcription-PCR using benign prostatic epithelia and adenocarcinoma (in 5 of 5 cases) isolated by laser capture microdissection, as well as bulk tissues (in 20 of 23 cases) from surgically resected human prostates. These findings suggest that CRISP-3 is a potential biomarker for prostate cancer.

Introduction

Prostate cancer is clinically silent and usually does not produce symptoms until the cancer has metastasized. The early detection of prostate cancer requires digital rectal examination, serum PSA3 test, and prostate biopsy. The widespread use of serum PSA testing as a screening tool for prostate cancer has resulted in the early detection and treatment of prostate cancer. However, an elevation in serum PSA is not specific for prostate cancer, and the serum PSA can be elevated for a number of reasons including prostatitis and benign prostatic hyperplasia (1). In addition, approximately 15–20% of men with prostate cancer have a normal serum PSA (1). A number of potential candidate proteins such as human kallikrein 2 (2) have been studied to improve the detection of prostate cancer; however, there remains a need for a more sensitive and specific serum and tissue marker of prostate adenocarcinoma.

In the last 10 years, considerable effort has been made in identifying tissue-specific genes by using cDNA libraries (3–5). Computer algorithms have been developed to group ESTs in clusters corresponding to distinct genes (6, 7). EST representation of a gene in normal versus cancer libraries from the same tissue can be used to decipher changes in gene expression as a result of malignant transformation. This is accomplished by comparing the number of ESTs corresponding to a gene originating from normal and cancer libraries. An expression profiling system has been developed to search the dbEST for EST clusters representing genes specifically found in prostate libraries (6, 8–11). In conjunction with experimental techniques, these expression profiling systems were used to identify genes that are specifically expressed in prostate and prostate cancer, and could be useful in the therapy of prostate cancer (8–10).

We searched the publicly available dbEST for potential diagnostic markers of prostate cancer. Novel bioinformatics algorithms were developed to examine publicly available databases for genes that display altered expression in prostate cancer. Our computational analysis revealed a number of up- and down-regulated genes, in some cases novel genes, associated with prostate cancer (12).

The most highly up-regulated gene based on our computational analysis was CRISP-3. The CRISP-3 gene belongs to a large family of proteins that are expressed in vertebrates, insects (13), plants (14), fungi, and yeast (15, 16). The three closely related members of the human CRISP family of genes are located on chromosome 6 (17), and all have in common a cysteine-rich domain at the COOH terminal (18), suggesting a common structural motif. The expression of CRISP genes has been characterized in several androgen-dependent murine tissues (19). In humans, CRISP-3 mRNA is expressed predominantly in salivary gland, prostate, and pancreas, and at much lower levels in epididymis, thymus, ovary, testis, colon (18), and lacrimal glands (20).

The purpose of this study was to investigate the potential of CRISP-3 as a biomarker for prostate cancer. We examined the secretory property of the CRISP-3 protein, and cellular and tissue specificity of CRISP-3 mRNA. We also determined and compared the expression levels of CRISP-3 and PSA transcripts in benign and neoplastic prostate tissues obtained from surgically resected human prostate samples.

Materials and Methods

Analysis of dbEST. EST map of CRISP-3 (Fig. 1) was generated by performing a Basic Local Alignment Search Tool
(BLAST) search of human CRISP-3 sequence (GI:5174674) against the dbEST. Relative expression levels of GAPDH, PSA, and CRISP-3 in normal and cancer epithelial cells were estimated according to the procedure described by Asmann et al. (12).

**CRISP-3 Protein Expression and Detection.** Construct pcDNA3.1GS-CRISP-3-V5-His6 was made by cloning CRISP-3 in pcDNA3.1GS-CRISP3-V5-His6 expression vector (Invitrogen, Carlsbad, CA). This expression vector places a V5 tag at the COOH terminus of the CRISP-3 protein. Another construct, pcDNA3.1GS-V5-His6 expression vector with no insert was used as negative control. The constructs were transiently transfected into HEK293 cells. HEK293 cells were cultured in DMEM (Life Technologies, Inc.) supplemented with 10% FCS, 2 mm l-glutamine, penicillin (100 units/ml), and streptomycin (50 units/ml). Cells were seeded into 10-cm plates the day before transfection. Transient transfections were carried out using a Calcium Phosphate Transfection kit (Promega). Cell culture medium was changed into serum-free DMEM 30 h after transfection. Culture medium and whole cell lysate of the transfected cells were collected 60 h after transfection, and analyzed by Western blot analysis using anti-V5 antibody. Ninety percent of cells were viable when they were harvested.

**In Situ Hybridization.** A Digoxigenin (DIG)-UTP-labeled 400-base riboprobe corresponding to nucleotides 757-1158 of CRISP-3 (GI:5174674) was synthesized in vitro using Ambion MEGAscript kit (Ambion, Austin, TX). The selected region of the sequence is very specific to the CRISP-3 gene according to BLAST searches in the hgs and nr databases, and does not include any Alu repeat elements. The in situ protocol was adopted from Braissant and Wahli (21) with minor modifications. Briefly, frozen prostate tissues in OCT were sectioned at 12 μm on Superfrost/Plus microscope slides (Fisher Part number 12–550-15) and treated with 4% paraformaldehyde in PBS for 20 min at room temperature, acetylated for 10 min in 0.25% acetic anhydride in 0.1% triethanolamine (pH 7.9), then washed in PBS for 15 min. Sections were equilibrated in 5× SSC and prehybridized at 59°C for 2.5 h. Prehybridization buffer contained 50% deionized formamide, 5× SSC, and 100 μg/ml salmon sperm DNA. Hybridization was performed at 59°C overnight with 250 ng/ml of DIG-labeled riboprobe in prehybridization buffer in a humid chamber containing 50% deionized formamide and 5× SSC. On day 2, slides were incubated...
for 30 min in 2× SSC at room temperature (Gleason Pattern-5 slides were treated with 1 μg/ml RNase A for 10 min) and stringently washed with 2× SSC and 0.1× SSC at 64°C for 1 h each. Sections were stained with alkaline phosphatase overnight at room temperature and mounted.

**Tissue Preparation and RNA Extraction.** Matched benign and prostatic adenocarcinoma tissues were collected fresh from human radical prostatectomy specimens and flash frozen. This study was approved by the Mayo Institutional Review Board. Experiments used samples of moderately differentiated (Gleason pattern 3) and poorly differentiated (Gleason patterns 4 and 5) prostatic adenocarcinoma after being reviewed by a urologic pathologist (J. C. C.) for diagnosis and Gleason pattern evaluation. No patients received preoperative therapy such as radiation or androgen deprivation. Flash-frozen benign and malignant prostate tissues embedded in OCT were sliced at 10 μm onto microscope slides (Gold Seal Products) and H&E stained. After dehydrating, slides were placed on a PixCell II LCM stage (Arcturus Engineering, Mountain View, CA). A CapSure LCM cap was placed on the area of interest on the tissue. A low-power infrared laser was pulsed at 65 mW for 1.2 ms (laser spot size 7–15 μm) to activate the transfer film resulting in adherence of the desired cells to the CapSure LCM cap. Nearly equal numbers of benign and malignant epithelial cells were collected from each sample. After collecting 2000–5000 cells, the CapSure LCM film was placed into a sterile 0.6-ml centrifuge tube containing 200 μl lysis buffer (RNeasy kit; Qiagen, Valencia, CA) supplemented with 1% β-mercaptoethanol and inverted for ~1 h at room temperature. Total RNA was isolated and eluted in 30 μl of nuclease free water following RNeasy kit instructions (Qiagen).

Experiments were also performed using tissue obtained directly from the frozen tissue block without prior LCM. In these tissue experiments, H&E sections were obtained from the benign prostate tissue and tissue containing the prostatic adenocarcinoma. These sections were reviewed by a urologic pathologist (J. C. C.) to insure appropriate diagnosis and Gleason pattern evaluation. In the frozen tissue blocks containing prostatic adenocarcinoma, the non-neoplastic tissue was manually cut away from the block to enrich for the content of adenocarcinoma. Approximately equal amounts of tissue (2–4 slices at 10 μm) were collected from cancer and benign samples in each case. Total RNA extraction was performed following the RNeasy kit instructions.

**RT-PCR.** Total RNA was treated with amplification grade DNase I (Life Technologies, Inc., Carlsbad, CA) and the companion buffer for 15 min at room temperature. DNase I was denatured by the addition of 2.5 mM EDTA (final concentration) and incubation at 65 degrees for 10 min. Reverse transcription was performed using DNase I-treated RNA template, following the RNeasy kit instructions (Qiagen). Experiments were also performed using tissue obtained directly from the frozen tissue block without prior LCM. In these tissue experiments, H&E sections were obtained from the benign prostate tissue and tissue containing the prostatic adenocarcinoma. These sections were reviewed by a urologic pathologist (J. C. C.) to insure appropriate diagnosis and Gleason pattern evaluation. In the frozen tissue blocks containing prostatic adenocarcinoma, the non-neoplastic tissue was manually cut away from the block to enrich for the content of adenocarcinoma. Approximately equal amounts of tissue (2–4 slices at 10 μm) were collected from cancer and benign samples in each case. Total RNA extraction was performed following the RNeasy kit instructions.

**Real-Time RT-PCR.** Real-time PCR was performed using a template from reverse transcription reaction, TaqMan Universal Mastermix (Applied Biosystems, Foster City, CA), gene-specific primers, and dual labeled probes (Integrated DNA Technologies, Skokie, IL) on a PE7700 system. Primers and dual-labeled probes were designed using Primer Express software (PE Biosystems, Foster City, CA). Special care was exercised in primer and probe design to insure that PSA and CRISP-3 primers did not cross-amplify other members of the human kallikrein and CRISP family, respectively. Primers and probe concentrations were 900 nM and 250 nM, respectively. Samples were run in duplicates of 25 μl volumes. Sequences of the primers and dual-labeled probes were as follows: CRISP-3 (product size: 89 bp), forward: 5′-cagagcttctcagaaataaca-3′, reverse: 5′-gtttcttttgcaggtggg-3′, PSA (product size: 506 bp), forward: 5′-attttgagggaggtgagg-3′, reverse: 5′-gctacaggctgagggaa-3′, and CRISP-3 (product size: 149 bp), forward: 5′-gcttggagcttacaggttg-3′, reverse: 5′-cattacccgcttcatagttc-3′.

**Tissue Expression Profile Using Multiple Tissue Dot Blot Assay.** Labeling of random probe was performed by incubating the PCR product from the 3′ untranslated region of CRISP-3 (~25 ng, 22 μl), 5 μl of 10× reaction buffer, 2 μl of 0.5 mM dATP, 2 μl of 0.5 mM dTTP, 2 μl of 0.5 mM dGTP, 5 μl of 3000 Ci/mmol [α-32P] dCTP, 10 μl of 0.5 μg/μl random primer, and 1 unit of T4 DNA polymerase at 37°C for 20 min. Labeling reaction was stopped by the addition of EDTA (5 μl of 250 mM) at 65°C for 5 min. MTE array (Clontech, Palo Alto, CA) was first prehybridized in 10 ml of prehybridization buffer (ExpressHyb Solution from Clontech, with 0.1 mg/ml sheared salmon testes DNA) for 30 min at 65°C. The array was hybrid-
ized in 5 ml prehybridization buffer with $2 \times 10^6$ cpm/ml purified probe overnight at 65°C. The array was washed four times for 20 min with continuous agitation in 2× SSC supplemented with 1% SDS at 65°C. Next, the array was washed two times for 20 min with continuous agitation in 0.1× SSC supplemented with 0.5% SDS at 55°C. Finally, the array was exposed to X-ray film at −70°C for 75 h.

Results

Bioinformatics Analysis Suggested Up-Regulation of CRISP-3 in Prostate Cancer. The number of ESTs derived from CRISP-3 in the prostate cancer libraries was compared with the number of ESTs in prostate normal libraries to determine the alteration of CRISP-3 expression in prostatic adenocarcinoma. We found a total of 54 ESTs in dbEST (Fig. 1) of which 37 ESTs derived from prostate libraries. Of these 37 ESTs, 32 originated from cancer microdissected libraries and only 5 from a normal bulk library, suggesting that CRISP-3 was overexpressed in prostate cancer relative to normal prostate tissue. Of the 32 prostate cancer ESTs, 2 came from a library constructed from microdissected malignant prostate cells and the remaining from metastatic prostatic carcinoma to bone. In addition to prostate libraries, EST analysis also identified 12 ESTs in bone marrow, 4 ESTs in blood (chronic myelogenous leukemia), and 1 EST in lung cancer libraries. All of the ESTs from nonprostate libraries are derived from cancer bulk libraries. The presence of CRISP-3 transcripts in cancerous bone marrow such as myelogenous leukemia is expected, because CRISP-3 cDNA was originally isolated from a human bone marrow cDNA library (22). Incidentally, in our dot blot experiment, we did not see any detectable levels of CRISP-3 message in leukemia cells or in normal lung (see below).

Electronic Expression Profiling of CRISP-3, and Comparison with PSA and GAPDH. Using EST data and with appropriate normalization, the expression level of any gene relative to the total expression of all of the genes in a particular cell can be estimated. By classifying the EST libraries to normal and cancer, we can estimate expression differences as a result of malignant transformation (12). According to this analysis, PSA mRNA expression in prostatic epithelial cells is ~1.3% of the total cellular mRNA, and expression of PSA mRNA is nearly identical in normal and cancer LCM libraries. Similarly, GAPDH gene expression is ~0.1% of the total cellular mRNA, and is similar in cancer and normal LCM libraries. CRISP-3 transcripts account for ~0.2% of total mRNA in cancerous prostate epithelial cells. The expression level of CRISP-3 in normal prostate epithelial cells could not be computed because there are no ESTs in the normal microdissected libraries. However, CRISP-3 ESTs are present in normal bulk libraries, and we could not exclude CRISP-3 mRNA expression in benign prostatic epithelial cells.

CRISP-3 Protein Is Secretory in a Transient Transfection Study. To be detected in bodily fluids, it is essential for a biomarker to be secreted. CRISP-3 has been reported to be a secretory protein. Computational analysis (23) using the online program SignalIP identified the first 20 amino acid residues of the protein as a secretory signal peptide. To investigate the secretory property of CRISP-3 experimentally, expression vector pcDNA3.1GS-CRISP-3-V5-His6, which includes CRISP-3 insert and a V5 tag at its COOH terminus, was transiently transfected into HEK293 cells. Sixty hours after transfection, the supernatant was collected and analyzed by Western blotting (Fig. 2). The putative Mr, 34,000 daltons secreted CRISP-3-V5-His6 protein in the

---

Fig. 3. RNA in situ hybridization on frozen tissue sections using DIG-labeled CRISP-3 riboprobe. a and c, the antisense riboprobe stained epithelial cells of Gleason pattern 3 adenocarcinoma (GP3) much stronger than the benign acini (BA). d and f, the antisense riboprobe also stained high-grade, Gleason pattern 5 tumor cells. b and d, the sense riboprobe signal was negative. High power magnification of the antisense riboprobe-stained tissues in a and d are shown in c and f, respectively.
medium was detected by anti-V5 antibody. The vector with no insert was used as negative control.

**In Situ Hybridization Indicates Expression of CRISP-3 in Epithelial Cells of Prostate Adenocarcinoma.** In situ hybridization studies using a DIG-labeled riboprobe with a sequence highly specific to CRISP-3 (Fig. 3) showed staining of the prostatic epithelial cells indicating epithelial cell specificity of the CRISP-3 transcript. The epithelial cells of Gleason pattern 3 and 5 adenocarcinoma (Fig. 3, a and c, respectively) showed intense cytoplasmic hybridization signal. A variable weak signal was seen in benign acini including benign prostatic hyperplasia. Sections that were hybridized with the sense CRISP-3 riboprobe were negative (Fig. 3, b and d).

**RT-PCR Suggests That CRISP-3 mRNA Is Increased in Prostate Cancer.** Because prostate cancer originates from prostatic epithelial cells, we investigated CRISP-3 expression in approximately 2000–5000 prostate epithelial cells using LCM and RT-PCR. We examined the expression of CRISP-3 in 5 cases of Gleason pattern 3 adenocarcinoma (Fig. 4). A housekeeping gene, GAPDH, as well as a putatively epithelial specific gene, PSA, displayed similar expression levels in normal and malignant cells. In the case of CRISP-3, the band representing gene expression in cancer cells was much brighter than the corresponding band for benign epithelial cells. These findings indicated there was an increase in CRISP-3 mRNA expression in prostate cancer relative to normal prostate epithelium.

**Real-Time RT-PCR Confirms RT-PCR Findings.** To obtain a quantitative estimate of the expression level of CRISP-3 gene, real-time RT-PCR was performed using TaqMan PE 7700 system. The cDNA derived from prostate epithelial cells captured by LCM was used as a template. The cycle number at which the amplified signal intensity exceeds a threshold value above the background ($C_T$) is used to determine the expression level of a gene. Relative mRNA concentration of a gene in different samples is estimated by comparing the $C_T$ values [$\Delta C_T = C_T_{\text{Benign}} - C_T_{\text{Cancer}}$]. PSA signal was used for normalization of CRISP-3 expression. That is because, similar to CRISP-3, PSA is an epithelial-specific transcript. Moreover, per cell expression of PSA is not significantly altered by pros...

---

**Fig. 4.** RT-PCR of total RNA from about 2000–5000 LCM captured normal or Gleason pattern 3 prostate epithelial cells (5 cases). The right most lane of each case is 100 bp ladder. The remaining lanes of each case (right to left from the lane next to 100 bp ladder lane) are: GAPDH in cancer prostate epithelial cells, GAPDH in normal prostate epithelial cells, PSA in cancer prostate epithelial cells, PSA in normal prostate epithelial cells, CRISP-3 in cancer prostate epithelial cells, and CRISP-3 in normal prostate epithelial cells, respectively.

**Fig. 5.** Expression of CRISP-3 in prostate adenocarcinoma. a, expression of CRISP-3 in Gleason pattern 3 epithelial cells was compared with that in a sample of normal or benign epithelial cells (Benign/GP3, $n = 5$). The cDNA templates were derived from epithelial cells collected by LCM. b, expression of CRISP-3 in bulk samples of Gleason pattern 3 (Benign/GP3, $n = 15$) and Gleason pattern 4+ (Benign/GP4+, $n = 8$) adenocarcinoma was compared with matched benign bulk samples. Expression ratios are related to $\Delta C_T$ in an exponential fashion; i.e., $\Delta C_T$ expression change $\sim e^{1.94 \Delta C_T}$. Approximate ranges of up and down (dn) regulation of CRISP-3 in prostate adenocarcinoma are indicated on the figure. In a majority of cases a significant up-regulation of CRISP-3 was observed. Data points in a and b represent differences in CT values between each sample pair and are normalized by PSA.
CRISP-3: A Potential Biomarker for Prostate Cancer

Comparisons of CRISP-3 and PSA Expression Regulation in Benign and Cancer Bulk Samples. We aimed to quantify the altered expression of CRISP-3 in prostate cancer with respect to PSA by calculating ∆ΔC(T) as follows: 

\[ \Delta \Delta C(T) = (C_{T-PSA-BENIGN} - C_{T-CRISP-3-CANCER}) - (C_{T-PSA-CANCER} - C_{T-CRISP-3-CANCER}) \]

where X is either CRISP-3 or PSA, and Y is cancer or benign. Histograms display distribution of CRISP-3 and PSA expression with respect to GAPDH in benign prostatic tissue and prostate adenocarcinoma. Each histogram was generated from 15 data points.

The histogram representing CRISP-3 expression in cancer displayed a rightward shift of ~5 cycles with respect to the benign sample histogram, suggesting a notable up-regulation of CRISP-3 in prostate cancer. On the average, the expression of CRISP-3 in cancer and normal samples was ~50-fold (~6 cycles) and ~4000-fold (~12 cycles) less than the expression of GAPDH in those samples, respectively. Some of the increase in expression of CRISP-3 in prostate cancer can be attributed to the higher percentage of epithelial cells in tumor samples compared with benign samples. However, we noted that the percentage of the epithelial cells in each benign bulk sample was comparable with the percentage of the epithelial cells in the corresponding cancer bulk sample. Taken together, these data suggest that the concentration of CRISP-3 transcript is significantly increased in prostate adenocarcinoma with respect to GAPDH transcript.

On the other hand, we observed a considerable overlap between the histograms representing PSA expression in adenocarcinoma and benign prostate tissues, with a mean difference between paired samples of <2-fold (<1 difference cycle in C(T)). The histograms representing PSA expression in benign prostatic epithelia and adenocarcinoma suggested 2–10-fold higher expression of PSA than GAPDH in most cases, and a narrower distribution of expression levels than CRISP-3. These data suggest that the level of PSA mRNA is not significantly increased in Gleason pattern 3 prostate cancer compared with benign. We obtained similar results from histograms of poorly differentiated adenocarcinoma using bulk tissue samples (data not shown).

Tissue Expression Profile Using Multiple Tissue Dot Blot Assay. To determine tissue specificity of human CRISP-3 on many human tissues, we performed a dot blot experiment using an MTE array. The MTE array is a nylon membrane to which polyadenylated RNAs from 76 different human tissues and cancer cell lines have been normalized and immobilized in separate dots. Because CRISP-3 has two highly homologous family members (CRISP-1 and CRISP-2), we chose the non-homologous 3’ untranslated region of CRISP-3 as the probe template for this experiment.

Our analysis revealed that CRISP-3 mRNA is present predominantly in prostate, pancreas, and salivary gland, and is much less abundant in ovary, thymus, fetal thymus, and descending colon (Fig. 7). By Northern blot experiments, Kratzschmar et al. (18) reported similar results. However, the MTE array used in the present study encompasses a much wider range of human tissues than what is included in previous reports.

Discussion

Expression profiling analysis of the prostate dbEST identified that the CRISP-3 gene is overexpressed in prostate cancer. In this study, we examined the potential of CRISP-3 as a biomarker for prostate cancer. Our study shows that the CRISP-3 transcript is relatively specific to prostatic epithelial cells. Furthermore, our quantitative real-time RT-PCR assays indicate that CRISP-3 mRNA is overexpressed in moderately and poorly differentiated prostatic adenocarcinoma relative to benign prostatic epithelium. We also detected an increase in CRISP-3 mRNA levels in a limited number of Gleason pattern 3 cancers compared with high-grade prostatic intraepithelial adenocarcinoma bulk samples by a housekeeping gene, GAPDH. Four histograms depicting expression with respect to GAPDH of CRISP-3 and PSA in benign and adenocarcinoma samples were generated.

The histogram representing CRISP-3 expression in cancer displayed a rightward shift of ~5 cycles with respect to the benign sample histogram, suggesting a notable up-regulation of CRISP-3 in prostate cancer. On the average, the expression of CRISP-3 in cancer and normal samples was ~50-fold (~6 cycles) and ~4000-fold (~12 cycles) less than the expression of GAPDH in those samples, respectively. Some of the increase in expression of CRISP-3 in prostate cancer can be attributed to the higher percentage of epithelial cells in tumor samples compared with benign samples. However, we noted that the percentage of the epithelial cells in each benign bulk sample was comparable with the percentage of the epithelial cells in the corresponding cancer bulk sample. Taken together, these data suggest that the concentration of CRISP-3 transcript is significantly increased in prostate adenocarcinoma with respect to GAPDH transcript.

On the other hand, we observed a considerable overlap between the histograms representing PSA expression in adenocarcinoma and benign prostate tissues, with a mean difference between paired samples of <2-fold (<1 difference cycle in C(T)). The histograms representing PSA expression in benign prostatic epithelia and adenocarcinoma suggested 2–10-fold higher expression of PSA than GAPDH in most cases, and a narrower distribution of expression levels than CRISP-3. These data suggest that the level of PSA mRNA is not significantly increased in Gleason pattern 3 prostate cancer compared with benign. We obtained similar results from histograms of poorly differentiated adenocarcinoma using bulk tissue samples (data not shown).

Tissue Expression Profile Using Multiple Tissue Dot Blot Assay. To determine tissue specificity of human CRISP-3 on many human tissues, we performed a dot blot experiment using an MTE array. The MTE array is a nylon membrane to which polyadenylated RNAs from 76 different human tissues and cancer cell lines have been normalized and immobilized in separate dots. Because CRISP-3 has two highly homologous family members (CRISP-1 and CRISP-2), we chose the non-homologous 3’ untranslated region of CRISP-3 as the probe template for this experiment.

Our analysis revealed that CRISP-3 mRNA is present predominantly in prostate, pancreas, and salivary gland, and is much less abundant in ovary, thymus, fetal thymus, and descending colon (Fig. 7). By Northern blot experiments, Kratzschmar et al. (18) reported similar results. However, the MTE array used in the present study encompasses a much wider range of human tissues than what is included in previous reports.

Discussion

Expression profiling analysis of the prostate dbEST identified that the CRISP-3 gene is overexpressed in prostate cancer. In this study, we examined the potential of CRISP-3 as a biomarker for prostate cancer. Our study shows that the CRISP-3 transcript is relatively specific to prostatic epithelial cells. Furthermore, our quantitative real-time RT-PCR assays indicate that CRISP-3 mRNA is overexpressed in moderately and poorly differentiated prostatic adenocarcinoma relative to benign prostatic epithelium. We also detected an increase in CRISP-3 mRNA levels in a limited number of Gleason pattern 3 cancers compared with high-grade prostatic intraepithelial

Fig. 6. Histograms of expression data for CRISP-3 and PSA derived from bulk Gleason pattern 3. Data were normalized by GAPDH as ∆ΔC(T) = (C_{T-GAPDH} - C_{T-PSA-BENIGN} - (C_{T-CRISP-3} - C_{T-PSA-CANCER})

Note that the altered expression of CRISP-3 in prostate cancer is related to ∆ΔC(T) in an exponential fashion, i.e., fold up-regulation ~ (1+amplification efficiency) ∆ΔC(T). We have determined that the amplification efficiencies for the transcripts in this study are 94% or higher (12).

We used LCM and real-time RT-PCR to compare the expression of CRISP-3 in Gleason pattern 3 adenocarcinoma against expression of CRISP-3 in benign epithelial cells. Fig. 5a illustrates the results in each pair of samples. CRISP-3 expression displayed significant up-regulation in Gleason pattern 3 adenocarcinoma compared with benign. In this group, ∆ΔC(T) values ranged from 4.8 to 11.6, indicating up-regulation of CRISP-3 by 20–2000-fold.

We expanded our sample size by performing additional experiments on bulk samples. We examined CRISP-3 expression in bulk samples of moderately differentiated (Gleason pattern 3) and poorly differentiated (Gleason pattern-4 and -5) prostatic adenocarcinoma (Fig. 5b), and observed an up-regulation of CRISP-3 in 20 of 23 cases compared with the expression of CRISP-3 in benign samples. In one case of poorly differentiated adenocarcinoma, CRISP-3 expression was suppressed relative to CRISP-3 expression in the benign sample. To insure the integrity of the data, we repeated the experiment on this case by LCM two additional times and obtained identical results.

Comparisons of CRISP-3 and PSA Expression Regulation in Benign and Cancer Bulk Samples. We aimed to quantify and compare the expression levels of CRISP-3 and PSA in benign and prostate cancer tissues. For that, we generated histograms depicting the distribution of expression levels of the two genes in benign and malignant prostate tissues (Fig. 6). The representation of data in this manner allowed us to examine expression of CRISP-3 and PSA in benign and malignant tissues separately. We normalized PSA and CRISP-3 expression data obtained from moderately differentiated adenocarcinoma.
neoplasia using epithelial cell samples collected by LCM (data not shown). However, a more extended data set is required to establish a difference in CRISP-3 expression between high-grade prostatic intraepithelial neoplasia and prostate adenocarcinoma. It is of note that the CRISP-3 mRNA level in 1 case of poorly differentiated adenocarcinoma was decreased relative to benign epithelial cells. The significance of this finding is unclear and will require analysis of additional cases.

The mature CRISP-3 protein consists of 225 amino acids. It contains one potential site for N-glycosylation (Asn 219) and 16 cysteine residues, of which 10 are concentrated in the COOH-terminal 56 amino acids of the protein. A number of homologous members of the family to CRISP-3 are allergens and toxins such as venom allergen-5 in hymenoptera (24) or pathogenesis-related-1 proteins (14) in plants. However, the function of the CRISP-3 protein and other members of the CRISP family are largely unknown. A survey of various organs where CRISP-3 is expressed suggests a role in local defense via excretion of exocrine glands such as salivary and lacrimal glands (25), or by the functions of leukocytes cell types such as murine pre-B cells (26). It will be important to determine whether the increased expression of CRISP-3 in cancer is part of a survival mechanism for the tumor cells or if it merely an epiphenomenon of carcinogenesis. Answers to such questions may help determine whether CRISP-3 is a prognostic marker of prostate cancer or a potential target for therapy.

PSA and CRISP-3 transcripts are expressed in about the same number of human tissue types (27–29), including prostate, pancreas (28), and salivary glands (28). Limited tissue distribution is essential for a specific biomarker. In addition, to be detected in bodily fluids, it is important for a biomarker to be secretory. Equine CRISP-3 protein is found in concentrations of up to 1 mg/ml in seminal plasma (30), underscoring the secretory property of this protein. Our Western blot analysis demonstrates that the human CRISP-3 is also a secretory protein. These findings, together with data regarding up-regulation of CRISP-3 in prostate adenocarcinoma, suggest that CRISP-3 has the salient characteristics of a promising and novel biomarker for prostate cancer.

By chemical castration of mice using a gonadotropin-releasing hormone antagonist, Haendler et al. (19) demonstrated that the level of CRISP-3 transcripts in mouse salivary gland is androgen dependent. However, to our knowledge no study regarding the effects of androgens on expression of CRISP-3 in humans has been performed. If CRISP-3 protein secretion is found to be dependent on androgens, it may be difficult to determine the basal level of CRISP-3 in the serum of people without prostate cancer. Also, in that case, special care has to be taken to interpret the serum measurements of CRISP-3 for patients who undergo androgen ablation therapy. These factors may affect the utility of CRISP-3 as a prostate cancer biomarker.

PSA has been widely used for diagnosis of prostate cancer because of the specific and high expression of PSA by the prostate gland. Our data at the mRNA level corroborate this observation (Fig. 6). CRISP-3 transcript is not as highly expressed in prostate as PSA. However, the concentration of CRISP-3 mRNA is increased in cancerous prostate tissues relative to normal. In patients with prostate cancer, we would expect that CRISP-3 protein levels will increase in the blood and semen because of an increase in the number of CRISP-3-secreting tumor cells in a manner similar to PSA. Unlike PSA, however, the increased expression of CRISP-3 in prostate cancer relative to benign tissues will potentially increase the specificity and sensitivity for CRISP-3 as a diagnostic marker of prostate cancer. In addition, if serum CRISP-3 levels increase with the development of prostate cancer, it will be important to determine whether CRISP-3 provides any information regarding tumor volume, stage, and outcome. We are currently undertaking studies examining the utility of CRISP-3 in the diagnosis of prostate cancer.

References
CRISP-3: A Potential Biomarker for Prostate Cancer


Cysteine-rich Secretory Protein-3: A Potential Biomarker for Prostate Cancer

Farhad Kosari, Yan W. Asmann, John C. Cheville, et al.


<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: <a href="http://cebp.aacrjournals.org/content/11/11/1419">http://cebp.aacrjournals.org/content/11/11/1419</a></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Cited articles</th>
<th>This article cites 28 articles, 9 of which you can access for free at: <a href="http://cebp.aacrjournals.org/content/11/11/1419.full#ref-list-1">http://cebp.aacrjournals.org/content/11/11/1419.full#ref-list-1</a></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Citing articles</th>
<th>This article has been cited by 7 HighWire-hosted articles. Access the articles at: <a href="http://cebp.aacrjournals.org/content/11/11/1419.full#related-urls">http://cebp.aacrjournals.org/content/11/11/1419.full#related-urls</a></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>E-mail alerts</th>
<th>Sign up to receive free email-alerts related to this article or journal.</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Reprints and Subscriptions</th>
<th>To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a>.</th>
</tr>
</thead>
</table>

| Permissions | To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org. |