Human β-Defensin-1, a Potential Chromosome 8p Tumor Suppressor: Control of Transcription and Induction of Apoptosis in Renal Cell Carcinoma

Carrie Q. Sun,[1] Rebecca Arnold,[5] Carina Fernandez-Golarz,[1] Amanda B. Parrish,[5] Tara Almekinder,[1] Ju He,[1] Shuk-mei Ho,[6] Pavel Svoboda,[3] Jan Pohl,[3] Fray F. Marshall,[1,3] and John A. Petros[1,2,3,4]

Departments of [1]Urology and [2]Pathology and [3]Winship Cancer Institute, Emory University; [4]Atlanta Veterans Affairs Medical Center, Atlanta, Georgia; [5]Department of Cell and Molecular Biology, Duke University, Durham, North Carolina; and [6]Department of Environmental Health, University of Cincinnati, Cincinnati, Ohio

Abstract

***Human β-defensin-1* (*hBD-1*) is a candidate tumor suppressor gene located on chromosome 8p23. Previously, we showed that cancer-specific loss of hBD-1 was found in 90% of renal clear cell carcinomas and in 82% of prostate cancers. To investigate the possible mechanisms of decreased gene expression and determine the function of *hBD-1* protein in urological cancers, we sequenced *hBD-1* gene coding regions in prostatic and renal cancer samples. We then analyzed the frequency distribution of promoter polymorphisms and determined the effect of these base changes on transcriptional activity of the *hBD-1* promoter. A polymorphism at −688 bases upstream of the ATG start codon affects *hBD-1* promoter activity, leading to a rate of reporter gene transcription that is 40% to 50% lower than the wild-type sequence when tested in either DU145 or TSU-Pr1 cell lines. In addition, a polymorphism at −44 bases was shown to enhance transcription up to 2.3 times more than the wild-type sequence in the same cell lines. In addition, three novel *hBD-1* promoter mutations were found in renal and prostate cancer clinical samples. An iso-5-aza-2′-deoxycytidine treatment was effective in transcription up-regulation in DU145, suggesting a possible upstream methylation-dependent effect. Synthetic hBD-1 peptide inhibited bladder cancer cell TSU-Pr1 proliferation. Overexpression of the *hBD-1* gene in renal cancer cells SW156 resulted in caspase-3-mediated apoptosis. These data support the hypothesis that *hBD-1* is a potential tumor suppressor gene for urological cancers. Promoter point mutations may be responsible for cancer-specific loss of hDB-1 expression.** (Cancer Res 2006; 66(17): 8542-9)

Introduction

Defensins are a family of antimicrobial peptides produced by WBCs and epithelial cells. Defensins are classified into α-defensins, β-defensins, and θ-defensins according to the size and binding patterns of disulfide bonds within mature peptides. To date, six β-defensins have been identified and mapped to a locus on chromosome 8p, an area that contains at least three separate tumor suppressor genes inactivated during the progression of human malignancies, including prostate and renal cancers (1–3). Previous studies have revealed that deletion of chromosome 8p is the most common genetic alteration in prostate cancer and the second most common genomic event in bladder and renal cancers (4). *Human β-defensin-1* (*hBD-1*), located in a defensin gene cluster on the short arm of chromosome 8, is constitutively expressed in prostate, kidney, and urogenital track luminal epithelium. Our previous studies have documented a cancer-specific loss of hBD-1 in 90% of renal clear cell carcinomas and in 82% of malignant prostate clinical samples, whereas high levels of expression are maintained in benign epithelium (5). For many years, defensins have been studied as biochemical barriers against invading pathogens, which join the innate immune system by killing and/or inactivating particular spectra of bacteria, fungi, and some enveloped viruses. Recent evidence has shown that human defensins also play a role in regulating cell-mediated, adaptive immunity by their chemotactic effect. Both hBD-1 and hBD-2 have been shown to induce the migration of immature dendritic cells and memory T cells. β-Defensin-2 can attract tumor necrosis factor-α–treated human neutrophils (6, 7), and murine β-defensin-2 may have the potential to trigger a type I immune response *in vivo* against tumor antigen by acting directly on the immature dendritic cells as an endogenous ligand for Toll-like receptor 4 (8). Currently, identification and characterization of the somatic genetic alterations that promote cancer are still a challenge in common cancers, such as prostate, renal, and bladder. To determine whether *hBD-1* is a tumor suppressor gene that is inactivated on chromosome 8p, we investigated a large number of clinical prostate and renal cancer samples and cell lines for the possible mechanisms of gene silencing and effect on tumor phenotype. Here, we report that *hBD-1* promoter mutations may be responsible for the down-regulated gene expression in clinical cases. The 5-aza-2′-deoxycytidine (5-aza-dCyd)–induced gene up-regulation in DU145, but not the other tested cell lines, suggests an indirect methylation-dependent suppression. About the biological function of hBD-1, we found that synthetic hBD-1 peptide can inhibit bladder cancer cell proliferation. Overexpression of the *hBD-1* gene resulted in near complete cell death in multiple human cancer cell lines. Furthermore, we show that hBD-1 can induce apoptosis in human renal carcinoma cells. Because of the role that has been identified in both innate and adaptive immunity, restoration of hBD-1 expression could theoretically be useful as a form of antitumor therapy especially for renal cell carcinoma, which is particularly sensitive to immunotherapy.

Requests for reprints: John A. Petros, Department of Urology, Emory University, 1365 Clifton Road, Building B4206, Atlanta, GA 30322. Phone: 404-778-4233; E-mail: jPetros@emory.edu.

doi:10.1158/0008-5472.CAN-06-0294

Materials and Methods

Cell culture. Human prostate cancer cell lines DU145, PC-3, and LNCaP and a bladder cancer cell line TSU-Pr1 were cultured in RPMI 1640 (Cellgro, Herndon, VA) with 10% fetal bovine serum (FBS). The renal cancer cell line SW156 was cultured in DMEM with 4.5 g/L glucose, L-glutamine, 10% FBS, and without sodium pyruvate (Cellgro). For transfections, medium was supplemented with 800 μg/mL G418 (Cellgro). To induce *hBD-1* gene transcription in T-Rex-inducing system (Invitrogen, Carlsbad, CA), the medium was supplemented with 1.5 μg/mL tetracycline (Invitrogen) for up to 48 hours. Baseline expression of hBD-1 varies in these cell lines, with DU145 having the highest level followed by TSU-Pr1 and PC-3 with LNCaP exhibiting the lowest levels (data not shown).

Clinical samples and RFLP analysis. Blood samples were obtained from 126 prostate cancer patients who had undergone radical prostatectomy at Emory University Hospital (Atlanta, GA). A "no-prostate-cancer" control group included 54 patients at least 50 years of age, whose prostate biopsy showed no evidence of cancer, and prostate-specific antigen levels were <4.0 ng/mL. The RFLPs were detected by PCR amplification of the genomic DNA extracted from these blood samples for the *hBD-1* exons 1 and 2 with published primers (9): exon 1, 5′-CTTGACTGTGGCACCTCCCTT-CAG-3′ and 5′-CAGCCCTGGGGATGGGAAACTC-3′ and exon 2, 5′-GCAGC-TACAAGCCATGAGTCTG-3′ and 5′-TGGCCCAAAGGAGGTATACTTC-3′. The PCR product was then digested by the restriction enzymes *Cac*8I, *Nla*IV, *Hga*I, and *Scr*FI, respectively, followed by 3% Metaphor agarose gel electrophoresis analysis. At positions −688 and −44 in the 5′-untranslated region (5′-UTR), the genomic sequence of the two alleles contained either a guanine or a cytosine according to the published sequence (10) that can be distinguished by restriction enzyme *Cac*8I for the nucleotide at position −688 and *Hga*I for −44. At positions −20 and −52 in the 5′-UTR, the nucleotide is either guanine or adenine. The variation can be detected by restriction enzyme *Scr*FI at position −20 and *Nla*IV at position −52 (9).

DNA extraction and mutational analysis. Genomic DNA was extracted from frozen prostate and renal cancer tissues according to the manufacturer's protocol of GenElute Mammalian Genomic DNA Miniprep kit (Sigma, St. Louis, MO). Extracting DNA from prostate cancer cells collected by laser microdissection was followed by the protocol that was previously published (11). For detection of point mutations, deletions, or genomic rearrangements, PCR was done for exons 1 and 2 with the primers listed above. The PCR products were sequenced with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Branchburg, NJ) using the ABI 3100 genetic analyzer.

Demethylation assay with 5-aza-dCyd followed by real-time PCR. Cells were subcultured in 100-mm dishes at 60% to 70% confluent for 24 hours. Fresh medium containing 5-aza-dCyd (Sigma) at 50 and 100 μmol/L was added to the cell culture. Total RNA was extracted from the cells after 72 hours of incubation with 5-aza-dCyd. The hBD-1 expression was measured by real-time reverse transcription-PCR (RT-PCR; Taqman, Applied Biosystems) using the iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad, Hercules, CA).

Bisulfite genomic sequencing. Bisulfite-modified DNA extracted from prostate, bladder cancer cell lines, and three pairs of prostate cancer and normal laser capture microdissected (LCM) samples was sequenced by first doing nested PCR with platinum Taq (Invitrogen) and two sets of primers designed by MethPrimer (sequences on request). All primer sequences were devoid of CpG dinucleotides to avoid biased amplification of the methylated alleles. The PCR product that contained the promoter region of hBD-1 was cloned into pGEM-T Easy vector (Promega, Madison, WI), and then DNA clones were picked for each sample and sequenced with M13 forward primer (Macrogen, Seoul, Korea). Methylation site was distinguished by bisulfite-dependent deamination: unmethylated cytosine changed to uracil, whereas methylated cytosines are resistant to this modification and remain as cytosines.

Cell proliferation assay. Cells were seeded in a 96-well plate with normal medium at 50% confluent (10,000 cells per well) and incubated at 37°C for the time required for the cells to attach. The medium was changed to fresh medium with different concentration of synthesized hDB-1 and control peptide. The synthetic hBD-1 (DHYNCVSSGGQCLYSACPIFTKIQGT-CYRGKAKCCK) was prepared in the Emory Microchemical and Proteomics Facility by standard t-butoxycarbonyl/benzyl solid-phase peptide synthesis. The all-reduced, hBD-1 was air oxidized to form the three intramolecular disulfides, high-performance liquid chromatography purified, and obtained in its trifluoroacetyl (TFA) salt; its mass was confirmed (3,925.78, monoisotopic). The control peptide used was a TFA salt of an IgA fragment (YYALSDAKEEEPRYKALRGENQDLREKERKYQDKIKKLEEKEKNLEKKSC).

The wells were emptied at the different end points by overturning onto paper towel followed by washing with 1× PBS twice and then emptied again. To completely lyse the cells, the plate was frozen and thawed thrice at −80°C and 37°C. The dsDNA in each well was quantified by adding 100 μL of aqueous Hoechst 33258 in TNE buffer per well (Molecular Probes, Eugene, OR). The fluorescence intensity was measured by the BioTek (Winooski, VT) FL600 Microplate Fluorescence Reader.

Construction of *hBD-1* reporter plasmids. Based on the sequence of *hBD-1* gene published in Genbank (12), progressive deletions of promoter sequences from 5′-flanking region, 1,140 bp immediately upstream from translation codon ATG were amplified by PCR from prostate cancer cell line DU145 and bladder cancer cell line TSU-Pr1 using Platinum Taq DNA polymerase (Invitrogen). The PCR products were then ligated into the TA cloning vector pCR2.1 vector (Invitrogen), transformed to INVaF′-competent *Escherichia coli* cells, and plated on Luria-Bertani/Ampicillin/5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside/isopropyl-L-thio-B-D-galactopyranoside plates. A white colony was selected and sequenced with BigDye Terminator v3.1 Cycle Sequencing kit using the M13 and T7 primers. The sequence-verified inserts were removed to pGL3-Basic Luciferase Reporter Gene vector (Promega) by cutting with restriction enzymes *Sac*I and *Xho*I.

Transfection and luciferase assay. Cells were cultured in a 24-well plate at 60% to 70% confluent overnight. The hBD-1 luciferase reporter constructs were mixed with transfection reagent Fugene 6 (Roche, Indianapolis, IN) at the ratio 2 μg/6μL (1:3) in 100 μL of serum-free medium before adding to the cell culture. The pSV-β-galactosidase control plasmid was cotransfected to the cells at one fourth of the concentration of luciferase construct. The intensity of luciferase was detected by LUMIstar Galaxy (BMG Labtech, Offenburg, Germany) at 48 hours after transfection. The β-galactosidase absorbance readings were used to normalize luciferase activity between different groups for transfection efficiency. The mean of triplicates was compared with different constructs.

Expression vectors and transfection. The full-length cDNA of *hBD-1* was removed from the commercial hBD-1 expression vector pINCY (Incyte, Wilmington, DE) by digesting with restriction enzymes *Eco*RI and *Not*I. This insert was then ligated into vectors of pcDNA 3.1(+) and pcDNA 4/TO (Invitrogen) and then confirmed by sequencing in both directions. To generate a stable cell line expressing hBD-1 protein, cells were cultured at 60% to 70% confluent in a six-well plate overnight. The vector pcDNA 3.1(+)/hBD-1 was transfected by mixing 2 μg plasmid DNA with 3 μL Fugene 6 per well. Selection drug G418 (800 μg/mL) was added to the cell culture at 48 hours after transfection. To establish a stable T-Rex-inducible system, the vector pcDNA4/TO/hBD-1 was cotransfected into SW156 cells with vector pcDNA6/TR at the concentration ratio 1:5. The expression clone was selected by 50 μg/mL Zeocin (Invitrogen) for 2 weeks.

Carboxymethyl extraction for cationic peptides hDB-1. Macro-Prep CM beads (Bio-Rad) were equilibrated in 25 mmol/L ammonium acetate (pH 6.8-7.2). Cell culture medium was collected and filtered by passing the medium through Whatman 4 filter paper. Carboxymethyl beads were added into the medium at the ratio 1:10 and stirred overnight at 4°C. Next day, the beads were centrifuged at 1,500 rpm for 3 minutes; the supernatant was discarded and the beads were washed twice with 25 mmol/L ammonium acetate (pH 7.8). For elution of the protein, two carboxymethyl bead volumes of 5% acetic acid were added and mixed by gentle shaking at room temperature 5 to 10 minutes, and then the beads were pelleted and the supernatant was transferred into an Eppendorf tube and dried in a SpeedVac overnight. The final sample was dissolved into 20 to 40 μL of Western loading buffer (13).

Western blot analysis. Whole-cell extracts were obtained by lysing cells with lysis buffer containing 50 mmol/L Tris base, 5 mmol/L EGTA,

150 mmol/L NaCl, and 1% Triton X-100 (pH 7.4). One tablet of protease inhibitor (Roche) was dissolved in 7 mL of lysis buffer. Total protein (30 μg/well) was loaded in 4% to 12% gradient NuPAGE MES SDS gel (Invitrogen) and transferred into Immun-Blot polyvinylidene difluoride membrane (Bio-Rad). The membrane was immunoblotted with anti-hBD-1 (13) at 1:1,000 dilution, anti-β-actin at 1:2,000 dilution (Sigma), anti–poly(ADP-ribose) polymerase (PARP) at 1:2,000 dilution (Cell Signaling, Beverly, MA), anti-caspase-8 at 1:1,000 dilution (Cell Signaling), anti-caspase-9 at 1:1,000 dilution (Cell Signaling), and DNA fragment factor at 1:1,000 dilution (Cell Signaling). Immunodetection was completed by using the corresponding secondary horseradish peroxidase (HRP)–conjugated antibodies (Amersham, Piscataway, NJ). HRP activity was detected using enhanced chemiluminescence from ECL Western Blotting Analysis System (Amersham).

Statistical analysis. χ^2 analysis was done for observed and expected occurrences of all single-nucleotide polymorphisms (SNP), with a $P < 0.05$ indicating significance. Student's t test was used to determine if the averages of two samples are different, with $P = 0.001$ indicating significance.

Results

Promoter-report analysis. To gain a better understanding of the cancer-specific loss of hDB-1 RNA and protein, we analyzed *hBD-1* promoter function. Two distinct promoters were tested for their ability to drive a luciferase reporter construct. The "wild-type" promoter contains the most common base (G) at position −688 and (C) at −44 in the 5′-UTR. The "mutant" promoter contains the second most common base (C) at position −688 and (G) at −44. These variants are prevalent in the population (1, 5). Promoter deletion constructs were made to include a variable amount of upstream sequence. In both wild-type and mutant promoters, activity was seen beginning at −144 bp (upstream of ATG) with full activity with the −192 bp promoter (Fig. 1*A* and *B*) In wild-type only, the longer promoter fragments up to −1,140 bp retained full activity. In the mutant promoter, the −433 bp fragment (containing the −44G mutation only) drove transcription with ∼40% greater efficiency ($P = 0.001$); however, this enhancement was inhibited by the polymorphism at −688 (C) existing upstream. The longer promoters of −726 and −1,140 bp (containing −44G and −688C) were significantly less effective ($P = 0.001$) in driving reporter transcription (Fig. 1*C* and *D*). This is in distinction to the wild-type sequence, where longer promoters functioned as well as the 433 bp promoter. These effects were observed with transient transfection

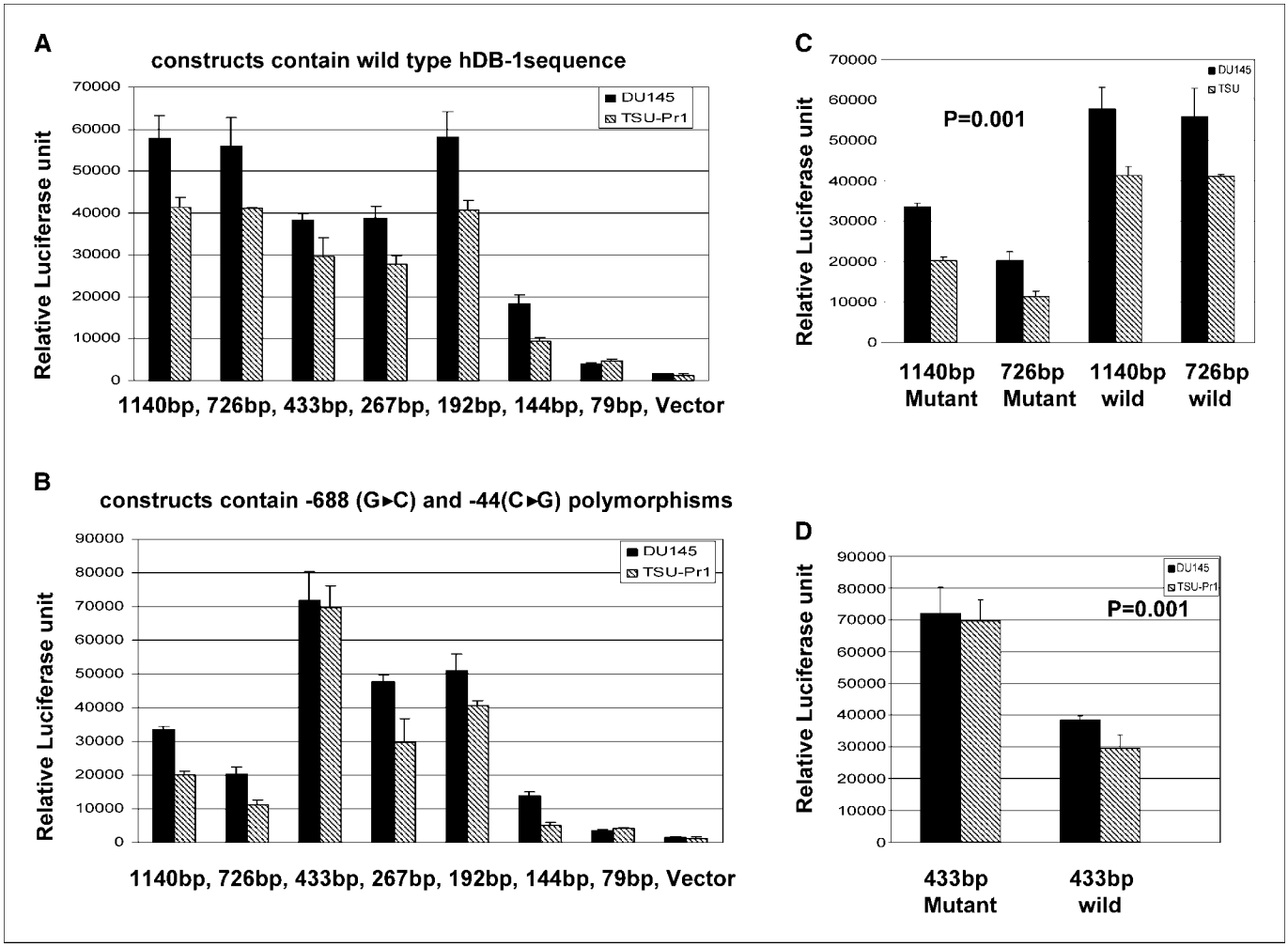

Figure 1. Polymorphism-associated hDB-1 promoter function was analyzed by luciferase transcriptional activity. *A,* relative luciferase unit of the progressive deleted wild-type hDB-1 promoter sequences in DU145 and TSU-Pr1 was measured at 48 hours after transient transfection. *B,* relative luciferase unit of the progressive deleted 5′-flanking region hDB-1 sequences with polymorphisms −688 (G/C) and −44 (C/G) in both DU145 and TSU-Pr1 was measured at 48 hours after transient transfection. *C,* comparison of the relative luciferase units of 1,140 and 726 bp of mutant (containing −688C and −44G) with the relative luciferase units of wild-type constructs in DU145 and TSU-Pr1. *D,* comparison of the relative luciferase unit of the 433 bp mutant (containing −44G only) with the wild-type constructs in both DU145 and TSU-Pr1.

Table 1. The frequency of promoter polymorphism of *hDB-1* in prostate cancer patients and normal control

Position	RFLP	Allele	Frequency in patients		Frequency in control	
			No.	Relative (%)	No.	Relative (%)
5′-UTR (−20 G/A)	*Scr*FI	1	20	16.1	12	22.2
		1+2	60	48.4	28	51.9
		2	44	35.5	14	25.9
5′-UTR (−44 C/G)	*Hga*I	1	80	64.5	41	75.9
		1+2	41	33.1	12	22.2
		2	3	2.4	1	1.9
5′-UTR (−52 G/A)	*Nla*IV	1	24	19.4	8	14.8
		1+2	52	41.9	25	46.3
		2	48	38.7	21	38.9
5′-UTR (−688 G/C)	*Cac*8I	1	66	53.2	32	59.3
		1+2	51	41.1	20	37
		2	9	7.1	2	3.7

NOTE: Allele 1 designates the absence of polymorphism; allele 2, the homozygotes for the polymorphisms; and allele 1+2, the heterozygotes for both alleles 1 and 2. Allele frequencies are given as absolute numbers and relative frequencies in a total of 124 patients and 54 controls.

into both the DU145 and the TSU-Pr1 cell lines but were more pronounced in DU145, which normally expressed hBD-1.

Promoter polymorphism frequency in patient with prostate cancer. To determine the frequency of promoter polymorphisms in patients with prostate cancer, these SNPs were identified using a separate PCR amplification followed by restriction enzyme digestion specific for each site. By this method, we determined the base in both alleles for each patient. Although a significant association between prostate cancer and the frequencies of either the −44 or the −688 mutation was not

Table 2. The frequency of single-base mutations of *hBD-1* promoter and coding regions in prostate and renal cancer patients

	Location and mutation	Wild-type frequency	Relative (%)	Mutant frequency	Relative (%)
Prostate					
Exon 1 (n = 23)	660 G/A	14	60.68	9	39.10
	668 C/G	17	73.91	6	26.08
	692 A/G	13	56.52	10	43.47
	322 T/A	18	78.26	5	21.73
Exon 2 (n = 30)	534 T/C	21	91.3	2	8.69
	1,754 A/G	3	10	27	90
Renal					
Exon 1 (n = 18)	660 G/A	8	44.44	10	55.56
	668 C/G	12	66.67	6	33.33
	692 A/G	4	22.22	14	77.78
	584 G/A	17	94.44	1	5.56
Exon 2 (n = 35)	1,754 A/G	2	5.71	33	94.28
	1,836 A/G	32	91.43	3	8.57

-52/660 G/A **-44/668 C/G** **-20/692 A/G**

gtcagctcag cctccaaag***g*** agccagc***c***tc tccccagttc ctgaaatcct g***a***gtgttgcc tgccagtcgc

catgagaact tcctaccttc tgctgtttac tctctgctta ctttgtctg agatggcctc aggtggtaac tttctcacag gccttggcca cagatctgat cattacaatt gcgtcagcag tggagggcaa tgtctctatt ctgcctgccc gatctttacc aaaattcaag gcacctgtta cagagggaag gccaagtgct gcaagtgagc tg***g***gagtgac cagaagaaat

gacgcagaag tgaaatgaac tt **1754 A/G**

NOTE: The mutation sites in a published *hBD-1* mRNA complete coding sequence (Genbank accession no. U73945). The italic region is the mRNA coding sequence. The base numbering system is based on the published genomic sequence (Genbank accession no. U50930). In this numbering system, base numbers 660, 668, and 692 (in exon 1) correspond to positions −52, −44, and −20 (relative to ATG) in the 5′-UTR. For exon 2, the base numbering system is based on the published genomic sequence (Genbank accession no. U50931).

found by χ^2 analysis when 126 prostate cancer patients were compared with the 54 controls, there was a trend toward higher frequency of patients with at least one mutant base compared with controls. As Table 1 indicates, 33.1% of prostate cancer patients had at least one mutant allele at −44 bp compared with 22.2% of controls. Similarly, 41.1% of patients had a mutant base at position −688 compared with 37% of controls. The frequencies of other promoter mutations (at −20 and −52 bp) not subjected to functional analysis are shown in Table 1.

Mutational analysis. To examine whether mutations within the *hBD-1* coding regions were responsible for the reduced gene expression, we analyzed 30 prostate cancer and 35 renal cell carcinoma clinical samples for *hBD-1* exons 1 and 2 by sequencing of PCR products with flanking exon-specific primers. According to the published genomic sequence (9), the polymorphism (A/G) at 692 in exon 1 corresponding to the position −20 in the 5′-UTR was found adenine in 43.47% of prostate cancer patients and 77.78% of renal carcinoma patients. The polymorphism at 1,754 (A/G) in exon 2 was found in 90% of prostate cancer patients and in 94.28% of renal cancer patients (Table 2). Furthermore, three additional novel point mutations, 322 (T/A) and 534 (T/C) in prostate cancer and 584 (G/A) in renal cell carcinoma, were found in *hBD-1* promoter with frequency distributions from a low of 5.56% to a high of 21.73% across all tumors (Table 2). No mutations were found in amino acid coding regions.

Effect of 5-aza-dCyd on hBD-1 gene expression. To determine whether methylation plays a role in *hBD-1* gene transcriptional down-regulation, we treated human prostate cancer cell lines (DU145, PC-3, and LNCaP), a bladder cancer cell line (TSU-Pr1), and a renal cancer cell line (SW156) with 50 and 100 μmol/L of the DNA methyltransferase inhibitor 5-aza-dCyd for 72 hours and measured the hBD-1 mRNA expression by real-time PCR. Whereas PC-3, LNCaP, TSU-Pr1, and SW156 showed no change in expression

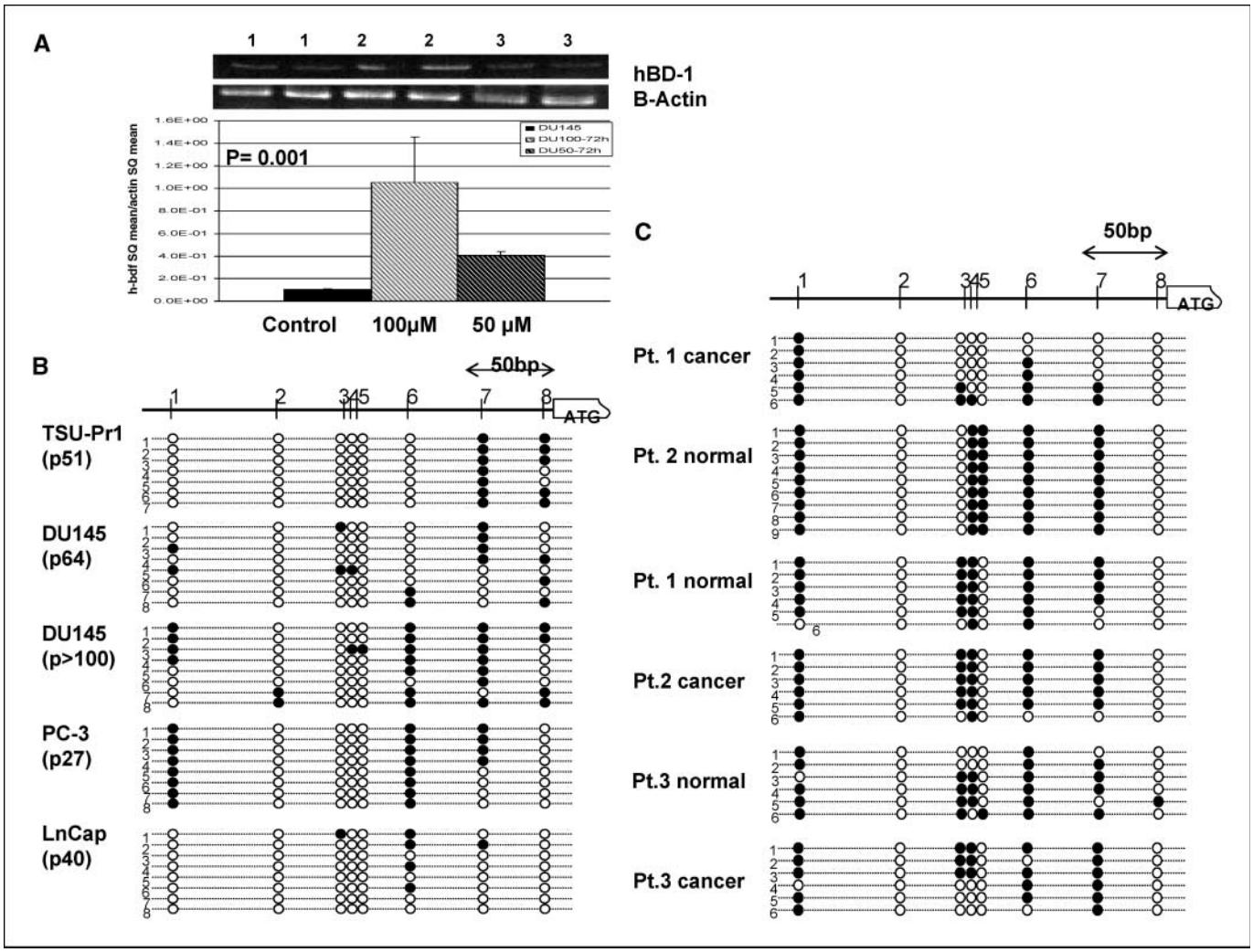

Figure 2. Methylation analysis in *hBD-1* promoter. *A,* real-time RT-PCR analysis of hBD-1 expression in DU145 treated with 50 and 100 μmol/L of 5-aza-dCyd for 72 hours followed by 1% agarose gel electrophoresis. Sample 1 is duplicate of nontreated cells, and samples 2 and 3 are duplicates of treated cells. *B* and *C,* representative CpG methylation patterns in *hBD-1* proximal promoter observed in individual cloned DNA fragments from cancer cell lines and LCM samples. *Row,* sequence allele. Six to eight alleles from each sample were cloned and sequenced. *Circle,* CpG site; *filled circle,* methylation; *open circle,* no methylation. *P,* cell line passage; *Pt,* patient. *B,* CpG methylation sites of *hDB-1* proximal promoter region in three prostate cancer cell lines (DU145, passages 64 and over 100; PC-3, passage 27; and LNCaP, passage 40) and in one bladder cancer cell line (TSU-Pr1, passage 51). *C,* comparison of CpG methylation sites in three pairs of normal and cancer LCM samples from prostate cancer patients.

with 5-aza-dCyd treatment (data not shown), DU145 showed ~3.9-fold or 10-fold ($P = 0.001$) induction of hBD-1 expression following 72 hours of treatment with 50 or 100 μmol/L 5-aza-dCyd, respectively (Fig. 2).

Bisulfite sequencing analysis of promoter methylation in cell lines and LCM samples. To investigate whether specific promoter CpG sites were methylated, we compared the extent of hBD-1 CpG island methylation among different cell lines and normal and malignant prostate tissues by bisulfite sequencing. As shown in Fig. 2*C*, patient 1 showed cancer-specific methylation of sites 4 to7, whereas patients 2 and 3 showed no significant difference in cancer compared with normal epithelium. In cell lines, TSU-Pr1 and DU145 showed methylation of sites 7 and 8 reside in exon 1 of *hBD-1*. DU145 also showed passage-dependent methylation at sites 1, 6, 7, and 8. PC-3 was methylated at sites 1, 6, and 7, and LNCaP was essentially unmethylated (Fig. 2*B*).

Effect of synthetic hBD-1. The TSU-Pr1 cell line was treated with either purified hBD-1 peptide or random control peptide at 50 μg/mL final well concentration and sampled at various time points over 48 hours. As shown in Fig. 3*D*, at all time points starting at 4 hours, a modest decrease in cell number was noted in hBD-1-treated cells compared with controls. This effect was most pronounced at 24 hours ($P < 0.001$) when cells were in log phase growth and was somewhat less after 48 hours when cells had nearly reached confluency. Other cell lines were not tested because of the limited peptide supply.

Effect of transfection of hBD-1 *in vitro*. Three cell lines, DU145, SW156, and TSU-Pr1, were transfected with full-length *hBD-1* cDNA and pcDNA3.1 vector control. Western blot analysis of culture medium confirmed presence of hBD-1 protein in transfected cells but not in controls at days 1 and 3 (Fig. 3*C*). Following 9 days, all three cell lines showed substantial cell death when compared with controls by standard phase-contrast light microscopy (Fig. 3*A* and *B*). A stable transfected clone never developed despite repeated attempts. Western blot analysis showed cleavage of caspase-3 and PARP in SW156 hBD-1-transfected cells at day 6 (data not shown).

Analysis of apoptosis in inducible system transfection. The renal cancer cell line SW156 was stably transfected with tetracycline-inducible hBD-1 expression vectors. Following 24 hours of induction with 1.5 μg/mL tetracycline, cell death was observed in culture (Fig. 4*B*). At 24 and 48 hours, cleaved caspase-8, caspase-9, PARP, as well as DNA fragment factor were also shown (Fig. 4*C*). At 48 hours, recombinant protein was present in large quantities in the culture medium (Fig. 4*D*).

Discussion

hBD-1 is a small soluble peptide secreted at epithelial surfaces, including the genitourinary and tracheobronchial tracts. The expression of hBD-1 is induced by cytokines under physiologic conditions of inflammation, and its increased secretion serves an antimicrobial host-defense function at these critical interfaces between host and environment (14). The β-defensins are part of the innate immune system and are chemotactic attractants for antigen-presenting dendritic cells, an effect that is mediated by specific cell surface receptors found on these antigen-presenting cells (15). *hBD-1* is one of the genes that reside in the area of the short arm of human chromosome 8 that is frequently deleted in renal and prostate cancer, a site likely to harbor one or more tumor suppressor genes. We have previously documented that, in clinical samples of both renal and prostate cancer, hBD-1 is commonly lost only in the cancer epithelium, consistent with what one would expect of a tumor suppressor protein (5). The work presented in this article was designed to test the hypothesis that *hBD-1*

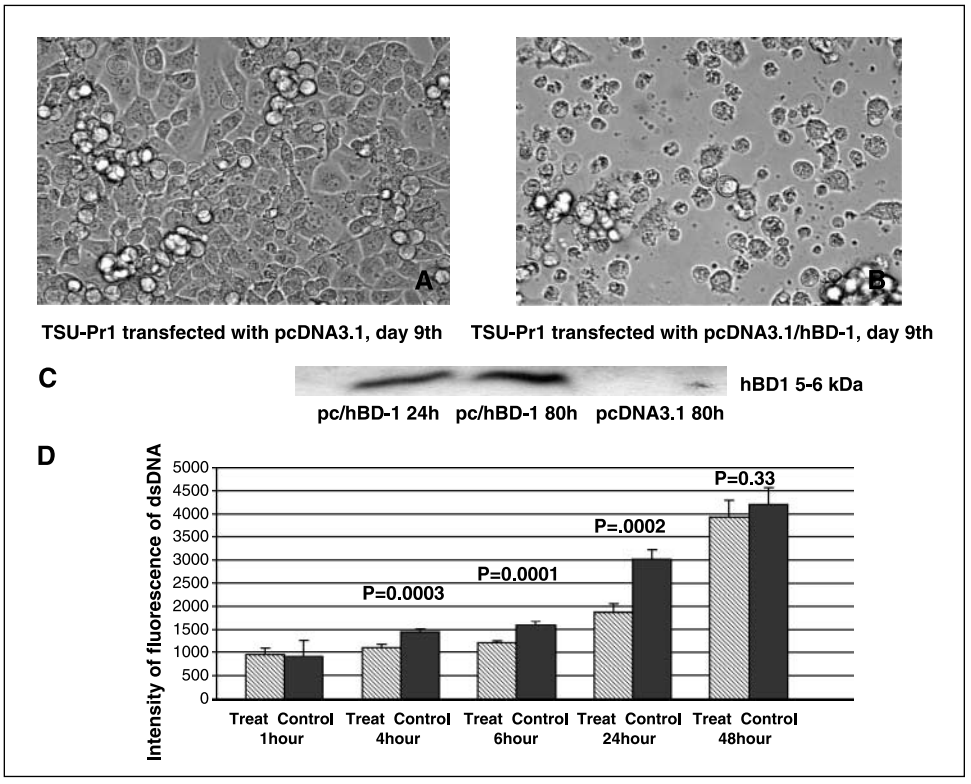

Figure 3. Effect of hDB-1 on bladder cancer cell line TSU-Pr1. Cells were transfected with pcDNA3.1/hBD-1 followed by G418 selection. *A,* on the 9th day, vector control cells started to form foci in selection medium. *B,* compared with the vector control, pcDNA/hBD-1-transfected cells showed floating and dying. *C,* Western blot analysis of hBD-1 protein in the medium of transfected cells at the indicated time points. *D,* TSU-Pr1 cells were treated with synthesized hBD-1 and random amino sequence control peptides (50 μg/mL). The cellular proliferation was measured by the intensity of Hoechst 33258 binding on the dsDNA at indicated time points. The average intensity calculation was based on four wells of each treatment group.

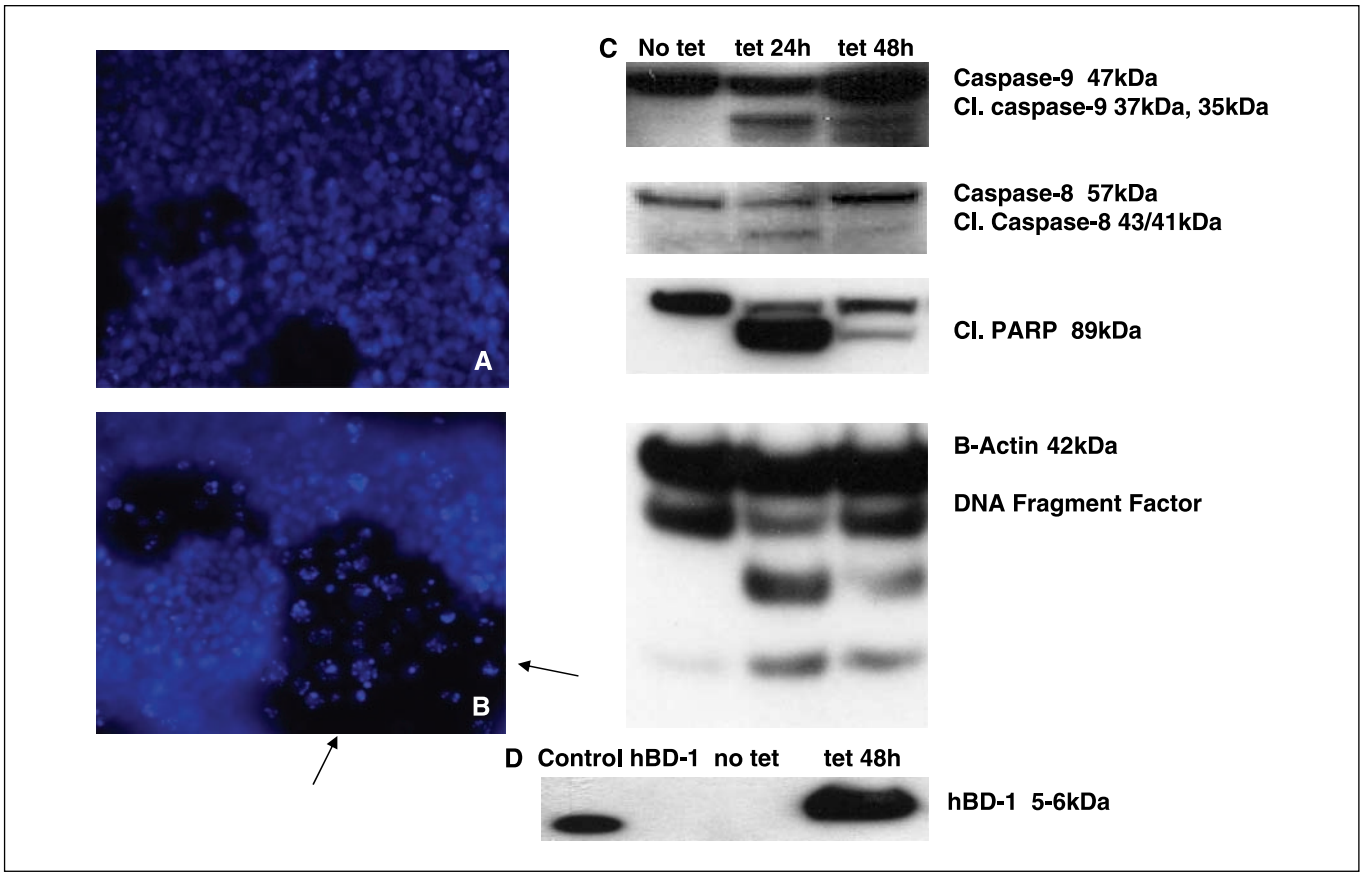

Figure 4. hBD-1 induced apoptosis in SW156 cells transfected with T-Rex/hBD-1. Photos of live cells stained with Hoechst dye. *A,* SW156 T-Rex/hBD-1-transfected cells without tetracycline inducing. *B,* SW156 T-Rex/hBD-1-transfected cells were induced with 1.5 μg/mL tetracycline. *Arrows,* after 24 hours, cell death was visualized. *C,* caspase-8, caspase-9, PARP cleavages, and DNA fragment factor were measured by Western blot analysis. β-Actin was used as loading control. *D,* hBD-1 protein in the culture medium was also measured by Western blot analysis at indicated time points.

functions as a tumor suppressor gene and, further, to examine promoter polymorphisms that may influence gene expression. We present data in support of two basic phenomena: first, that overexpression of hBD-1 induces cancer cell apoptosis and second, that promoter polymorphisms are common in these cancers and when studied in promoter-reporter constructs actually alter the transcriptional activity of the *hBD-1* promoter. As for the tumoricidal effects of hBD-1, we have shown that the addition of exogenous synthetic hBD-1 peptide causes a small but consistent inhibition of tumor cell proliferation (Fig. 3*D*). There is a much more dramatic effect when hBD-1 is overexpressed in cells by either stably transfected inducible expression constructs (Fig. 4) or transient transfections (Fig. 3). In these cases of endogenous overexpression, apoptosis is induced that is evident in both cell loss (Figs. 3*B* and 4*B*) and the appearance of DNA fragmentation as well as caspase-8, caspase-9, and PARP cleavage (Fig. 4*C*). Because each of these experiments included vector control arms, we are confident that the induced cell death is due to the expression of hBD-1. With respect to the possible mechanisms whereby hBD-1 expression is regulated, we have done transient transfections of both mutant and wild-type promoters of different lengths into multiple different cancer cell lines. All promoters were cloned from existing cell lines that have the same mutations (or polymorphic variants) found in actual clinical tumors and sequence verified. As shown in Fig. 1, the longer promoters (−1,140 and −726 bases upstream of ATG) are sensitive to the polymorphisms at positions −44 and −688, with the mutant promoters failing to efficiently drive transcription of the luciferase reporter. It is interesting that these apparently functional polymorphisms are also overrepresented in patients with cancer compared with no-cancer controls (Table 1). Thus, it is possible that patients with promoter polymorphic variants are more susceptible to cancer-associated loss of expression of hBD-1. Because gene expression can also be regulated by promoter methylation, we studied 5-aza-dCyd treatments of various cancer cell lines. The prostate cancer cell line DU145 was the only line that showed substantial expression of hBD-1 on treatment with the demethylating agent 5-aza-dCyd (Fig. 2*A*). The results of bisulfite sequencing were also somewhat equivocal with no consistent methylation patterns noted in the cell lines and no dramatic differences in methylation patterns when an individual's cancer epithelium was compared with the surrounding normal epithelium (Fig. 2*B* and *C*). Those results suggest that promoter methylation of hBD-1 may not play an important role in hBD-1 gene regulation. However, the 5-aza-dCyd-dependent substantial induction of hBD-1 expression in the DU145 cell line suggested that methylation might play a role in regulating hBD-1 expression indirectly.

In summary, our data support the hypothesis that *hBD-1* is a tumor suppressor gene whose reexpression inhibits cancer cell proliferation, although definitive proof will require additional studies. We have also shown that common polymorphic variants in the *hBD-1* promoter regulate transcription and may predispose

individuals with the mutant variants to the development of cancer. It is already established that the hBD-1 peptide is frequently lost in a cancer-specific fashion and that the gene is within the minimal overlap of loss on 8p23.2 thought to contain a tumor suppressor gene involved in both prostate and renal cancers. Thus, *hBD-1* as a candidate 8p tumor suppressor may be interesting to study in genitourinary and other cancers.

Acknowledgments

Received 1/24/2006; revised 5/31/2006; accepted 6/27/2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Lily Yang (Department of Surgery, Emory University) and Dr. Thomas Ganz (Department of Medicine and Pathology, University of California at Los Angeles, Los Angeles, CA) for their valuable technical assistance, and Dr. Leland Chung for collaboration and provision of critical resources.

References

1. Boniotto M, Hazbon MH, Jordan WJ, et al. Novel hairpin-shaped primer assay to study the association of the −44 single-nucleotide polymorphism of the DEFB1 gene with early-onset periodontal disease. Clin Diagn Lab Immunol 2004;11:766–9.
2. Vatta S, Boniotto M, Bevilacqua E, et al. Human β defensin 1 gene: six new variants. Hum Mutat 2000;15:582–3.
3. Schnapp D, Reid CJ, Harris A. Localization of expression of human β defensin-1 in the pancreas and kidney. J Pathol 1998;186:99–103.
4. Wu S, Hafez G, Zhang J, et al. Identification of the prostate cancer micro-foci with chromosome 8p deletion at the tumor interface area by histopathological-FISH parallel examination. J Oncology 2001;19:1143–7.
5. Donald C, Sun Q, Lim S, et al. Cancer-specific loss of β-defensin 1 in renal and prostatic carcinomas. Lab Invest 2003;83:501–5.
6. Niyonsaba F, Ogawa H, Nagaoka I. Human β-defensin-2 functions as a chemotactic agent for tumor necrosis factor-α-treated human neutrophils. Immunology 2004;111:273–81.
7. Yang D, Biragyn A, Kwak L, Oppenheim J. Mammalian defensins in immunity: more than just microbicidal. Immunology 2002;23:291–6.
8. Biragyn A, Ruffini PA, Leifer CA, et al. Toll-like receptor 4-dependent activation of dendritic cells by β-defensin 2. Science 2002;298:977–9.
9. Dork T, Stuhrmann M. Polymorphisms of the human β-defensin-1 gene. Mol Cell Probes 1998;129:171–3.
10. Liu L, Zhao C, Heng HQ, Ganz T. The human β-defensin-1 and α-defensins are encoded by adjacent genes: two peptide families with disulfide topology share a common ancestry. Genomics 1997;43:316–20.
11. Aldridge BA, Lim SD, Baumann AK, et al. Automated sequencing of complete mitochondrial genomes from laser-capture microdissected samples. Biotechniques 2003;35:6006–7.
12. Liu L, Wang L, HP J, et al. Structure and mapping of the human β-defensin HBD-2 gene and its expression at sites of inflammation. Gene 1998;222:237–44.
13. Serensen OE, Thapa DR, Rosenthal A, Liu L, Roberts AA, Ganz T. Differential regulation of β-defensin expression in human skin by microbial stimuli. J Immuno 2005;174:4870–9.
14. Vankeerberghen A, Nuytten H, Dierickx K, Quirynen M, Cassiman JJ, Cuppens H. Differential induction of human β-defensin expression by periodontal commensals and pathogens in periodontal pocket epithelial cells. J Periodontol 2005;76:1293–303.
15. Yang D, Chertov O, Bykovskala SN, et al. β-Defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science 1999;286:525–8.

Human β-Defensin-1, a Potential Chromosome 8p Tumor Suppressor: Control of Transcription and Induction of Apoptosis in Renal Cell Carcinoma

Carrie Q. Sun, Rebecca Arnold, Carina Fernandez-Golarz, et al.

Cancer Res 2006;66:8542-8549.