DNA methylation of the *RIZ1* gene is associated with nuclear accumulation of p53 in prostate cancer

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The retinoblastoma protein-interacting zinc finger gene, *RIZ1*, is thought to be a tumor suppressor gene. *RIZ1* is inactivated by mutation, deletion and DNA methylation in several human cancers. In the present study, the relationship between DNA methylation of *RIZ1* and mutation of *p53* was investigated in prostate cancer (PCa). In total, 47 cases of node-negative PCa (stages I–III) were analyzed. DNA methylation of the *RIZ1* gene was detected in 20 (42.6%) of the 47 PCa tissues by methylation-specific polymerase chain reaction. DNA methylation of the *RIZ1* gene was not associated with clinicopathological features. DNA methylation of *RIZ1* tended to be present more frequently in PCa specimens with a high Gleason score (16/30, 53.3%) than in those with a low Gleason score (4/17, 23.5%); however, this tendency was not statistically significant ($P = 0.0675$). Nuclear accumulation of p53 was observed in four (8.5%) of 47 PCa specimens by immunostaining. All four PCa specimens with nuclear accumulation of p53 were stage III disease and showed DNA methylation of *RIZ1*. However, of the remaining 43 cancers without nuclear accumulation of p53, DNA methylation of *RIZ1* was observed in only 16 (37.2%) specimens ($P = 0.0272$). Of the three PCa cell lines, only the PC3 cell line showed loss of *RIZ1* mRNA due to DNA methylation, and this loss was rectified by treatment with a demethylating agent, 5-Aza-2′-deoxycytidine. These results suggest that transcriptional inactivation of *RIZ1* by aberrant DNA methylation may contribute to prostate carcinogenesis. Genetic alterations are likely associated with epigenetic alterations in PCa. (*Cancer Sci* 2007; 98: 32–36)

Prostate cancer (PCa) is one of the most common cancers and the second leading cause of cancer death in men in the USA.[1] An understanding of the genetic and epigenetic pathways involved in the pathogenesis of PCa is essential for development of improved diagnostic and treatment modalities. A variety of genetic and epigenetic alterations are associated with PCa.[2,3] Epigenetic changes, such as DNA methylation of CpG islands, are detected commonly in human cancers. Hypermethylation of CpG islands is associated with silencing of many genes, especially defective tumor-related genes, and has been proposed as an alternative way to inactivate tumor-related genes in human cancers.[4,5] Identification of methylated genes may be useful in the diagnosis and treatment of PCa and may provide insight into prostate carcinogenesis. Prior studies have shown that DNA hypermethylation is a crucial mechanism in transcriptional silencing of tumor-related genes in PCa.[6,7]

The retinoblastoma protein-interacting zinc finger gene, *RIZ1*, was isolated with a functional screen for retinoblastoma (Rb)-binding proteins.[8] Domain analysis suggested that RIZ1 is a histone methyltransferase (HMT) specific for the lysine 9 residue of histone H3, an activity known to be linked with transcriptional repression.[9] *RIZ1* is considered to be a tumor suppressor gene because it can induce G_2–M arrest and apoptosis of several types of cancer cells.[10,11] *RIZ1* plays an important role in human cancers, as evidenced by genetic mutations.[12–14] The *RIZ1* gene is located on human chromosome 1p36, a region deleted in many human cancers,[15] and chromosome 1p36 is a potential hereditary PCa susceptibility locus.[16] In addition to genetic alterations, DNA methylation of *RIZ1* has been shown to be a common mechanism for inactivation of *RIZ1* expression in human cancers.[17,18] In PCa, DNA methylation of *RIZ1* is present in 31% of tumor tissues.[19]

A knockout study showed that *RIZ1* is a tumor susceptibility gene in mice.[14] *RIZ1* and *p53* deficiencies likely cooperate in tumor formation in mice and are expected to occur in human cancers as well.[14] In fact, many sporadic human cancers carry both *p53* mutations and a silenced *RIZ1* gene.[10,14] The *p53* gene is involved in the tumorigenesis of many human cancers,[20] including PCa.[21] p53 functions as a transcriptional regulator involved in G_1 phase growth arrest of cells in response to DNA damage. p53 also has roles in regulation of the spindle checkpoint, centrosome homeostasis and G_2–M phase transition.[22] Several lines of evidence suggest associations between genetic and epigenetic alterations. *p53* mutations have been found frequently in colorectal and gastric cancers without DNA methylation.[23,24] However, the association between genetic and epigenetic alterations has not been investigated in PCa.

In the present study, we investigated the relationship between *RIZ1* methylation status and *p53* mutation status in 47 PCa tissues. To determine whether transcriptional silencing of the *RIZ1* gene is caused by DNA hypermethylation, we compared the methylation status with expression of *RIZ1* mRNA in PCa cell lines.

Materials and Methods

Tissue samples. Subjects were 47 patients with PCa who were referred to the Department of Urology, Hiroshima University Hospital (Hiroshima, Japan). Forty-seven PCa tissues from these 47 patients were analyzed for DNA methylation of *RIZ1* and localization of p53. PCa samples were obtained by radical prostatectomy, and all PCa cases were confirmed to be node negative by pathological examination. None of the 47 patients with PCa received preoperative treatment. All 47 specimens were archival, formalin-fixed, paraffin-embedded tissues. It was confirmed microscopically that the tumor specimens consisted mainly (>50%) of cancer cells. Tumor staging was according to the TNM classification system.[25] In the present study, PCa were graded by the reporting pathologists on the radical surgery specimen, according to the system of the Gleason score.[26] After prostatectomy, the serum prostate-specific antigen (PSA) level was measured by *E*-test Tosoh II Assay (Tosoh, Tokyo, Japan). Patients were followed up by PSA measurement monthly during the first 6 months after prostatectomy and then every 3 months

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thereafter. Biochemical relapse was defined as a PSA level of 0.2 ng/mL or greater. Because written informed consent was not obtained, for strict privacy protection, identifying information for all samples was removed before analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

Cell lines and drug treatment. LNCaP, PC3 and DU145 PCa cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained in RPMI-1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (Whittaker, Walkersville, MD, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells were treated with a final concentration of 1 μM 5-aza-2′-deoxycytidine (Aza-dC; Sigma Chemical, St Louis, MO, USA) for 5 days before they were harvested for DNA or RNA extraction.

Genomic DNA extraction and methylation-specific polymerase chain reaction. For extraction of DNA from the archival, formalin-fixed, paraffin-embedded tissue samples, PCa samples were dissected manually from different sets of 10 serial, 10 μm-thick, formalin-fixed, paraffin-embedded tissue sections with a fine needle. The dissected samples were lysed by incubation in 200 mg/mL proteinase K at 55°C for 3 days. Genomic DNA was purified by three rounds of phenol–chloroform extraction followed by ethanol precipitation. For DNA extraction from cell lines, genomic DNA was extracted with a Genomic DNA Purification Kit (Promega, Madison, WI, USA). To examine the DNA methylation pattern, genomic DNA was treated with 3 M sodium bisulfite, as described previously.[27] For analysis of DNA methylation of the *RIZ1* gene, methylation-specific polymerase chain reaction (MSP) was carried out as described previously.[17] Polymerase chain reaction (PCR) products (15 μL) were loaded onto 8% non-denaturing polyacrylamide gels, stained with ethidium bromide, and visualized under ultraviolet light.

Immunohistochemistry. Formalin-fixed, paraffin-embedded samples were sectioned, deparaffinized, and stained with hematoxylin–eosin to ensure that the sectioned block contained tumor cells. Adjacent sections were then stained immunohistochemically. For immunostaining of p53, a Dako LSAB Kit (Dako, Carpinteria, CA, USA) was used in accordance with the manufacturer's recommendations. In brief, sections were pretreated by microwaving in citrate buffer for 30 min to retrieve antigenicity. After peroxidase activity was blocked with 3% H_2O_2–methanol for 10 min, sections were incubated with normal goat serum (Dako) for 20 min to block non-specific antibody binding sites. Anti-p53 antibody (DO7, 1 : 100; Novocastra, Newcastle, UK) was incubated with tissue samples for 60 min at room temperature followed by incubations with biotinylated antimouse IgG and peroxidase-labeled streptavidin for 10 min each. Staining was completed with a 10-min incubation with the substrate–chromogen solution. The sections were counterstained with 0.1% hematoxylin. p53 staining was classified according to the percentage of stained cancer cells. When more than 10% of cancer cells were stained, the immunostaining was considered positive.

PCR–single-strand conformation polymorphism analysis. Exons 5–8 of the *p53* gene were examined for mutations by PCR–single-strand conformation polymorphism (SSCP) analysis with 10 sets of primers, as described previously.[28] Each target sequence was amplified in a 20-μL reaction volume containing 10–20 ng genomic DNA, 0.2 μM dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.3 μM of each primer and 0.2 μL of Ampli Taq Gold (Applied Biosystems, Foster City, CA, USA). PCR amplification consisted of 35 cycles of 94°C for 30 s, 60°C or 55°C for 30 s, and 72°C for 30 s after the initial activation step of 94°C for 10 min. PCR products were diluted 10-fold with formamide dye solution, denatured at 85°C for 10 min, and separated by electrophoresis on 6% polyacrylamide gels. Gels were stained, and bands were visualized with a Silver Staining II kit (WAKO, Osaka, Japan).

Reverse transcription–polymerase chain reaction. Expression of *RIZ1* mRNA was analyzed by reverse transcription (RT)-PCR. Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and 1 μg of total RNA was converted to cDNA with a First-Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA). Primer sequences and amplification conditions were as described previously.[18] RT-PCR products were subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide, and examined under ultraviolet light. *ACTB*-specific PCR products were amplified from the same RNA samples and served as internal controls.

Statistical methods. Associations between clinicopathological parameters and DNA methylation of *RIZ1* were analyzed by Fisher's exact test. A *P*-value of less than 0.05 was considered statistically significant.

Results

DNA methylation of *RIZ1* and p53 mutation status in PCa tissues. DNA methylation status of the *RIZ1* gene was examined in a total of 47 PCa tissue specimens from 47 patients. DNA methylation of *RIZ1* was detected in 20 (42.6%) of 47 PCa tissues. Representative results of MSP for *RIZ1* are shown in Fig. 1A. No association was detected between the methylation status of

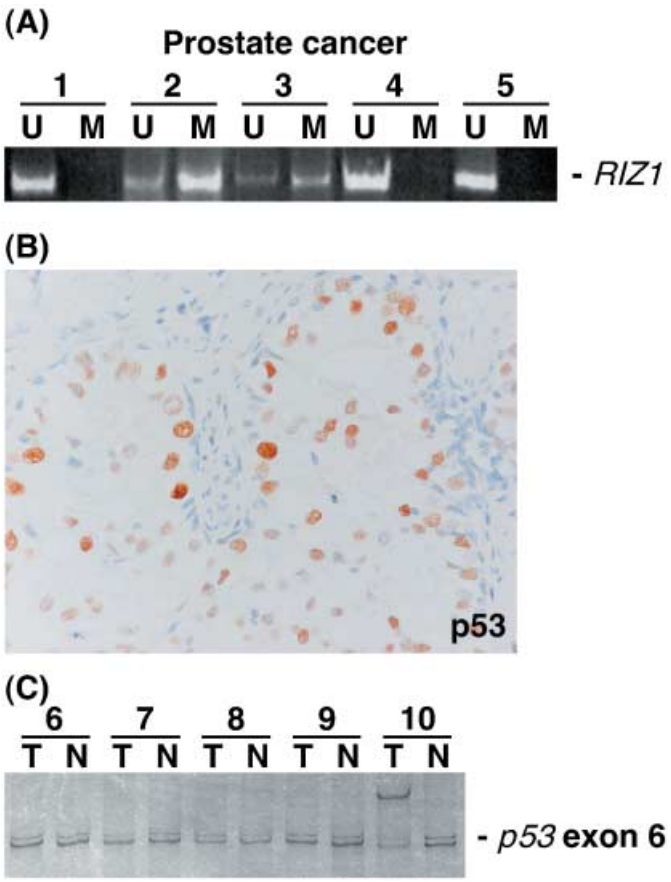

Fig. 1. (A) Methylation-specific polymerase chain reaction (PCR) of *RIZ1* in prostate cancer (PCa). Methylated *RIZ1* was detected in two cases (cases 2 and 3) of PCa. U, unmethylated PCR product; M, methylated PCR product. (B) Immunostaining of p53 in PCa. Nuclear accumulation of p53 was observed in PCa cells. Original magnification, ×400. (C) PCR–single-strand conformation polymorphism analysis of *p53*. A *p53* mutation was detected in one case (case 10).

RIZ1 and age ($P = 1.000$), T grade ($P = 0.1425$), stage ($P = 0.1425$), preoperative PSA concentration ($P = 0.7674$), or relapse ($P = 0.3917$) (Table 1). DNA methylation of *RIZ1* tended to occur more frequently in PCa cases with a high Gleason score (16/30, 53.3%) than in those with a low Gleason score (4/17, 23.5%); however, the difference was not statistically significant ($P = 0.0675$; Table 1). We next investigated the nuclear accumulation of p53 in the 47 PCa tissues by immunostaining. Nuclear accumulation of p53 typically indicates the presence of *p53* gene mutations.[29] Immunostaining revealed nuclear accumulation of p53 in four (8.5%) of 47 PCa tissues (Fig. 1B).

Table 1. Association between *RIZ1* methylation status and clinicopathological features and nuclear accumulation of p53 in prostate cancer

Feature	*RIZ1* methylation status			*P*-value
	Methylated	n%	Unmethylated	
Age (years)				
>70	10	43.5	13	1.0000
≤70	10	41.7	14	
Tumor grade				
T1/2	7	30.4	16	0.1425
T3	13	54.2	11	
Stage†				
I/II	7	30.4	16	0.1425
III	13	54.2	11	
Gleason score‡				
2–6	4	23.5	13	0.0675
7–10	16	53.3	14	
Preoperative PSA (ng/mL)§				
<10	13	44.8	16	0.7674
>10	7	38.9	11	
Relapse¶				
Positive	7	35.0	13	0.3917
Negative	13	48.1	14	
Nuclear accumulation of p53				
Positive	4	100.0	0	0.0272
Negative	16	37.2	27	
p53 mutation determined by PCR-SSCP				
Mutant-type	2	100.0	0	0.1758
Wild-type	18	40.0	27	

†Tumor stage according to TNM classification. ‡Tumor grade according to Gleason criteria. §Prostate-specific antigen (PSA) concentration was determined as described in the Materials and Methods. ¶Relapse was defined as serum PSA concentration of 0.2 ng/mL or higher. PCR-SSCP, polymerase chain reaction–single-strand conformation polymorphism.

We also carried out PCR-SSCP analysis of *p53*. Representative results are shown in Fig. 1C. Of the four PCa specimens with nuclear accumulation of p53, two (50.0%) exhibited a *p53* mutation. No mutation was found in the 43 PCa specimens without nuclear accumulation of p53. All PCa specimens with nuclear accumulation of p53 showed DNA methylation of *RIZ1* whereas only 16 (37.2%) of 43 PCa specimens without nuclear accumulation of p53 showed DNA methylation of *RIZ1* ($P = 0.0272$, Fisher's exact test; Table 1). We found no association between the methylation status of the *RIZ1* and *p53* mutations determined by PCR-SSCP analysis ($P = 0.1758$, Fisher's exact test; Table 1).

DNA methylation status and expression of *RIZ1* in PCa cell lines. To determine whether DNA hypermethylation of *RIZ1* inactivates transcription of the gene, DNA methylation and expression of *RIZ1* were investigated in three PCa cell lines (Fig. 2A). MSP revealed DNA hypermethylation of *RIZ1* in PC3 cells, whereas hypermethylation of *RIZ1* was not detected in LNCaP or DU145 cells. To study the relationship between DNA methylation status and *RIZ1* expression levels, we carried out RT-PCR of mRNA from PC3 cells. Transcriptional inactivation of *RIZ1* was observed in PC3 cells with DNA hypermethylation (Fig. 2B). LNCaP and DU145 cells expressed *RIZ1*. To investigate whether transcriptional inactivation of *RIZ1* was caused by DNA methylation in PC3 cells, we treated PC3 cells and LNCaP cells (unmethylated control) with Aza-dC and carried out MSP (Fig. 2C) and RT-PCR (Fig. 2D) analyses. Unmethylated *RIZ1* was detected in PC3 cells after Aza-dC treatment. Expression of *RIZ1* was restored in PC3 cells after treatment with Aza-dC. *RIZ1* expression in LNCaP cells was not changed significantly by Aza-dC treatment.

Discussion

A variety of genetic and epigenetic alterations are associated with human cancers. Although there have been several reports regarding genetic and epigenetic changes in various genes in PCa, in most of these studies, the alteration was investigated for just a single gene. In the present study, the relationship between DNA methylation of *RIZ1* and mutation of *p53*, as measured by nuclear accumulation of p53, was investigated, and we found that PCa tissues with nuclear accumulation of p53 also showed DNA methylation of the *RIZ1* gene.

In the present study, DNA methylation of *RIZ1* was found in 42.6% of PCa cases analyzed, a frequency slightly higher than that reported previously.[19] In the PCa cell lines, DNA hypermethylation of *RIZ1* was detected in PC3 cells, which expressed undetectable levels of the *RIZ1* mRNA. After 5 days of Aza-dC treatment, unmethylated *RIZ1* was observed, and expression of *RIZ1* mRNA followed. Thus, hypermethylation of *RIZ1* plays an

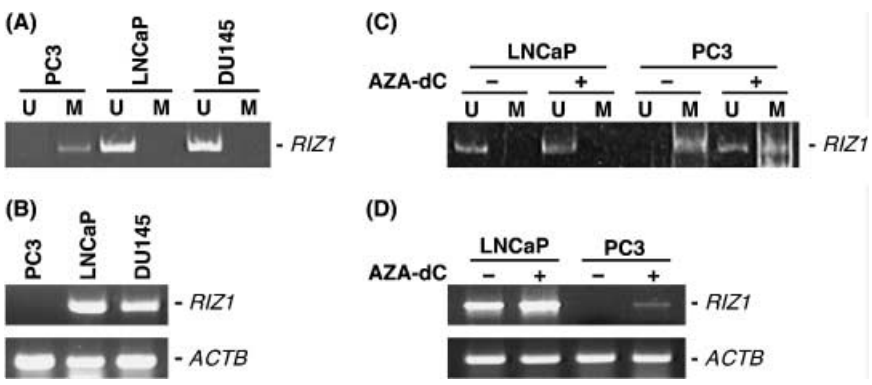

Fig. 2. DNA methylation status and expression of *RIZ1* in prostate cancer (PCa) cell lines. (A) Methylation-specific polymerase chain reaction (MSP) of *RIZ1*. The methylated allele was detected only in the PC3 cell line. U, unmethylated polymerase chain reaction (PCR) product; M, methylated PCR product. (B) Reverse transcription–PCR of mRNA from PCa cell lines. *RIZ1* is not expressed in the PC3 cell line. (C) Effect of 5-Aza-2′-deoxycytidine (Aza-dC) treatment. Aza-dC-treated LNCaP and PC3 cells and untreated LNCaP and PC3 cells were analyzed by MSP. The unmethylated allele was detected in Aza-dC-treated PC3 cells but not in untreated PC3 cells (D) Expression of *RIZ1* was analyzed in Aza-dC-treated LNCaP and PC3 cells and untreated LNCaP and PC3 cells. RIZ1 mRNA is expressed in Aza-dC-treated PC3 cells but not in untreated PC3 cells.

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important role in inactivation of the *RIZ1* gene. Previous studies have demonstrated that CpG island hypermethylation in the region examined was well correlated with epigenetic silencing of the *RIZ1* gene.[17,18] In the present study, DNA methylation of the *RIZ1* gene was not associated with clinicopathological features. Because DNA methylation of *RIZ1* is a rare event in non-malignant prostate tissues,[19] these results suggest that DNA methylation of the *RIZ1* gene may be associated with prostate carcinogenesis.

It has been reported that *p53* is mutated in late-stage PCa.[21] In the present study, nuclear accumulation of p53 was observed only in stage III PCa, and all PCa tissues with nuclear accumulation of p53 showed DNA methylation of *RIZ1*. In the present study, of the four PCa specimens with nuclear accumulation of p53, only two exhibited a *p53* mutation. These inconsistent results between immunohistochemistry and PCR-SSCP may be due to methodological differences. Because only exons 5–8 were examined by PCR-SSCP in the present study, the mutation of *p53* may be in the remaining exons. It is also possible that the sensitivity of PCR-SSCP may not be sufficient to detect a small number of mutant alleles in a background of wild-type alleles from stromal cells and normal epithelial cells. It has been reported that the correlation between nuclear accumulation of p53 and the presence of *p53* gene mutations can vary.[30]

RIZ1 has HMT activity.[9] HMT activity is thought to be important to the tumor suppression function of RIZ1, because this activity is reduced by *RIZ1* mutations found in human cancers.[9] Because histone modification is thought to affect chromatin structure directly,[31] aberrant chromatin structure may induce mutation of the *p53* gene. In fact, Suv39h (an HMT) -deficient mice display severely impaired viability and chromosomal instabilities.[32] A subset of PCa cells with mutations in *p53* may arise from PCa cells with DNA methylation of *RIZ1*. Therefore, DNA methylation of *RIZ1* may predict development of *p53* mutant PCa cells. In the PCa cell lines, *p53* mutation status has been described previously (PC3, mutant-type *p53*; LNCaP, wild-type *p53*; DU145, mutant-type *p53*).[33] Because DNA hypermethylation of *RIZ1* was detected in PC3 cells, mutation of the *p53* gene may be induced by inactivation of *RIZ1*. In contrast, DNA hypermethylation of *RIZ1* was not detected in DU145 cells despite the presence of *p53* mutation. Whether silencing of *RIZ1* induces *p53* mutation should be verified experimentally in the near future.

Treatment options for the early stages of PCa have been limited to local treatment. Treatments for more advanced disease rely on suppression of testosterone production, primarily with hormonal therapy. Until the introduction of luteinizing hormone-releasing hormone agonist therapy, estrogen therapy was often used for hormonal manipulation of PCa.[34] Because estradiol treatment produces a selective decrease in *RIZ1* expression,[35] it is interesting to investigate the *RIZ1* expression in PCa tissues from patients who received preoperative hormone treatment.

In conclusion, our results suggest that genetic alterations are associated with epigenetic alterations and that these alterations are not random events in PCa. To better understand the development of PCa at the molecular level, molecular classification of PCa based on genetic and epigenetic alterations may be useful.

Acknowledgments

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