RTVP-1, a Tumor Suppressor Inactivated by Methylation in Prostate Cancer

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ABSTRACT

We previously identified and characterized a novel p53-regulated gene in mouse prostate cancer cells that was homologous to a human gene that had been identified in brain cancers and termed RTVP-1 or GLIPR. In this report, we document that the human RTVP-1 gene is also regulated by p53 and induces apoptosis in human prostate cancer cell lines. We show that the expression of the human RTVP-1 gene is down-regulated in human prostate cancer specimens compared with normal human prostate tissue at the mRNA and protein levels. We further document epigenetic changes consistent with RTVP-1 being a tumor suppressor in human prostate cancer.

INTRODUCTION

We identified a novel mouse gene, mouse RTVP-1, as a p53 target gene using differential display PCR and extensive promoter analysis. Comparison of the deduced protein sequence of mouse RTVP-1 with known protein sequences revealed that this gene had 68% identity and 75% homology to the human RTVP-1 gene (1). Human RTVP-1 had been identified in human glioblastoma and was referred as GLIPR (glioma pathogenesis-related protein; Ref. 2) or RTVP-1 (related to testes-specific, vespid, and pathogenesis protein; Ref. 3). RTVP-1 and GLIPR have also been identified as a marker of myelomonocytic differentiation in macrophages (4). The RTVP-1/GLIPR protein has high amino acid homology with human testis-specific protein, TPX1, contains a putative signal peptide sequence and transmembrane domain, and is structurally similar to group 1 of plant pathogenesis-related (PR-1) proteins that are implicated in plants defense response to viral, bacterial, and fungal infection (2, 3, 5). Because the mammalian testis proteins, plant PR-1 proteins, and the insect venom Ag-5 proteins are all secreted, it has been speculated that RTVP-1 is a secretary protein and may play a role in the human immune system (5).

Our recent studies demonstrated that mouse RTVP-1 was up-regulated by p53 and was also induced by DNA-damaging agents such as γ-irradiation or doxorubicin (1). Functional analysis of mouse RTVP-1 demonstrated that overexpression induced apoptosis in multiple mouse and human cancer cell lines, including prostate cancer cell lines (1). Because the mouse RTVP-1 gene induced more apoptosis in cancer cell lines than normal cell lines, we evaluated its therapeutic potential in a preclinical model for metastatic prostate cancer (6). These studies confirmed that adenoviral vector-mediated mouse RTVP-1 delivery to orthotopic prostate cancers suppressed tumor growth and metastasis, and these effects were mediated by proapoptotic, antiangiogenic, and immunostimulatory activities. In this report, we extend these functional studies to the human RTVP-1 gene and present evidence that indicates the RTVP-1 gene should be considered a tumor suppressor gene based on its regulation by p53, induction of apoptosis and growth inhibition, and down-regulation in prostate cancer compared with normal prostate tissues. Epigenetic rather than genetic changes appear to be the prevalent mechanism of down-regulation of human RTVP-1 based on analysis of the methylation status of CpG dinucleotides in the promoter region of the human RTVP-1 gene. Additional evidence for epigenetic regulation is increased expression in cancer cell lines by treatment with a demethylating agent.

MATERIALS AND METHODS

p53 Binding Site Analysis. Oligonucleotides for the putative p53-binding site A (5′-AGGCATGTCGACATGCAAAGAGCTTTT-3′) and B (5′-GCACACCGCAGGCTATCAGGAAAGTTTG-3′) were used for luciferase reporter constructs (lowercase bases indicate nucleotides different from consensus p53-binding sequence). Each pair of oligonucleotides was annealed and subcloned into the Smal site of the pGL3-promoter vector (minimal SV40 promoter; Promega, Madison, WI). Clones containing a single copy or two concatenated copies were used for transfection into human prostate cancer cell line TSU-P1 or mouse prostate cancer cell line 143B-IPA, as described previously (1). All constructs were confirmed by DNA sequencing. An electropherotic mobility shift assay was performed with an oligonucleotide probe representing p53-binding site A prepared by annealing 5′-ttacAGGCAT- GTCGCAGAGTTCTTgatgat-3′ with 5′-atacAAATTACGCTggacagTGACATGCTTgtaa-3′. A p53 consensus binding site (cbs) probe was prepared by annealing 5′-ctgacAGGCATGTCGACATGCAAAGAGCTTTT-3′ and 5′-AGGCATGTCGACATGCAAAGAGCTTTT-3′.

Functional Analysis of Human RTVP-1. Adenoviral vectors Adβgal and AdhRTVP-1 were generated as described previously (1, 7). Cells were infected with Adβgal or AdhRTVP-1 at a multiplicity of infection of 100 with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining, 4′,6-diamidino-2-phenylindole (DAPI) staining, and DNA fragmentation analysis 48 h later as described (1). The percentage of cell death was determined from the ratio of MTT-negative cells to total cells from three randomly selected fields. Apoptotic morphology was analyzed with fluorescence microscopy after DAPI staining. The percentage of apoptosis was determined by the ratio of apoptotic nuclei to total nuclei in three randomly selected microscopic fields. DNA fragmentation analysis was performed with a Cell Death ELISA kit (Roche, Indianapolis, IN) with a modified protocol described previously (1). For clonogenic survival, cells were trypsinized, and a single-cell suspension was prepared 24 h after infection. One percent of the original cells were reseded on a 10-cm plate and incubated for 10–14 days. Colonies were stained with MTT and counted by computer-assisted image analysis. Data are from three or more independent infections.

Human Prostate Tissue Samples. All samples of human prostate tissues were obtained with informed consent and maintained by the Baylor Specialized Program of Research Excellence in the prostate cancer tissue bank (8). Fresh frozen tissue punches of normal and tumor tissue were obtained at the time of radical prostatectomy. The pathological status was confirmed before processing, and the tumor samples had a tumor cell percentage of 80–100% with a Gleason score of 6–8. Formalin-fixed, paraffin-embedded specimens were also obtained from the Baylor Specialized Program of Research Excellence in the prostate cancer tissue bank.
Analysis of Human RTVP-1 Expression by Quantitative Real-Time Reverse Transcription-PCR (RT-PCR). Total RNA was isolated from prostate tissue samples by using the Ultraspec RNA reagent (Biotec, Houston, TX) and treated with an RNaseasy mini kit and an RNase free DNase kit (Qiagen). TaqMan One-Step PCR Master Mix reagent (Applied Biosystems) was used for reverse transcription and PCR with 40 ng of total RNA. Total RNA from prostate cancer cell line PC3 diluted from 1 μg/gl to 1 ng/gl in serial 10-fold dilutions was used as an external standard. Tagman PCR was done with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer’s instructions. The primers and probes were manufactured by Assays-on-Demand Service (Applied Biosystems), human RTVP1 Assay ID: Hs00199268_ml and 18S rNA Assay ID: Hs99999901_s1. The relative expression of human RTVP-1 mRNA was determined by using threshold cycle comparisons to the standard curve method described by the manufacturer and normalized to 18S RNA in the same cDNA.

Statistical analysis was performed using paired t test.

In Situ Hybridization. Human primary prostate cancers (n = 16) and lymph node metastatic deposits (n = 5), as well as histologically normal prostate tissue (n = 6), were used for in situ hybridization with an antisense human RTVP-1 probe. The specimens were frozen in liquid nitrogen immediately after surgical removal and then cut in 6-μm sections with a cryostat. The sections were air-dried and fixed in a solution containing 30% of 4% paraformaldehyde, 40% of ethanol, and 30% of acetic acid for 20 min. Antisense cRNAs and a corresponding sense RNA probe were made by run-off transcription from a linearized vector pCR2.0 (Invitrogen, Carlsbad, CA) containing a 265-bp fragment of human RTVP-1. Antisense and sense probes were prepared using the DIG-LAB labeling kit (Roche Diagnostics Corp., Indianapolis, IN). Sections were hybridized and detected as described previously (9). Briefly, the sections were prehybridized in 50% formamide, 5× SSC, 5× Denhardt’s solution, 250 μg/ml yeast tRNA, 4 mM EDTA, and 1 mg/ml salmon sperm DNA at 37°C for 60 min and hybridized in the prehybridization buffer (without the sperm DNA) containing 5 ng/ml digoxigenin (DIG)-labeled cRNA probes at 48°C overnight. The sections were then rinsed in 4× SSC (2 × 10 min), 2× SSC (10 min), 1× SSC (10 min), and 0.1× SSC (30 min at 48°C). The DIG-labeled RNA hybrids on sections were immunocytochemically detected by applying mouse anti-DIG IgG, followed by signal amplification with a DIG-conjugated antibody to the mouse IgG Fab fragment, and finally detected by anti-DIG IgG conjugated with fluorescein (Roche Diagnostics Corp., Indianapolis, IN). Sections from normal prostate and cancer as well as metastatic cancer deposits were always processed in parallel under the same conditions and using the same batches of antisense or sense probes and reagents. The sections labeled by the antisense probe were evaluated under a fluorescence microscope at power of ×400, and the RTVP-1 mRNA levels were scored according to the relative fluorescence intensities in cytoplasm of the great majority (>75%) of cancer cells, with reference to the adjacent control sections hybridized with the sense probe: 0, no signal above background level detectable; 1, weak, but identifiable over the background; 2, moderate, evidently recognizable signal level; and 3, strong signal level. Statistical analysis using Kruskal-Wallis rank test was performed to determine the significance of the differences in the fluorescence scores in different tissues. P < 0.05 was considered statistically significant.

Immunohistochemistry. The same set of human prostate tissues as well as lymph nodes with metastatic deposits that were used for in situ hybridization were snap-frozen in liquid nitrogen, and 6-μm cryostat sections were cut for immunostaining. An additional set of paraffin sections from 66 primary prostate cancers, 22 matched, adjacent histologically normal prostate tissue, and 5 lymph node metastases (one matched with a primary cancer) were immunostained. A polyclonal rabbit antibody generated against a peptide for human RTVP-1 amino acids 27–43 was used for immunostaining at a dilution of 1:400 in PBS with 0.5% normal horse serum with detection using the routine ABC technique (10). To verify the specificity of the immunoreactions, some sections were incubated in normal rabbit serum, PBS, or in the antibody preabsorbed with a synthetic human RTVP-1 polypeptide (2 mg/ml), in place of the primary antibody. The RTVP-1 immunostaining of cancer and normal prostate epithelia was evaluated under a microscope in 5–10 microscopic measuring fields, randomly selected for each specimen, at a power of ×200. Positively labeled cells were counted and expressed as a labeling percentage (%). The overall immunostaining score for each specimen was designated as: 3, ≥75%; 2, >25 and <75%; and 1, ≤25% of the cells stained. Statistical analysis was with the Kruskal-Wallis rank test.

Loss of Heterozygosity (LOH) Analysis. Genomic DNA was extracted from a set of 42 matched samples of primary prostate cancer and normal prostate tissue with the QIAamp DNA mini kit (Qiagen). Eleven polymorphic microsatellite markers from 12q14 to 12q21 were selected for analysis. One of each primer pair was end labeled with [γ-32P]ATP using T4 polynucleotide kinase (Strategene, Menasha, WI). PCR products were separated on a denaturing sequencing gel with 6% polyacrylamide (19:1) containing 7 m urea, followed by autoradiography.

Methylation Analysis. Genomic DNA from tissue samples was purified with the QIAamp DNA kit (Qiagen) and treated with sodium bisulfite to convert nonmethylated cytosines to uracil (11). Each bisulfite-treated DNA sample was then amplified by PCR. A primer pair (sense primer 5′-GGAAT-GAATTATGTGTTA-3′ and antisense primer 5′-TAAACCCTAAATCAATACAC-3′) was designed to avoid any CpG dinucleotides in the region of −474 to +236 of the human RTVP-1 gene. PCR reaction conditions were: 95°C for 15 min to activate HotStarTaq polymerase, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final 72°C for 10 min. PCR product was cloned into AdvanTage PCR cloning vector (Clontech, Palo Alto, CA), and 10 independent clones from each sample were sequenced with an ABI 310 automatic sequencer. To confirm the efficiency of the bisulfite reaction, 10 non-CpG cytosine residues were evaluated for conversion to uracil residues. Only 27 of 2630 (1%) nucleotides evaluated in the patient samples were not converted. All CpG dinucleotides (designated A–M) were independently scored as 0 to 1, with 1 indicating 10 of 10 clones having a methylated C. The frequency of methylation at each CpG dinucleotide was expressed as a mean frequency of methylation in 13 pairs of normal or tumor tissue samples.

Demethylation Analysis. LNCaP, TSU-Prl, and 148-P1 cancer cells were treated with 0, 1, 5, or 10 μM 5-aza-2′-deoxycytidine for 4 days with media replacement every 2 days. RNA was prepared, and RT-PCR was performed using primers 5′-ACTCGGCGAATCACCTTC-3′ and 5′-TGG-CAATTTGGGTAGTCTGGGT-3′ for human RTVP-1 and 5′-TTCTTT-GGTGACCTGGCTTT-3′ and 5′-GCTTGGGACCTTGACCATTT-3′ for human HPRT. Primers for mouse RTVP-1 and murine β-actin were as described previously (1). PCR conditions were: 94°C 15 min, followed by cycling at 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s. Cycle numbers were 29 for human RTVP-1 in TSU-Prl, 31 for human RTVP-1 in LNCaP, 25 for human HPRT in both cell lines, 25 for mouse RTVP-1, and 22 for murine β-actin. For each RT-PCR, 200 ng of total RNA were reverse transcribed using oligo(dT)18 and Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer, Boston, MA). Statistical analysis was performed using paired t test.

RESULTS

**Human RTVP-1 Is a p53 Target Gene.** To verify that human RTVP-1, similar to mouse RTVP-1, is a p53 target gene we evaluated the published human RTVP-1 genomic sequence in National Center for Biotechnology Information human genome database (NT_029419) for potential p53-binding sites. Two potential p53-binding sites with ≥90% identity compared to the p53 cbs (12) were found in the human RTVP-1 sequence from −2 kb through intron 2 (Fig. 1A). Site A is located in the promoter region at −1.8 kb, with one base altered in each half-site compared with the consensus p53-binding site. Site B is located in exon 2, with one base altered in each half-site and a two-base insertion in the second half-site (Fig. 1A). To test these two potential p53-binding sites, we constructed four luciferase reporter constructs with either one (1X) or two (2X) copies of site A or site B in a luciferase vector with a minimal promoter (pGL3-pro). In human cancer cell line TSU-Prl (Fig. 1B), cotransfection with wild-type p53 strongly activated the transcription of site A-mediated luciferase activity (−10-fold for 1X A and −17-fold for 2X A compared with luciferase activity in control vector pGL3-pro transfected cells). Mutant p53 and empty vector failed to activate site A-mediated luciferase activity. Wild-type p53 showed only a small effect (−2-fold) on the transcription of site B-mediated luciferase activity. In the p53 null mouse prostate cancer cell line 148–1PA (Ref. 13; Fig. 1C), the
transcriptional activation of site A-mediated luciferase activity by wild-type p53 was 18-fold for 1× A and 35-fold for 2× A. As with TSU-Pr1, mutant p53 and empty vector failed to activate site A-mediated luciferase activity, and wild-type p53 showed only a small effect (<5-fold) on site B-mediated luciferase activity. To confirm p53-binding to site A of human RTVP-1, we performed electrophoretic mobility shift assay using synthetic site A sequence as a probe. A p53-specific protein-DNA complex was observed when pure p53 protein was incubated with 32P-labeled site A probe (Fig. 1D, Lane 2). This complex was absent in probe only (Lane 1) or when cold p53 probe competitor DNA (Lane 3) or cold site A probe competitor (Lane 4) was used. These data indicate that site A p53-binding site is responsible for the transcriptional activation of human RTVP-1.

Human RTVP-1 Induces Apoptosis and Suppresses Colony Growth in Prostate Cancer Cells. We demonstrated previously that, similar to mouse RTVP-1, overexpression of human RTVP-1 by transfection of a human RTVP-1-expressing plasmid led to apoptosis in the human cancer cell line TSU-Pr1 (1). In this study, we extended these results by determining both transient apoptosis-induced cell death and long-term survival by colony formation in TSU-Pr1 and human prostate cancer cell line LNCaP. Forty-eight h after infection of cells with an adenoviral vector expressing human RTVP-1 (AdhRTVP-1) or a control vector Adβ-gal, MTT-negative dead cells in human RTVP-1-expressing LNCaP and TSU-Pr1 cells were ~70 and 45%, respectively, compared with ~7% MTT-negative dead cells in control Adβ-gal-infected cells (Fig. 2A). DAPI staining identified ~43% LNCaP and ~35% TSU-Pr1 apoptotic cells after AdhRTVP-1 infection compared with ~7–8% apoptotic cells in Adβ-gal-infected cultures (Fig. 2B). DNA fragmentation as determined by Cell Death ELISA indicated ~3-fold increase in human RTVP-1-expressing cells compared with β-gal-expressing cells for both LNCaP and TSU-Pr1 (Fig. 2C). After infection with AdhRTVP-1, colony formation was only 4 and 17% compared with Adβ-gal-infected cells in LNCaP and TSU-Pr1 cells, respectively (Fig. 2D). These data suggest that human RTVP-1 may exert its tumor suppressor functions, in part, by inducing apoptosis and suppressing cell growth/survival.

Human RTVP-1 Is Down-Regulated in Prostate Cancer. To evaluate expression of human RTVP-1 in prostate cancer, we performed quantitative real-time PCR of human RTVP-1 gene expression in 11 matched pairs of human normal and tumor prostate tissues (Fig. 3A). In all but one patient (no. 3), the expression of human RTVP-1 was lower in the prostate cancer tissue than in the normal tissue after normalization using 18S RNA quantitation. Similar results were obtained independently using normalization with β-actin (data not shown). Statistical analysis indicated that human RTVP-1 is down-regulated in prostate tumors compared with normal prostate tissue from the same patient (P = 0.0287, paired t test).

To confirm this result and extend the observation to metastatic prostate cancer, we determined the mRNA expression profiles of human RTVP-1 in a panel of human prostate cancer tissue specimens by in situ hybridization using a fluorescent riboprobe (Fig. 3, B–E). In normal human prostate (Fig. 3B), human RTVP-1 mRNA was localized predominantly in prostatic epithelial cells, among which the basal cells exhibited the strongest signal level. Some isolated stromal cells showed a moderate human RTVP mRNA level as well. A moderate level of human RTVP-1 mRNA was also present in primary cancer cells (Fig. 3C). Interestingly, all metastatic cells in lymph nodes (Fig. 3D) revealed much lower levels of signal when compared with normal prostate or primary cancers. Sections from human prostate cancers that were incubated with the sense riboprobes gave rise to negative results (Fig. 3E). Semiquantitative analysis of a panel of fresh frozen human prostate cancer tissue specimens revealed that human RTVP-1 mRNA levels were significantly reduced in metastases relative to normal prostate epithelium or primary prostate cancer cells (Table 1; P = 0.0041, Kruskal-Wallis rank test).

Immunohistochemical analysis of the same frozen specimens showed that human RTVP-1 protein was predominantly expressed in
infection of 100 and were evaluated for apoptosis, viability, and clonogenic survival.

**A**, DNA fragmentation was determined by absorbance at 405 nm with a reference at 490 nm.

**B**, viable and dead cells were counted by phase contrast microscopy after MTT staining 48 h.

**C**, nuclei were counted, and apoptosis was expressed as a percentage of apoptotic nuclei.

**D**, apoptosis enumeration after DAPI staining. Apoptotic and nonapoptotic glandular epithelial cells in normal human prostate, whereas a lower level of expression was present in isolated cells within the stroma (Fig. 4A). Antibody specificity was demonstrated by blocking with peptide (Fig. 4A, inset). Cancer cells in the prostate (primary tumor) expressed a lower level of human RTVP-1 compared with normal glandular epithelial cells (Fig. 4, B and C), whereas the human RTVP-1 levels in lymph node metastatic deposits were dramatically reduced (Fig. 4D). Semiquantitative analysis of immunohistochemical staining revealed that metastatic cancer cells were labeled significantly less than primary cancer cells or normal glandular epithelia (Table 1; \( P = 0.0115 \), Kruskal-Wallis rank test). Immunohistochemical analysis of a larger series of formalin-fixed, paraffin-embedded specimens confirmed that human RTVP-1 protein expression was highest in glandular epithelial cells in adjacent normal human prostate with a lower level of expression in prostate cancer cells and even lower expression in cancer cells in lymph node metastases (Fig. 4E; \( P < 0.0001 \), \( \chi^2 \) test).

**Genetic Changes in the Human RTVP-1 Gene in Prostate Cancer Are Relatively Infrequent.** To evaluate the frequency of genetic changes in the human RTVP-1 gene as a mechanism of down-regulation, we evaluated the frequency of LOH of human RTVP-1 in prostate cancer using 41 pairs of normal and tumor prostate tissues and 11 polymorphic microsatellite markers from chromosome 12q14 to 12q21 flanking the human RTVP-1 locus. Among these 41 patients, 4 patients (9.8%) had LOH on chromosome 12q (primary data not shown). The tumor samples from two patients (nos. 15 and 21) demonstrated LOH in D12S1601, D12S92, D12S337, and D12S102, respectively, whereas tumor samples from two other patients (nos. 29 and 45) demonstrated LOH in D12S102 and/or D12S335 (Fig. 5). We further evaluated the paired normal prostate and primary prostate cancer samples used for RT-PCR for evidence of mutations in the human RTVP-1 cDNA by DNA sequencing and did not find any mutations, further suggesting that genetic changes in the human RTVP-1 gene are not common in primary prostate cancer. Overall, these data suggest that genetic changes in the human RTVP-1 gene are infrequent and are likely responsible for the suppression of human RTVP-1 expression in only a subset of patients.

**Epigenetic Changes Are Responsible for Down-Regulation of the RTVP-1 Gene in Prostate Cancer.** We therefore speculated that the suppression of human RTVP-1 expression in tumors might be the result of aberrant human RTVP-1 gene methylation at its locus on chromosome 12. To evaluate this possibility, we performed methylation analysis on 13 pairs of matched normal and tumor prostate tissue samples that included the 11 pairs that were used for RT-PCR analysis. All 13 CpG dinucleotides located in the region of \(-474 \) to +236 (labeling from A–M) are shown in Fig. 6A. The mean frequency of methylation at individual CpG dinucleotides in genomic DNA is summarized in Fig. 6B. DNA from tumor tissue samples had a higher frequency of methylation in all of the CpG dinucleotides, with the most striking differences in patients 1, 5, 11, and 12. Importantly, the increase in tumor methylation (Fig. 3) is inversely correlated (\( R^2 = 0.569, P = 0.0046 \)) with the decrease of human RTVP-1 expression in the tumor samples (Fig. 6C), suggesting that methylation in CpG dinucleotides in the promoter region of human RTVP-1 is largely responsible for the down-regulation of human RTVP-1. To exclude the possibility that the down-regulation of human RTVP-1 involved mutation of the p53 gene, we sequenced the entire p53 cDNA from the 13 pairs of matched normal and tumor tissues. The results failed to detect mutations in the p53 gene in these samples and were consistent with numerous studies showing that p53 mutations are infrequent events in the primary tumor but are selected for and clonally expanded in prostate cancer metastases (reviewed in Ref. 14).
To determine whether demethylation could reverse the suppression of RTVP-1 expression in cancer, we treated two human cancer cell lines, LNCaP and TSU-Pr1, and a mouse prostate cancer cell line, 148-1PA, with the demethylation reagent 5-aza-2’-deoxycytidine for 4 days. The results presented in Fig. 6D show dose-dependent increases in RTVP-1 expression, with ~13-fold increase in LNCaP, ~3-fold in TSU-Pr1, and ~7-fold in 148-1PA. To confirm that these results were the result of demethylation rather than from a stress or damage induction pathway, we transfected a human RTVP-1 promoter luciferase construct into the cancer cells and treated with 5-aza-2’-deoxycytidine for 24 or 48 h and measured luciferase activity. No significant increase over basal levels was detected within this time period (data not shown). Overall, these data demonstrate that methylation in the RTVP-1 gene is largely responsible for the down-regulation of RTVP-1 in prostate cancer and potentially other malignancies.

**DISCUSSION**

In this study, we demonstrate that human RTVP-1, similar to mouse RTVP-1, is a p53 target gene with a single site (A) that confers p53 inducibility and binds p53 in vitro (Fig. 1). In the mouse RTVP-1 gene, the p53 response element is located in intron 1 (1), whereas in the human RTVP-1 gene is located ~1.8 kb from the initial ATG site. We further demonstrate that human RTVP-1 is capable of inducing apoptosis and suppressing colony formation when overexpressed with an adenoviral vector. We demonstrate that two human cancer cell lines [LNCaP, an androgen-responsive prostate cancer cell line, and TSU-Pr1, a cell line widely used as a prostate cancer model but recently considered to be derived from bladder (15)] have a similar response to human RTVP-1. These results are in agreement with our previous results with mouse RTVP-1 (1, 6).

We used three techniques to analyze fresh-frozen normal prostate and prostate cancer tissue taken at the time of surgery: quantitative RT-PCR, in situ hybridization, and immunostaining. The results of all three approaches were congruent and showed that in human prostate samples, human RTVP-1 expression is down-regulated in cancerous tissue. An extensive analysis of fixed specimens by immunohistochemistry also confirmed down-regulation of human RTVP-1 in prostate cancer.

Multiple aberrant gene activities can drive tumorigenesis and pro-

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**Table 1** Semiquantitative analysis of human RTVP-1 expression by in situ hybridization and immunohistochemistry on matched frozen specimens

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* The relative fluorescence intensity following in situ hybridization scored as: 0, no signal detected; 1, low level; 2, moderate; or 3, high level of RTVP-1 mRNA.

**Fig. 3.** RTVP-1 mRNA expression is down-regulated in prostate tumor tissue. A, quantitive real time RT-PCR was performed for human RTVP-1 gene expression on normal and tumor tissue from 11 patients. The relative expression was determined by calculation of threshold cycles and normalized to 18S RNA as an internal control. In situ hybridization detected human RTVP-1 mRNA expression as strongest in the basal and glandular epithelial cells of normal human prostate (B), whereas prostate cancer cells exhibited only a moderate level of human RTVP-1 mRNA in primary prostate tumors (C). In contrast, much lower levels of RTVP-1 mRNA were found in the metastatic deposits in lymph nodes (D). Control sections of human prostate cancer that were incubated with sense riboprobes had minimal staining (E). B-E, ×200.
The metastatic and drug/hormone-resistant phenotype of certain cancers such as prostate cancer may result from epigenetic events, such as aberrant gene methylation (16–19), and/or genetic events such as point mutation and LOH (20, 21). Our sequencing analysis for human RTVP-1 involved 13 pairs of matched normal and primary tumor specimens, and we did not detect any point mutations or deletions. For the LOH analysis, we used a much larger number of specimens, i.e., 41 matched normal and primary tumors and detected 4 cases in which regional, i.e., 12q14 to 12q21, LOH occurred. In one case, the LOH flanked the human RTVP-1 gene. In the other three cases, more distant alterations on only one side of the human RTVP-1 locus were observed. Although it is conceivable that relatively distant alterations of this type could disrupt the normal expression pattern of the gene, we did not confirm this in these three specimens. The presence of additional LOH in the chromosome 12q region could also be suggestive of dysfunction of other critical genes in this region (22), perhaps related to RTVP-1. Overall, we concluded from our genetic analysis that DNA mutation likely occurs in only a subset of primary prostate cancers. Because our mutation/LOH analysis did not include

LOH on chr 12q (76.5 cM - 92.5 cM) in PCa: 4/41 (9.8%)}
metastatic tissues, we must leave open the possibility that DNA mutations do affect human RTVP-1 expression during progression. This possibility is supported by the progressive reduction in human RTVP-1 mRNA and protein that we observed using in situ hybridization and immunostaining in metastatic tissues (see Figs. 3 and 4).

We did find that the human RTVP-1 promoter was extensively methylated in tumor tissue compared with normal prostate in 13 pairs of normal and tumor specimens. These data were remarkable in that the increase in methylation in a specific region flanking the transcriptional start site in all of the tumor specimens (Fig. 6C) was correlated ($R^2 = 0.569, P = 0.0046$) with the decreased level of expression, as analyzed by quantitative real-time PCR (Fig. 3A). We conclude that down-regulation of human RTVP-1 in prostate cancer is mainly attributable to promoter methylation. Because RTVP-1 is a direct p53 target gene, we also analyzed the mutation status of p53 using cDNA sequencing in these specimens and found no mutations. This was also supported by immunostaining for p53 protein (not shown). Although our analysis did not include a complete assessment of all possible mechanisms of suppression of p53 function, e.g., ARF/p14 mutation (23), our results are in agreement with the rather extensive literature that demonstrates p53 mutations are infrequent in primary prostate cancer but are selected for during metastatic progression (reviewed in Ref. 14). Our results also raise the interesting possibility that during prostate cancer progression, inactivation of a key target gene, i.e., human RTVP-1, through methylation occurs before p53 mutation. If further studies confirm this possibility, one could envision the use of a panel of p53 target genes that includes human RTVP-1 together with p53 for biomarker analysis in patient specimens to provide specific information regarding the status of prostate cancer progression.

In summary, we propose that human RTVP-1 is a tumor suppressor that undergoes epigenetic inactivation in prostate cancer. The role of human RTVP-1 in other cancers awaits further studies. The mechanism of action of RTVP-1 is likely to be complex, but certainly the pro-apoptotic functions we have identified in this and previous studies (1, 6) may contribute significantly to the tumor suppressor activity. The generation and analysis of mice with homozygous deletions of the RTVP-1 gene should provide additional insight into the role of RTVP-1 as a tumor suppressor (24). An additional level of complexity that will require further studies in vivo is the role of RTVP-1 in immune regulation, suggesting that immune surveillance may contribute to tumor suppressor activities.

ACKNOWLEDGMENTS

Portions of the work were conducted in facilities provided by the Houston Veterans Affairs Medical Center. We thank Anna Frolov for statistical analysis.

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