Hypoexpression and Epigenetic Regulation of Candidate Tumor Suppressor Gene CADM-2 in Human Prostate Cancer

Guimin Chang1, Shuping Xu1, Rajiv Dhir2, Uma Chandran3, Denise S. O’Keefe1, Norman M. Greenberg4, and Jeffrey R. Gingrich1

Abstract

Purpose: Cell adhesion molecules (CADM) comprise a newly identified protein family whose functions include cell polarity maintenance and tumor suppression. CADM-1, CADM-3, and CADM-4 have been shown to act as tumor suppressor genes in multiple cancers including prostate cancer. However, CADM-2 expression has not been determined in prostate cancer.

Experimental Design: The CADM-2 gene was cloned and characterized and its expression in human prostatic cell lines and cancer specimens was analyzed by reverse transcription-PCR and an immunohistochemical tissue array, respectively. The effects of adenovirus-mediated CADM-2 expression on prostate cancer cells were also investigated. CADM-2 promoter methylation was evaluated by bisulfite sequencing and methylation-specific PCR.

Results: We report the initial characterization of CADM-2 isoforms: CADM-2a and CADM-2b, each with separate promoters, in human chromosome 3p12.1. Prostate cancer cell lines, LNCaP and DU145, expressed negligible CADM-2a relative to primary prostate tissue and cell lines, RWPE-1 and PPC-1, whereas expression of CADM-2b was maintained. Using immunohistochemistry, tissue array results from clinical specimens showed statistically significant decreased expression in prostate carcinoma compared with normal donor prostate, benign prostatic hyperplasia, prostatic intraepithelial neoplasia, and normal tissue adjacent to tumor (P < 0.001). Adenovirus-mediated CADM-2a expression suppressed DU145 cell proliferation in vitro and colony formation in soft agar. The decrease in CADM-2a mRNA in cancer cell lines correlated with promoter region hypermethylation as determined by bisulfite sequencing and methylation-specific PCR. Accordingly, treatment of cells with the demethylating agent 5-aza-2′-deoxycytidine alone or in combination with the histone deacetylase inhibitor trichostatin A resulted in the reactivation of CADM-2a expression.

Conclusions: CADM-2a protein expression is significantly reduced in prostate cancer. Its expression is regulated in part by promoter methylation and implicates CADM-2 as a previously unrecognized tumor suppressor gene in a proportion of human prostate cancers.

Prostate cancer is the most common malignancy detected in men in the United States, and is the second leading cause of cancer mortality today (1). The molecular mechanisms underlying the progression of prostate cancer remain poorly understood, particularly due to the extreme heterogeneity of primary tumors. However, the shift in balance between tumor suppressor genes and oncogenes likely drives both the genesis and progression of this disease. Earlier studies have shown that loss of heterozygosity (LOH), mutation, and gene promoter methylation all contribute to the inactivation of tumor suppressor genes in prostate cancer (2). DNA methylation abnormalities, however, have emerged as the most frequent molecular changes in prostate neoplasms (3) such that many tumor suppressor genes undergo CpG hypermethylation and subsequent loss of expression. Examples include the APC gene (4), the CD44 gene (5), and the E-cadherin (CDH1) gene (6).

Recent studies suggest that cell adhesion molecules (CADM), a newly identified family of proteins, might serve as tumor suppressors (7). Most of the CADMs belong to an immunoglobulin superfamily whose members express three extracellular immunoglobulin-like loops, a transmembrane region, and an intracellular domain. Multiple normal tissues express CADMs. However, a variety of cancers express either lack CADMs or express them at reduced levels. For example, transcriptional silencing of the

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CADM-1 gene (also known as Necl-2/TSLC1/SynCam1/IGSF-4A) occurs in lung cancer (8), prostate cancer (9), and esophageal cancer (10) as a result of promoter methylation. Fukuhara and colleagues reported that the upstream regions of the CADM-3 (Necl-1/TSSL1/SynCam-3/ IGSF4B) and CADM-4 (Necl-4/TSSL2/SynCam4/IGSF4C) genes consist of areas rich in guanine and cytosine residues that meet the criteria of CpG islands, suggesting that the promoters of CADM-3 and CADM-4 may also undergo methylation (11). A recent study showed a decrease in CADM-4 protein expression in prostate tumors as compared with normal prostate tissue, with evidence suggesting that CADM-4 might suppress tumorigenicity (12). More recently, several articles have reported that CADM-3 and CADM-4 also function as tumor suppressors in multiple cancer cells (13–16), and that CADM1 also regulates epidermal adhesion and wound healing as well as being involved in epithelial cell structure (17, 18).

In this study, we report our analysis of CADM-2 in prostate cancer, a member of the CADM family which has not been previously well characterized in cancer. The gene is also called Necl-3, IGSF4D, and SynCAM 2 (19, 20) and maps to chromosome 3p12.1, a region which, interestingly, has been shown to undergo loss of heterozygosity in 56% of prostate tumors using microarray analysis (21). More recently, CADM-2 has been characterized as a bona fide adhesion molecule that engages in homophilic and heterophilic interactions with other CADM family members, leading to cell aggregation and organization of functional synapses through heterophilic adhesion. Other reports have shown that CADM-2 is expressed in the nervous system of developing zebrafish (22), suggesting that CADM-2 is a conserved gene evolutionarily and may be implicated in a multitude of physiologic and pathologic processes. However, thus far there have been no reports regarding the expression of CADM-2 in human cancer cells. In this article, we show that expression of CADM-2 in human prostate cell lines and patient specimens is reduced in part as a result of its promoter hypermethylation, implicating CADM-2 as a tumor suppressor gene in prostate cancer.

Materials and Methods

RNA isolation and reverse transcription-PCR

RNA was isolated using RNAzol (Biotech Laboratories, Inc.). Human prostate and brain RNA libraries were purchased from Clontech. Reverse transcription-PCR (RT-PCR) analysis was done: CADM-2a upstream, 5′-CAGCCAGGATCCACAGTGTACCTGTTCTGTGTTGCAAC-3′; downstream, 5′-GAATTCCAGCTGACTGTC-3′—360 bp product; CADM-2b upstream, 5′-CGGAATTCCGACGATGATTTGGAAA/CADM-2-r as above. A construct replacing the signal peptide of CADM-2a with CADM-2b was constructed. Recombinant pcDNA3-CADM-2a-8 was constructed by inserting the PCR fragment amplified by the primer set CADM-2a-f CGGAATTCCGACGATTTGGAAA and CADM-2b-r CCAGATGGAGATTTGGAAA into pcDNA3 (Invitrogen) and pEGFP-N1 (Clontech). CADM-2a-b-m8 were cloned into pcDNA3 (Invitrogen) and pEGFP-N1 (Clontech).

Recombinant CADM-2 vectors

CADM-2a-FL, CADM-2b-FL, CADM-2a-m8 (missing exon 8), and CADM-2b-m8 were cloned into pcDNA3 (Invitrogen) and pEGFP-N1 (Clontech). CADM-2b, CADM-2b-f CGGAATTCCGACGATTTGGAAA and CADM-2b-r CCAGATGGAGATTTGGAAA into pcDNA3 (Invitrogen) and pEGFP-N1 (Clontech). A construct replacing the signal peptide of CADM-2a with CADM-2b was constructed. Recombinant pcDNA3-CADM-2a-8 was constructed by inserting the PCR fragment amplified by the primer set CADM-2a-f CGGAATTCCGACGATTTGGAAA and CADM-2b-r CCAGATGGAGATTTGGAAA into pcDNA3 (Invitrogen) and pEGFP-N1 (Clontech). The resultant clones were confirmed by sequencing.

Cell culture

Cell lines were obtained from the American Type Culture Collection, except for PrEC (Cambrex BioScience, Inc.) and BPH-1, which were provided by Dr. Changqing Ma. Cell lines were propagated in RPMI 1640 (Life Technologies, Inc.) containing 10% fetal bovine serum (Bio Whittaker), whereas HEK-293 was cultured in DMEM containing 10% fetal bovine serum. RWPE-1 and RWPE-2 were maintained in unfiltered keratinocyte serum-free medium with 5 ng/mL of human recombinant epidermal growth factor and 0.05 mg/mL of bovine pituitary extract. PrEC was maintained in PrE GM BulletKit media (Cambrex BioScience). All media contained 100 units/mL of penicillin and 100 μg/mL of streptomycin (5% CO₂ at 37°C).

Translational Relevance

This article shows for the first time CADM-2 expression pattern both in vitro and in vivo in prostate cancer. Lost expression of the normal isoform through its promoter hypermethylation and/or histone deacetylation implicates CADM-2 as a previously unrecognized tumor suppressor gene in the progression of prostate cancer. Further investigation of the correlation between CADM-2 expression and staging and/or Gleason score in a larger number of prostate cancer specimens will help to develop CADM-2 as an epigenetic marker of prostate cancer. For example, detection of CADM-2 promoter hypermethylation in the biopsied prostate cancer. Restoration of CADM-2 expression may also be applied to prostate cancer treatment through epigenetic therapy or virus-mediated gene therapy. In addition, understanding the biological functions of CADM-2 might lead to further insight into other potential therapeutic targets.
**Peptide synthesis and antibody generation**

Synthetic peptides corresponding to the following hydrophilic segments of CADM-2 were produced by Research Genetics, Inc.: NH₂-terminal (CADM-2a, 165-183) and COOH terminus (CADM-2a, 426-444). Horseradish-peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) was used as a secondary antibody. Mouse anti-GFP, human α-tubulin, and goat anti-human β-actin were obtained from Santa Cruz Biotechnology.

**Recombinant adenovirus transduction**

Recombinant adenovirus was generated with the AdEasy System (23). Recombinant vector was transfected into 293 cells and plaques purified. Recombinant Ad-CADM-2 was used after purification, characterization, and titration of the viral infectivity by fluorescence assay. Adenovirally mediated transduction of DU145 was done as stated previously (24).

**Transient transfections and Western blot analysis**

Cells were transfected using LipofectAMINE plus (Invitrogen) for 4 hours. For Western blots, 5 × 10⁵ transfected cells/well suspended in 250 μL of ice-cold lysis buffer containing protease inhibitor (human prostate and brain protein medleys from BD Bioscience), and run on 10% to 12% SDS-PAGE acrylamide. Blots were incubated (primary antibodies for 60 min) at room temperature and bands visualized (ECL detection system or Pierce).

**Antibody specificity determination by peptide competition assay**

A peptide competition assay was done (25). CADM-2 COOH-terminal antibody diluted 1:2,000 (1 μg/mL) in 5% (w/v) bovine serum albumin/TBST was preincubated for 1 hour at room temperature in serial dilutions of the competing COOH-terminal homologous peptide (diluted NH₂-terminal peptide was used as a secondary antibody. Mouse anti-GFP, human α-tubulin, and goat anti-human β-actin were obtained from Santa Cruz Biotechnology.

**Patient material and tissue microarray construction**

A 176-patient tissue array containing multiple replicates of normal, high-grade prostatic intraepithelial neoplasia (PIN), and prostate cancer was used to evaluate CADM-2 expression (26). The tissue microarray (TMA) set included progression TMAs (109 cases with different Gleason grades and stages), foci of metastatic prostate cancer, PIN, normal adjacent to tumor, benign prostatic hyperplasia (BPH), and “true normal” prostatic tissue from organ donors (647 samples, 176 patients, ages 15-85 y).

**Immunohistochemistry and TMA analysis**

TMA slides were stained for CADM-2 using the COOH-terminal antibody in an Autostainer. Staining was scored from 0 to 4 depending on the amount of staining present. The analysis scale was based on the percentage of cells with whole cell positivity (0 = none, 1 ≤11%; 2 = 10-25%; 3 = 25-50%; 4 >50%). A high immunohistochemistry score was defined as 3 or more. Patients with more than one tissue sample were averaged. There were 11 donors, 17 benign tissues (BPH), 38 high-grade PIN, 42 normal adjacent to tumor, 109 prostate adenocarcinoma, 6 metastatic lymph node tissues, and 10 metastatic tissues. Mean values from each group were compared using the Mann-Whitney rank sum test and P < 0.05 values were considered significant. Kruskal-Wallis one-way ANOVA on ranks compared mean values within Gleason scores and stages of prostate cancer. P < 0.05 values were considered significant. All pairwise multiple comparison procedures were conducted using the Dunn method.

**In vitro proliferation and colony formation**

DU145 at 50% confluence were infected with virus at various multiplicities of infections (MOI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were done after 3 and 5 days. DU145 cells were transduced at MOIs of 5 for colony formation assays (27). Every 3 days, normal growth medium was gently layered over the cultures and colonies counted after 15 days using an inverted microscope.

**Bisulfite sequencing methylation analysis**

Bisulfite sequencing and T-A cloning were done as previously described (9, 28). Briefly, genomic DNA was extracted from cells by DNA-Bee reagent (TEL-TEST, Inc.) and purified with the QiAamp DNA kit (Qiagen, Inc.). DNA was denatured with 0.3 mol/L of NaOH for 15 minutes followed by treatment with 3.1 mol/L of sodium bisulfite and 0.8 mmol/L of hydroquinone (pH 5.0; Sigma), at 50°C for 16 hours. DNA was then treated with 0.2 mol/L of NaOH for 10 minutes at 37°C. The modified DNA was purified in a total volume of 20 μL, and 1 μL was used for genomic sequencing and methylation-specific PCR (MSP). Modified DNA (100 ng) was amplified by PCR (sense, 5′-ATCTATCCCTAACCGAAATAAAAACGAAA-3′; antisense, 5′-CCCTTCTCTAAAACACTAAAAAAA-3′). Primers were based on the MethPrimer Program (15). PCR reaction conditions: 95°C for 15 minutes followed by 50 cycles (94°C for 1 min, 56°C for 1 min, and 72°C for 1 min; final incubation 72°C for 10 min). The PCR product was purified and sequenced. Ten non-CpG cytosine residues were evaluated for conversion to uracil residues, and repeated independently three times. To quantify the methylation rate of CpG sites in specific cells, the PCR product was cloned into pCR2.1-TA cloning vector (Invitrogen); six positive clones were picked from each cell line for sequencing.

**MSP analysis**

Genomic DNA (100 ng) was subjected to sodium bisulfite modification as above. Based on the promoter sequence of CADM-2a, MSP and unmethylation-specific PCR primers were designed using Serologicals CpGware software: MSP sense, 5′-ATCATATCCTAAGCGCAAATAAAGCGAA-3′; antisense, 5′-AAGTAAATTTTTGTCGTCGTTGC-3′;
and unmethylation-specific PCR sense, 5'-ATATCTATCCCC-TAACAAAAATAAAAAACAAA-3'; antisense, 5'-AGTAA-GTATGATTTGGTTGTTT-3'; product sizes 251 and 256 bp, respectively. CADM-2a 5'-untranslated region (UTR) from -442 to -659 was chosen for MSP analysis. MSP analysis was done according to previous reports (29). DNA isolated from tissue sections was subjected to bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research). Primers for MSP were CADM-2 Meth F2 (sense), 5'-TITTGCCGCGTTTTGC-3' and CADM-2 METH R (antisense), 5'-TAATATCCTCCTCCCGACG-3'. Bisulfite-modified DNA was amplified by PCR: 10 minutes at 94°C denaturation, 40 cycles of amplification (94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 30 seconds). The absence of a DNA template served as a negative control. Products were analyzed in nondenaturing 8% polyacrylamide gels stained with ethidium bromide. DNA from human genomic DNA (Chemicon International, Inc.) was bisulfite-converted and used as a positive control for unmethylated genes. DNA from a primary human fetal cell line (Chemikon) treated in vitro with M.SssI bacterial Cpg methylase (New England Biolabs, Inc.) was bisulfite converted and used as a positive control for methylated alleles.

Demethylation analysis

Cells were plated at 10^6 per 100 mm dish, grown for 24 hours, then 5-aza-2'-deoxycytidine (5-aza-dC; Sigma) was added daily for 4 days at 0, 2.5, or 5 μmol/L. Cells cultured with both trichostatin A (TSA; Cayman Chemical Company) and 5-aza-dC were initially treated with 5-aza-dC for 72 hours followed by an additional dose of 5-aza-dC and then TSA 8 hours later (TSA concentration, 0.5-150 ng/mL). PCR conditions were 94°C for 5 minutes, followed by 50 cycles (TSU-pr1 cells) or 40 cycles (PPC-1, DU145, and LNCaP cells) at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 45 seconds (forward, 5'-ACCATGATTTGGAAACGCAG-3'; reverse, 5'-GAATTCCAGTGTTACTG-3') for a 365-kb product with β-actin control.

Results

Cloning and characterization of CADM-2

The full-length cDNA clone of CADM-2 was obtained by RT-PCR using total RNA extracted from human prostate. Analysis revealed a predicted open reading frame of 442 to 256 bp, respectively. CADM-2a 5'-untranslated region (UTR) from -442 to -659 was chosen for MSP analysis. MSP analysis was done according to previous reports (29). DNA isolated from tissue sections was subjected to bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research). Primers for MSP were CADM-2 Meth F2 (sense), 5'-TITTGCCGCGTTTTGC-3' and CADM-2 METH R (antisense), 5'-TAATATCCTCCTCCCGACG-3'. Bisulfite-modified DNA was amplified by PCR: 10 minutes at 94°C denaturation, 40 cycles of amplification (94°C for 30 s, 53°C for 30 s, and 72°C for 30 s). The absence of a DNA template served as a negative control. Products were analyzed in nondenaturing 8% polyacrylamide gels stained with ethidium bromide. DNA from human genomic DNA (Chemicon International, Inc.) was bisulfite-converted and used as a positive control for unmethylated genes. DNA from a primary human fetal cell line (Chemikon) treated in vitro with M.SssI bacterial Cpg methylase (New England Biolabs, Inc.) was bisulfite converted and used as a positive control for methylated alleles.

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Results

Cloning and characterization of CADM-2

The full-length cDNA clone of CADM-2 was obtained by RT-PCR using total RNA extracted from human prostate. Analysis revealed a predicted open reading frame of 1,311 bp and a 437-amino acid protein with an estimated molecular weight of 47.7 kDa. A high degree of homology of peptides exists between CADM-2 and the other CADM proteins: human CADM-3 (Ncl-1/human TSL1; 44.4% homology), human CADM1 (Ncl-2/human IgSF4/TSLC1; 40.8%), and CADM-4 (human Necl-4/human TSL2; 33%). The mRNA sequence was submitted to GenBank as CADM-2 (Ncl-3), accession number AF538973. Searches identified another mRNA sequence, accession number AL834270 differing primarily in the amino acids encoding a putative signal peptide (94.7% homology). Searching the human genome database with each signal peptide sequence revealed that each matched separate regions in the genomic contig NT_022459.12 of chromosome 3p12.1, suggesting the existence of two isoforms designated CADM-2a and CADM-2b respectively. In addition to divergent signal peptides, analysis revealed other differences. CADM-2a includes 27 bp found in the 3' end of the first exon of CADM-2b, designated exon V1b. Furthermore, the isoforms have different start codons, indicating that they have different promoters. The CADM-2b promoter seems to reside in the intron between exons 1a and 1b.

Putative domains of CADM-2

Domain analysis was done with Simple Modular Architecture Research Tool software and transmembrane segments TMHMM2 software, and showed that CADM-2 has a predicted extracellular domain containing one immunoglobulin-like V-type loop and two immunoglobulin-like C2-type loops, a single hydrophobic transmembrane domain, and a short intracellular domain containing a protein 4.1-binding motif and a terminal PDZ-binding motif. The extracellular domain of CADM-2a bears six NX(S/T) motifs for N-linked glycosylation and one proximal membrane O-glycosylation site predicted by web site-based software, NetOGlyc 3.1.

Splice variants of CADM-2

Forward primers designed to hybridize to the first exon sequence of either CADM-2a or CADM-2b and a reverse primer incorporating the stop codon were used to amplify both isoforms from whole brain and total prostate RNA. Prostate and brain tissue expressed both CADM-2a and CADM-2b, whereas only the prostate expressed an isoform of CADM-2a lacking the sequence for 41 amino acids in the membrane-proximal region which includes a putative O-glycosylation site. This sequence region corresponds to exon 8 as determined by the GeneScan program. This isoform missing exon 8 has been designated CADM-2a-m8.

Expression patterns of CADM-2a and CADM-2b mRNA and protein in prostate cancer cell lines

Expression of CADM-2a and CADM-2b was evaluated by RT-PCR in various prostate cancer cell lines, in the bladder cancer cell line TSU-pr1, and in the "normal" prostate cell line RWPE-1, which has been immortalized by HPV18. Normal brain and prostate tissues were also examined. Nonquantitative PCR showed relatively uniform CADM-2b mRNA expression in all cell lines and tissues examined (Fig. 1A). In contrast, CADM-2a mRNA varied substantially with the highest levels in normal brain and prostate tissue, PPC-1 cells, and RWPE-1 cells. The metastatic prostate cancer cell lines PC3, LNCap, and DU145 as well as metastatic TSU-pr1 cells had low or nearly absent levels of CADM-2a mRNA. RWPE-2 cells, a tumorigenic derivative of RWPE-1 cells transformed by K-ras, also displayed decreased levels of CADM-2a mRNA (Fig. 1A). We noted the presence of two distinct CADM-2a bands in normal
prostate tissue. Sequencing results showed that the two bands represented CADM-2a and CADM-2b-m8.

To determine the endogenous expression of CADM-2 in several cell lines, we developed two antibodies against synthetic peptides from the amino and carboxyl termini: the NH2 terminus and the COOH terminus. The presence of CADM-2 protein in cell lines was determined using affinity-purified antibodies. The predicted molecular weight of endogenous CADM-2 is approximately 50 kDa. Multiple nonspecific bands were detected by NH2-terminal antibodies in all cell lines and tissues (data not shown), indicating that the NH2-terminal antibody was not specific. CADM-2 protein expression in HEK-293, Caco-2 cell lines (Fig. 1B) detected by COOH-terminal antibody was quite high, although significantly lower in all “normal” prostate cell and cancer cell lines (Fig. 1B). CADM-2 expression in HEK293 and Caco-2 showed a single clear band with double the protein size when compared with that in mouse liver, suggestive of probable dimerization which results in protein degradation. Therefore, to determine whether the different signal peptides result in differential processing of CADM-2, the signal peptide of CADM-2a-m8 was replaced with the signal peptide from CADM-2b, creating CADM-2b-m8. Transient transfection with pN1-CADM-2b-m8 resulted in a very weak band at 30 kDa consistent with protein degradation. More interestingly, there are some small bands detected by GFP antibody especially CADM-2a isoforms (Fig. 2A), suggesting that CADM-2-GFP fusion protein may be cleaved by some specific enzymes. Detailed mechanisms warrant further investigation.

Determination of CADM-2 antibody specificity using peptide competition assay

To further test the specificity of anti–CADM-2 antibodies, several peptide competition assays were done. We used exogenous adenovirally mediated CADM-2a-m8 proteins in DU145 cells and endogenous CADM-2 in 293 cells for peptide competition assays. Figure 2B shows that the protein amounts of both CADM-2a full-length and CADM-2a-m8 expression increased with the higher adenoviral MOIs. However, the expression patterns of the two vectors were different, the bands of CADM-2a-m8 having a broad range from 50 to 100 kDa because of N-glycosylation (data not shown). CADM-2a had two isolated major bands, possibly due to O-glycosylation modification, as CADM-2a-m8 is missing exon 8 which encodes potential O-glycosylation sites. The peptide competition assay generated similar results between ectopic
CADM-2 expression at 5 MOI adenovirally mediated CADM-2a-m8 in DU145 cells (Fig. 2C) and endogenous 293 cells (Fig. 2D). The density of the CADM-2 band detected by COOH-terminal antibody, 110 kDa in 293 cells and 55 kDa in Ad-CADM-2a-m8, was reduced with an increase of homologous COOH-terminal peptide, and was even absent with an increase of COOH-terminal peptide up to 10 μg/mL concentration, indicating that the homologous COOH-terminal peptide inhibited binding. As expected, under identical conditions, heterologous NH2-terminal peptide did not block the binding of COOH-terminal antibody, suggesting no competitive inhibition by the NH2-terminal peptide. Thus, the specificity of anti–CADM-2 COOH-terminal antibody was confirmed.

**CADM-2 expression in normal tissue and human prostate cancer specimens**

No significant CADM-2 protein expression in prostate cell lines prompted us to determine if expression could be detected in human normal tissues. Results showed that CADM-2 protein was highly expressed in normal brain and prostate (Fig. 3A). The COOH-terminal antibody reacted with multiple protein bands detected in brain and prostate tissue (Fig. 3A). The multiple bands might be due to complex N- and/or O-glycosylation because five potential X(S/T) motifs for N-linked glycosylation, and one potential proximal O-glycosylation site, were predicted by the software stated above (data not shown).

To further evaluate CADM-2 protein expression, prostate tumor tissue microarrays were examined. Immunohistochemical analysis revealed that adenocarcinoma exhibits significantly lower levels (ANOVA, \( P < 0.001 \)) of CADM-2 protein compared with normal adjacent tissue, BPH, PIN, and normal donor tissue (Fig. 3B; Table 1). However, there was no statistically significant difference in CADM-2 expression by stage or Gleason score, indicating that CADM-2 expression is reduced or lost during prostate carcinogenesis. Evaluation of CADM-2 levels according to Gleason scores revealed that the differences in the median values among the various scoring groups lacked statistical significance and thus differences may have resulted from random sampling variability.

**Ectopic expression of CADM-2a suppresses DU145 cell proliferation**

To investigate whether CADM-2a affects prostate cell proliferation, DU145 cells were transiently transduced...
with Ad-CADM-2a-FL, Ad-CADM-2a-m8, and Ad-CMV. The number of viable cells at 3 and 5 days after transduction with adenoviruses at various MOIs was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The viability of transduced cells was compared with that of untransduced DMEM-treated control cells. The results presented in Fig. 4A show that cell viability was markedly reduced for DU145 cells transduced by Ad-CADM-2a-FL and Ad-CADM-2a-m8 at both time points and that inhibition of cell viability was dose-dependent. Additionally, after 5 days of transduction at 50 MOI, both Ad-CADM-2a-FL and Ad-CADM-2a-m8 killed almost 84% of DU145 cells. In contrast, the growth of cells treated with control virus containing no inserts, Ad-CMV, was only slightly affected. There are significant differences between the Ad-CADM-2a-FL or Ad-CADM-2a-m8 groups and the control viral group Ad-CMV at day 3 and day 5 posttransduction, respectively (P < 0.01). The mechanisms of this inhibition warrant further investigation.

**Effect of CADM-2a on colony formation in soft agar**

Introduction of tumor suppressor genes into tumorigenic cells could reverse the ability of these cells to grow in an anchorage-independent manner (30). Therefore, we assessed whether exogenous CADM-2a affected anchorage-independent growth in DU145 cells. DU145 cells (1.0 × 10⁴), transiently transduced at 5 MOI by Ad-CADM-2a-FL, Ad-CADM-2a-m8, and Ad-CMV were seeded in soft agar and allowed to grow for 15 days. DU145 parental cells and cells with Ad-CMV vector alone served as the control group. Introduction of either the CADM-2a-m8 or CADM-2a-FL by viral vectors significantly decreased the number of colonies able to form in soft agar compared with DU145 parental cells and cells with Ad-CMV vector alone (P < 0.01). This finding, combined with the cell proliferation assay, suggested that CADM-2 could function as a putative tumor suppressor in prostate cancer.

**Table 1. CADM-2 protein expression in prostate tissue by TMA**

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<td>0.53</td>
<td>0.688</td>
<td>0.371</td>
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</table>

NOTE: TMA slides were stained for CADM-2 expression using a polyclonal antibody for CADM-2. Each core was then examined for expression and assigned a number from 0 to 4 depending on the intensity of CADM-2 immunostaining as determined by a pathologist. Abbreviation: NAT, normal adjacent tissue.

*These groups were significantly different, ANOVA on ranks (P < 0.001); adenocarcinoma vs. other groups is different in pairwise comparisons.
Bisulfite sequencing analysis of CADM-2a
5′-UTR methylation

According to RT-PCR analysis, CADM-2a RNA expression generally decreased in prostate cancer cell lines compared with more normal prostate cell lines and tissue. Therefore, CADM-2a and CADM-2b sequences were analyzed by MethPrimer Program software for possible methylation sites (31). Based on GC content (64.4%) and the ratio of observed to expected CpG dinucleotides (0.674), the 5′-UTR of CADM-2a contains two CpG islands: one located between −682 bp and −58 bp relative to start codon ATG, and a second one located between −1,002 bp and −832 bp. In contrast, the 5′-UTR of CADM-2b has no putative CpG islands. Note that the identification of the CADM-2a promoter and its first exon in human chromosome 3 was based on the FirstEF program (32). The results of this analysis indicated that the putative transcription start site was 928 bp upstream of the ATG start codon.

Bisulfite genomic sequencing was used to test for CADM-2a promoter hypermethylation. Genomic DNA from RWPE-1, RWPE-2, PPC-1, DU145, LNCaP, PC-3, and TSLU-pr1 cells was isolated and modified with bisulfite, which converts cytosine residues to uracil while leaving 5-methylcytosine unaltered. From each cell line, a 237 bp fragment spanning −237 bp to 0 bp relative to ATG within the putative CpG island was amplified by PCR, subcloned into a TA vector, and then six clones per cell line were sequenced. Almost all of the 22 CpG dinucleotides within the fragment were methylated in DU145 (79.5%), LNCaP (71.2%), and TSLU-pr1 (82.6%) cells, although methylation was not complete. In contrast, none of the sites in clones from RWPE-1 and PPC-1 DNA exhibited methylation (0%; Fig. 4A). Interestingly, DNA from PC-3 and RWPE-2 cells displayed more heterogeneity; with the CpGs in some of the clones heavily methylated whereas other clones were sparsely or totally unmethylated. The overall methylation frequency of DNA from...
RPWE-2 and PC-3 cells was 61.4% and 43.9%, respectively (Fig. 5A). There was also a trend of decreasing methylation frequency towards the 3′ end of the CpG islands in many of the cancer cells. Combined with the transcript expression studies, this suggested an inverse relationship between promoter methylation and gene transcription.

MSP analysis of the CADM-2a 5′-UTR in prostate cancer cell lines and patient specimens

To confirm the findings in tissue culture cell lines, we used MSP analysis to further evaluate CADM-2a methylation in the cell lines and patient specimens. The MSP analysis corroborated with bisulfite sequencing: the 5′-UTR was predominantly methylated in LNCaP and DU145 cells; partially methylated in RWPE-2, PC-3, and TSU-pr1 cells; and unmethylated in RWPE-1 and PPC-1 cells (Fig. 5B). Methylation of the CADM-2a 5′-UTR was present in three of the nine patient tumors (Fig. 5C). Of note, all three tumors positive for MSP were high-grade disease. In addition, of the four patients that developed recurrent disease, two had tumors with methylated CADM-2a (Table 2).

Restoration of CADM-2a expression through treatment with 5-aza-dC and/or TSA

To validate the role of DNA methylation in silencing CADM-2a, the ability of the DNA methylation inhibitor 5-aza-dC and the histone deacetylase inhibitor TSA to restore CADM-2a expression in cell lines was tested. After
treatment with 5-aza-dC, expression of CADM-2a mRNA in PPC-1 cells remained consistent although expression was restored in a dose-dependent manner in LNCaP cells (Fig. 5Da and b). Although 5-aza-dC weakly reactivated CADM-2a expression in DU145 cells, it had no effect on the expression in TSU-pr1 cells. The combination of 5-aza-dC with TSA showed the re-activation of gene expression levels in DU145 and TSU-pr1 cells (Fig. 5Dc and d). These data suggest that repression of CADM-2a expression occurs, at least in part, through CADM-2a hypermethylation as well as histone deacetylation.

**Discussion**

The CADM family is a recently described family of genes whose protein expression and function has not been completely characterized. We cloned CADM-2 from normal prostate to assess any potential role that this gene may have in the development and progression of prostate cancer. We have shown that the CADM-2 gene encodes two isoforms, CADM-2a and CADM-2b, each driven by an independent promoter. CADM-2a and CADM-2b differ primarily in signaling peptides, likely resulting in differential cellular localization, cell processing, and potentially in function. We have been unable to detect or show stable protein expression in spite of clear mRNA expression.

Prostate and bladder cancer cell lines obtained from metastases such as DU145, PC-3, LNCaP, and TSU-pr1 cells show decreased levels of CADM-2a mRNA compared with levels in RWPE-1 cells, and normal prostate and brain tissues. This finding correlates with the hypermethylation of the promoter, suggesting that silencing of CADM-2a might be an important event in the development or the progression of prostate cancer. RWPE-2 cells, the tumorigenic derivative of RWPE-1 cells transformed by K-ras and PPC-1, also expressed diminished levels of CADM-2a mRNA. In contrast, CADM-2b mRNA levels remained relatively similar in all cell types examined. Differential regulation through hypermethylation of the CADM-2a promoter likely accounts for this difference in expression pattern. Interestingly, CADM-2a expression in RWPE-2 cells was significantly decreased compared with RWPE-1, implicating a connection between Ki-Ras expression and CADM-2a hypermethylation. Indeed, one group reported that activated Ras, in combination with the SV40 T antigen, immortalized normal human bronchial epithelial cells that then formed colonies in soft agar. The transformation corresponded with high levels of DNA methyltransferase (DNMT3b) activity such that methylation and subsequent silencing of several tumor suppressor genes occurred, including CADM-1 (33).

CADM-2a expression was restored in selected cell lines after 5-aza-dC and/or TSA treatment, further confirming that hypermethylation is a major mechanism for silencing CADM-2a. 5-aza-dC mediated inhibition of DNA methyltransferase, which alone maintains the genomic de novo methylation of cytosines, was able to induce CADM-2a gene expression appreciably in LNCaP cells, but only weakly in DU145 cells and not at all in TSU-pr1 cells (Fig. 5D). TSA treatment of DU145 and TSU-pr1 cells perhaps weakly induced some expression (Fig. 5D). Interestingly, a recent report (34) illustrated that TSA could induce DNA demethylation in the absence of 5-aza-dC. However, the combination of TSA and 5-aza-dC synergistically induced CADM-2a transcription in both DU145 and TSU-pr1 cells (Fig. 5D). The ability of these two inhibitors to work in concert to restore expression has been shown for many genes, including the estrogen receptor (35) and metallothionein 1G (MT1G; ref. 36), and is consistent with the recently uncovered mechanisms by which methylation regulates gene expression.

To understand the mechanism by which this downregulation occurs in adenocarcinoma of the prostate, we have

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**Table 2. Clinicopathologic factors of nine prostate cancer patients and one normal donor**

NOTE: Entries in boldface in this table indicate that 3 of the 9 patients’ tumors had CADM-2 promoter methylation, and all 3 tumors positive for MSP were high grade and/or recurrent disease.

Abbreviation: PSA, prostate-specific antigen.
investigated CADM-2 expression by immunohistochemical staining. Using prostate TMA slides, we show a significant downregulation of CADM-2 in prostate cancer that does not correlate with tumor grade and progression in our prostate TMA. Except CADM-3, which is highly expressed in the neural system, all CADM molecules are expressed in multiple tissues including prostate. Accordingly, loss of expression of Necl-2 in PPC-1 is due to its promoter methylation. Bisulfite sequencing assay determined that promoter methylation was found in 32% primary prostate tumors, and showed no significant association with the Gleason score and tumor-node-metastasis staging (9). In one report (37), using a TMA and immunohistochemical staining, the frequency of downregulated or lost expression of CADM-1 in metastatic nasopharyngeal carcinoma lymph node was significantly higher than that in primary nasopharyngeal carcinoma, suggesting that CADM-1 is a tumor suppressor gene in nasopharyngeal carcinoma. CADM-4 (Necl-4/IGSF4C) is mainly expressed in the prostate, kidney, and bladder in addition to the brain according to one report. Its expression is reduced or lost in prostate cancer cell lines such as PPC-1 and DU145. Introduction of CADM-4 into PPC-1 strongly suppresses subcutaneous tumor formation in nude mice (12). These findings indicate that alteration of these three CADMs is associated with prostate tumor genesis and/or metastasis.

The increased methylation of CADM-2a and the subsequent decrease in expression in prostate cancer cells suggests that CADM-2a acts as a novel tumor suppressor, especially given that other CADMs serve as tumor suppressors in a variety of cancers. Adenovirus-mediated overexpression of CADM-2a statistically suppressed DU145 cell growth in vitro, indicating that restoration of CADM-2 expression attenuates prostate cancer growth functioning like tumor suppressor. The ability of CADM-2a to diminish colony formation in soft agar further supports its role as a tumor suppressor (Fig. 4B) because the ability of cells to grow in an anchorage-independent manner represents a hallmark of tumorigenesis (Fig. 4). Mao et al. (38) reported that adenovirus-mediated CADM-1 (TSLC1) inhibits non–small-cell lung cancer growth through induction of apoptosis. Detailed mechanisms of CADMs family to suppress tumor growth needs to be further investigated.

In conclusion, based on the data in this study, we propose that CADM-2 might act as a tumor suppressor in the progression of transformed prostate cancer cells to invasive and metastatic prostate cancer. Moreover, the silencing of CADM-2a is accomplished at least in part through promoter hypermethylation and may be associated with more aggressive prostate cancer. These conclusions warrant confirmatory investigations in a larger number of patients over a wider spectrum of diseases. In addition, they suggest that understanding the biological function of CADM-2 might lead to further insights into the development and progression of prostate cancer, as well as potential therapeutic targets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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