Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths among men in the United States and Europe (1). The incidence of prostate cancer increases with aging (2). Once a tumor has metastasized, the long-term prognosis is poor because no curative therapy is available. Cancer development and metastasis are multistep processes that, among others, involve the inactivation of tumor suppressor genes. When normal expression levels of these growth-inhibitory proteins are suppressed, uncontrolled cell cycling and growth can result. Identifying such specific molecular changes may contribute to improved diagnosis, clinical management, and outcome prediction of newly diagnosed prostate cancers (3).

Silencing of cancer-associated genes by hypermethylation of CpG islands within the promoter and/or 5’ regions is a common feature of human cancer and is often associated with partial or complete transcriptional block (4). This epigenetic alteration provides an alternative pathway to gene silencing in addition to gene mutation or deletion. Moreover, the finding of promoter methylation of several genes in small biopsies and bodily fluids of cancer patients has proven to be useful as a molecular tool for cancer detection (5, 6).

The RAS-association domain family 1 has seven different isoforms that are produced by alternative splicing and transcription from two different promoters with CpG islands (7, 8). The RAS-association domain family 1, isoform A (RASSFIA) is a tumor suppressor gene in the RAS pathway that can regulate proliferation, induce apoptosis, and bind to and stabilize microtubules (9). Inactivation of RASSFIA is frequently observed in a number of solid tumors and epithelial cancers, including prostate cancer (3, 10–17).
In the present study, we investigated the chromatin changes involved in the inactivation of the RASSF1A gene in prostate cancer samples through the analysis of CpG methylation in the promoter regions, histone acetylation (acetyl-H3, acetyl-H4), dimethyl-H3-K4 (H3K4me2), and dimethyl-H3-K9 (H3K9me2) methylation associated with the RASSF1A promoter region.

### Materials and Methods

**Clinical samples.** A total of 131 newly diagnosed prostate cancer tissues from radical prostatectomy and 65 pathologically proven benign prostate hypertrophy (BPH) samples from transurethral resection (TUR-P). The pathologic background of the prostate cancer patients included Gleason sum (GS) <7 (75 cases) and GS ≥7 (56 cases); pT2 (85 cases), pT3 (44 cases), and pT4 (2 cases); and preoperative serum PSA ≤4.0 (18 cases), PSA 4.0-10.0 (63 cases), and PSA >10.0 (50 cases). The median follow-up time was 35.5 months, with a range from 0.7 to 91.4 months. Serum PSA levels after radical prostatectomy was used as a surrogate end point, with a level ≥0.2 ng/mL designated as PSA failure. The median age of prostate cancer patients included Gleason sum (GS) <7 (75 cases) and GS ≥7 (56 cases); pT2 (85 cases), pT3 (44 cases), and pT4 (2 cases); and preoperative serum PSA ≤4.0 (18 cases), PSA 4.0-10.0 (63 cases), and PSA >10.0 (50 cases).

**Cell lines.** RWPE-1 and PWR-1E, a nontumorigenic human prostate epithelial cell line, and the human prostate cancer cell lines LNCaP and PC3 were obtained from the American Type Culture Collection. RWPE-1 and PWR-1E cells were maintained in keratinocyte serum-free medium (Life Technologies) and followed the manufacturer's protocols (Upstate Biotechnology) and MRI, which allowed accurate localization of prostate cancer before radical prostatectomy (19). Each tissue sample was fixed in 10% buffered formalin (pH 7.0) and embedded in paraffin wax. For histologic evaluation, 5-μm-thick sections were used for H&E staining. All of the samples were microscopically dissected and analyzed for methylation (20). In BPH samples, high-grade prostate intraepithelial neoplasia and cancer were ruled out by microscopic analysis.

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation (ChIP) assays were done on cell line DNA using an EZ ChIP Universal Methylated DNA (Intergen) was used as a positive control for methylation. The obtained MSP and USP products were analyzed by electrophoresis in 3% agarose gels and stained with ethidium bromide.

**Bisulfite DNA sequencing.** Bisulfite-modified DNA was amplified using a pair of universal primers. Direct bisulfite DNA sequencing of the PCR products using either forward universal primer or reverse primer was done according to the manufacturer’s instructions (Applied Biosystems). The primer sequences (22) and PCR conditions are shown in Table 1. For semiquantitative analysis, a preliminary suitable number of PCR cycles for each primer and PCR conditions are shown in Table 1.

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation (ChIP) assays were done on cell line DNA using a EZ ChIP (Upstate Biotechnology) and followed the manufacturer's protocols with some modifications. Formaldehyde was added to the cells in a culture dish to a final concentration of 1% and incubated at 37°C for 10 min. The cells were washed in 1 ml of ice-cold PBS with protease inhibitors, scraped, and resuspended in 400 μL of SDS lysis buffer. Lysates were sonicated for 10 s nine times on ice and centrifuged at 15,000 rpm for 10 min at 4°C. Supernatants were

### Table 1. Primer sequences and PCR conditions

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<th>Primer type</th>
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loaded on 1% agarose gels and determined to have reduced DNA lengths between 200 and 1,000 bp. The sonicated samples were precleaned with salmon sperm DNA/protein A agarose beads (Upstate Biotechnology). The soluble chromatin fraction was collected, and 8 μL of antibody for acetyl-H3, acetyl-H4, dimethyl-H3-K4 (H3K4me2), or 12 μL of antibody for dimethyl-H3-K9 (H3K9me2), or no antibody, was added and incubated overnight with rotation (23). All antibodies were purchased from Upstate Biotechnology. After rotation, chromatin–antibody complexes were collected using salmon sperm DNA/protein A agarose beads and washed according to the manufacturer’s protocol. Immunoprecipitated DNA was recovered using a QIAquick PCR Purification Kit (Qiagen) and analyzed by PCR. We used previously reported (23, 24) primers designed to separately amplify four regions in the RASSF1A promoter area (Fig. 1A). The primer pairs used for ChIP assays are shown in Table 1. One additional primer set was used to amplify a 166-bp fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal control. Each PCR reaction was initially set up using different amounts of ChIP sample with varying PCR cycle numbers, and we selected the final PCR conditions accordingly. PCR products were analyzed on 3.0% agarose gels and visualized by UV illumination. Densitometric analysis of the observed bands was done using ImageJ software. Relative enrichment was calculated by taking the ratio between the net intensity of the RASSF1A PCR product from each primer set and the net intensity of the GAPDH PCR product for the bound sample and dividing this by the same ratio calculated for the input sample (25). The value of each point was calculated as the average from two independent ChIP experiments and a total of four independent PCR analyses.

Statistical analysis. The relationship between clinicopathologic findings and methylation status of the RASSF1A gene was done using the χ² test and Fisher’s exact test. For each clinicopathologic finding, the association with PSA failure-free probability was determined using Kaplan-Meier curves, and a log-rank test was used to determine significance. P values of <0.05 were regarded as statistically significant. All statistical analyses were done using StatView version 5.0 for Windows.

Fig. 1. A, schematic representation of the promoter region of the human RASSF1A gene. Vertical lines, location of CpG dinucleotides; arrow, approximate position of the transcription start site. Doubled horizontal line, region examined by MSP. Four horizontal bars, location of the DNA fragments amplified by PCR done on the DNA recovered from ChIP assay. B, typical MSP results in BPH and prostate cancer samples are shown. Methylated bands were detected in 97 (74.0%) of the prostate cancer samples and in only 12 (18.5%) of the BPH samples. C, bisulfite DNA sequencing of unmethylated (top) and partially methylated (bottom) samples. In unmethylated samples, every CpG site was unmethylated. In partially methylated samples, there was a “T” peak along with a “C” peak at the CpG sites. Bars under the sequence, CpG sites. The results of the bisulfite DNA sequencing were consistent with those obtained by MSP.
Results

Methylation status of RASSF1A gene. Typical examples of MSP and USP bands obtained during methylation analysis of the RASSF1A promoter are shown in Fig. 1B. To confirm the methylation status of the RASSF1A promoter, bisulfite-modified DNA was amplified and sequenced. Typical bisulfite-modified DNA sequencing of prostate cancer and BPH samples are shown in Fig. 1C. In BPH samples, most CpG sites were not methylated, whereas in prostate cancer samples, most CpG sites were methylated in the promoter region. In the total group of prostate cancer and BPH, there was no correlation between age and methylation. In the 131 prostate cancer samples, positive RASSF1A methylation was detected in 97.

Fig. 2. Correlation of clinicopathologic features with methylation frequency of the RASSF1A promoter. A, the difference in the frequency of RASSF1A promoter methylation was significant between prostate cancer and BPH samples. B, there was significant correlation between methylation frequency of RASSF1A and high GS or clinical stage. Methylation frequency of RASSF1A increased with higher GS and high stage. The total number of patients was 131, but some patients were lacking clinicopathologic findings, and then data were not included. Cap, capsular invasion; SV, seminal vesicle involvement; pN, lymph node invasion; ly, lymphatic vessel invasion. N and P, negative and positive, respectively.

Fig. 3. Kaplan-Meier PSA failure-free survival curves of prostate cancer patients after radical prostatectomy, grouped according to the evaluated variables. A, methylation frequency of RASSF1A; B, GS; C, preoperative PSA; D, pT category. Follow-up ranged from 0.7 to 91.4 mo, with a median of 35.5 mo.
The mRNA transcript of RASSF1A was significantly increased after 5-aza-dC treatment alone or with a combination of 5-aza-dC and TSA in PWR-1E and the two prostate cancer cell lines. In contrast, TSA treatment failed to re-express the RASSF1A gene in these cell lines. GAPDH expression served as a loading control. B. MSP analysis of RASSF1A. MSP analysis showed partial demethylation of the RASSF1A promoter region after 5-aza-dC treatment in prostate cancer cell lines. M, reactions specific for methylated DNA; U, reactions specific for unmethylated DNA.

Table:

<table>
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<th></th>
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<td>GAPDH</td>
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**Effects of 5-aza-dC and TSA on RASSF1A gene expression in prostate cancer cells.** To clarify the role of epigenetic suppression of the RASSF1A gene, we treated prostate cancer cell lines with 5-aza-dC and TSA. At baseline, expression of the RASSF1A mRNA transcript was detected in the normal prostate cell line (RWPE-1), but was negative in PWR-1E and prostate cancer cell lines (LNCaP and PC3). With TSA treatment, RASSF1A re-expression was not detected in prostate cancer cell lines. However, the expression level was significantly increased in both prostate cancer cell lines after treatment with the demethylating agent 5-aza-dC or combined treatment with azaC and TSA (Fig. 4A). To determine the effects of the demethylating agent 5-aza-dC, we examined the methylation status in LNCaP and PC3 cells after 5-aza-dC treatment. In both cell lines, partial demethylation was detected by MSP (Fig. 4B).

**ChIP assay.** The association of RASSF1A promoter methylation and gene silencing in relation to chromatin remodeling has not been reported previously in prostate cancer. To establish this functional link, we examined local histone acetylation and H3 methylation in the chromatin associated with the RASSF1A promoter region using a ChIP assay. The histone-associated DNAs, immunoprecipitated with antibodies against acetyl-H3, acetyl-H4, H3K4me2, or H3K9me2, were individually amplified with four primer sets covering the RASSF1A promoter region (Fig. 1A). The results in Fig. 5 show marked differences in the levels of histone acetylation and H3 methylation. Enhancement of histone acetylation and H3K4me2 methylation was observed in RWPE-1 cells, in which the RASSF1A promoter is unmethylated and transcriptionally active; however, there was no acetylation or methylation of these same sites in the hypermethylated, transcriptionally silenced promoters (PWR-1E, LNCaP, and PC3). In contrast, H3K9me2 was enriched along the entire hypermethylated and transcriptionally inactive promoters.

To investigate the effect of DNA methyltransferase inhibitor and HDAC-1 in the histone modifications of RASSF1A promoter, we treated LNCaP cells with 5-aza-dC and/or TSA (Fig. 6A and B). The ChIP assays revealed that TSA treatment alone did not induce any alteration of histone modification. These data show that in addition to being unable to reactivate expression of a hypermethylated, silenced RASSF1A gene, TSA alone was unable to evoke obvious change in key parameters of the histone code in the RASSF1A promoter (29). In contrast, we observed a complete reversal of the histone modifications after 5-aza-dC treatment alone or the combination of 5-aza-dC and TSA. Acetyl-H3, acetyl-H4, and H3K4me2 levels were higher, whereas H3K9me2 levels were lower in the promoter region.

**Discussion**

Transcriptional gene silencing by hypermethylation of CpG islands in the promoter region is becoming recognized as a common mechanism for the inactivation of tumor suppressor genes in human malignancies (26–28). In recent years, the list of tumor suppressor genes that are inactivated by epigenetic events rather than classic mutation/deletion events has been growing (7). Unlike mutational inactivation, methylation is reversible, and demethylating agents and inhibitors of HDACs are being used in clinical trails (7).

DNA methylation is an important mechanism in prostate cancer and is involved in the inactivation of various essential genes such as E-cadherin (29), MDR1 (30), and glutathione S-transferase P1 (31). In this study, we found that the RASSF1A...
gene was methylated in 74% of prostate cancer cases examined. Liu et al. (15) has also reported that RASSF1A methylation was frequently observed in prostate cancer (71%). RASSF1A functions as a tumor suppressor gene through several distinct pathways, including microtubule stability (32, 33) and cell cycle regulation (8, 16, 34). The RASSF1A gene has several isoforms, including RASSF1A and RASSF1C that are transcribed from two different promoters containing CpG islands (10, 11). Selective promoter methylation of the RASSF1A promoter, but not RASSF1C, is frequent in many cancers, including lung, breast, ovarian, and renal cell carcinomas (10–13). In prostate cancer, inactivation of the RASSF1A gene was reported to be related to carcinogenesis, and Maruyama et al. (3) described a significant correlation between methylation status, GS, PSA levels, and stage. The results of the present study also showed that RASSF1A promoter methylation was associated with high GS and clinical stage. Currently, Rosenbaum et al. (35) reported that the methylation status of selected genes (GSTPI, APC) in prostate cancer specimens may be predictive for time to recurrence in patients undergoing prostatectomy. We also analyzed PSA failure-free probability as disease-free survival. Of the clinicopathologic features considered, only GS was significantly associated with poor outcome.

The mechanisms of RASSF1A epigenetic change in relation to prostate tumorigenesis, especially chromatin structural changes during the silencing of the genes, are not known. Thus, we examined the molecular mechanisms of inactivation of the RASSF1A gene by analysis of chromatin remodeling such as CpG methylation in the promoter regions, histone acetylation (acetyl-H3, acetyl-H4), dimethyl-H3-K4 (H3K4me2) and dimethyl-H3-K9 (H3K9me2) methylation associated with the RASSF1A promoter region. Histone acetylation and H3K4me2 methylation were increased in the unmethylated RWPE-1 RASSF1A promoter; however, there was no acetylation or methylation of these same sites in the hypermethylated LNCaP and PC3 promoter. In contrast, H3K9me2 was enriched along the entire hypermethylated and transcriptionally inactive promoter in LNCaP and PC3 cells. Similar results has been reported in breast cancer (23, 24). These results support the idea that DNA methylation-mediated gene silencing is closely linked with repressive histone modifications at the gene promoter in cancer cells (23, 25, 36).

In this study, we also investigated the effect of a DNA methyltransferase inhibitor (5-aza-dC) and/or a HDAC-inhibitor (TSA) on chromatin remodeling. We treated LNCaP cells with TSA alone, but there was no change in histone acetylation and H3

Fig. 5. ChIP assays of the RASSF1A CpG island. Chromatin DNA was immunoprecipitated with antibodies specific for acetyl-H3, acetyl-H4, dimethyl-H3-K4 (H3K4me2), and dimethyl-H3-K9 (H3K9me2), respectively. DNA fragments corresponding to RASSF1A promoter regions 1, 2, 3, and 4 (see Fig. 1A) were amplified by PCR. Enhancement of histone acetylation and H3K4me2 methylation was observed in the RWPE-1 promoter. However, there was no acetylation or methylation of these same sites in PWR-1E, LNCaP; and PC3 cells. In contrast, H3K9me2 was enriched in these cell lines. A, PCR analyses of ChIP assay on RWPE-1, PWR-1E, LNCaP, and PC3 cells in the four promoter regions. B, points, enrichment data calculated from the corresponding DNA fragments amplified by PCR; bars SD.
methylation. In contrast, after 5-aza-dC treatment alone or a combination of 5-aza-dC and TSA, there was increased accumulation of acetylated histones and H3K4me2 methylation concomitant with reactivation of the methylated RASSF1A promoter. These results favor the idea that DNA methylation is more important compared with histone deacetylation in maintaining a silent state at hypermethylated promoters because 5-aza-dC can reactivate genes silenced with aberrant promoter hypermethylation, but TSA alone did not reactivate these genes (37). This change in histone modification upon inhibition of DNA methyltransferase suggests that in prostate cancer cells, DNA hypermethylation, or another activity mediated by DNA methyltransferase, may also be essential for maintaining repressive histone modifications at gene promoters silenced by aberrant DNA hypermethylation (25). Furthermore, the observation that 5-aza-dC can both reactivate expression of the silenced RASSF1A gene and completely reverse key histone modifications surrounding the gene promoter strengthens the idea that interdependence exists between these two events.

In conclusion, this is the first report suggesting that chromatin remodeling, such as reduced histone acetylation and H3K4me2 methylation, play a critical role in the maintenance of promoter DNA methylation-associated gene silencing in prostate cancer.

Fig. 6. Effects of 5-aza-dC and TSA on the histone modifications of the RASSF1A promoter. ChIP assays were done on LNCaP cells after treatment with 5 μmol/L 5-aza-dC for 48 h and/or 300 nmol/L TSA for 24 h. TSA treatment alone did not induce any alteration of histone modification. In contrast, complete reversal of the histone modifications were observed after 5-aza-dC treatment alone or the combination of 5-aza-dC and TSA. Acetyl-H3, acetyl-H4, and H3K4me2 levels were higher, whereas H3K9me2 levels were lower in this region of the promoter. A, PCR analyses of ChIP assays. B, points, enrichment data calculated from the corresponding DNA fragments amplified by PCR; bars, SD.

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Epigenetic Modifications of RASSF1A Gene through Chromatin Remodeling in Prostate Cancer

Ken Kawamoto, Steven T. Okino, Robert F. Place, et al.


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