[CANCER RESEARCH 64, 5956–5962, September 1, 2004]

CpG Hypermethylation of *MDR1* Gene Contributes to the Pathogenesis and Progression of Human Prostate Cancer

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ABSTRACT

Multidrug resistance 1 (*MDR1*) gene encodes for P-glycoprotein (P-gp), a Mr 170,000 transmembrane calcium-dependent efflux pump that is inactivated in prostate cancer. We hypothesize that inactivation of the *MDR1* gene through CpG methylation contributes to the pathogenesis and progression of prostate cancer. To test this hypothesis, CpG methylation status of the *MDR1* promoter and its correlation with clinicopathological findings were evaluated in 177 prostate cancer samples and 69 benign prostate hypertrophy (BPH) samples. Cellular proliferation index and apoptotic index were determined by proliferating cell nuclear antigen (PCNA) and single-strand DNA immunostaining, respectively. After 5-aza-2′-deoxycytidine treatment, increased expression of *MDR1* mRNA transcript was found in prostate cancer cell lines (DU145, DuPro, and ND1). *MDR1* methylation frequency was significantly higher in prostate cancer samples compared with BPH samples (54.8 *versus* 11.6%, respectively, $P < 0.001$). Logistic regression analysis revealed that PC patients are 11.5 times more likely to have *MDR1* methylation than BPH patients (95% confidence interval 4.87–27.0) and that *MDR1* methylation is independent of the age. Significant correlation of *MDR1* methylation was observed with high pT category ($P < 0.001$), high Gleason sum ($P = 0.008$), high preoperative prostate-specific antigen ($P = 0.01$), and advancing pathological features. In addition, PCNA-labeling index were significantly higher in methylation-specific PCR (MSP)-positive than in MSP-negative prostate cancer samples ($P = 0.048$). In contrast, no significant difference in apoptotic index was found between MSP-positive and -negative prostate cancer samples. These findings suggest that CpG hypermethylation of *MDR1* promoter is a frequent event in prostate cancer and is related to disease progression via increased cell proliferation in prostate cancer cells.

INTRODUCTION

Prostate cancer is one of the most common malignancies among men (1, 2). In organ-confined prostate cancer, treatments such as radical prostatectomy (1), radiotherapy (2), and chemotherapy (3) are effective. However, multiple drug resistance often develops in patients with advanced disease. Multidrug resistance 1 (*MDR1*) gene encodes for P-glycoprotein (P-gp), a Mr 170,000 transmembrane calcium-dependent efflux pump, which is expressed in various tissues (4–6). Aberrant expression of P-gp is associated with various cancers. Recent studies have shown that hypermethylation of normally unmethylated CpG dinucleotides (CpG islands) located in the promoter regions is involved in gene silencing at the transcriptional level (7, 8). In some cancers, one of the mechanisms underlying down-regulation of *MDR1* is thought to be CpG hypermethylation of the *MDR1* promoter (9–11). Recent publications have indicated another functional role of P-gp as a marker for disease progression, in addition to the conventional role as a drug resistance marker (12–16). In hepatocellular carcinoma and osteosarcoma, it has been demonstrated that the frequency of P-gp overexpression is higher in patients with localized disease than in metastatic diseases (12, 13). It is not known whether expression of P-gp is associated with disease progression in prostate cancer (14–16).

We hypothesize that inactivation of *MDR1* genes through CpG methylation is responsible for the pathogenesis and progression of prostate cancer. To address this issue, we analyzed mRNA levels and promoter methylation status of the *MDR1* gene in prostate cancer samples and related these findings to clinical and pathological outcomes. In addition, we also evaluated the imbalance between cell proliferation and apoptosis and its association with methylation status of *MDR1* promoter in human prostate cancer tissues.

MATERIALS AND METHODS

Tissue Samples. A total of 177 newly diagnosed prostate cancer tissues from radical prostatectomies (136 retropubic and 41 perineal approaches) and 69 pathologically proven benign prostate hypertrophy (BPH) samples from transurethral resection was obtained from Shimane University Hospital (Izumo, Japan). The patients' background and clinicopathological characteristics are summarized in Table 1. The pathological findings of prostate cancer samples were determined by the general rule for Clinical and Pathological Studies on prostate cancer by the Japanese Urological Association and the Japanese Society of Pathology (17). Our routine diagnostic work-up for prostate cancer includes serum prostate-specific antigen (PSA) level, transrectal ultrasonography, color Doppler ultrasonography, and magnetic resonance imaging, which enable us to speculate more accurate localization of prostate cancer before radical prostatectomy (18). As a control, 10 samples of normal peripheral blood lymphocytes were used.

Tissue Preparations. All of BPH samples and half of each prostate cancer samples were fixed in 10% buffered formalin (pH 7.0) and embedded in paraffin wax. For histological evaluation, 5-μm thick sections were used for H&E staining. The remaining half of each prostate cancer sample was immediately frozen and stored at −80°C for RNA extraction. All of the samples were microscopically dissected and analyzed for methylation. In BPH samples, high-grade prostate intraepithelial neoplasia and cancer were ruled out by microscopically analysis.

Cell Culture. Human prostate cancer cell lines of Du145 and DuPro were obtained from American Type Culture Collection (Manassas, VA). The other human PC cell line, ND1, was developed in our laboratory (19). The media used in this study for Du145, DuPro, and ND1 were RPMI 1640 supplemented with 10% fetal calf solution.

Nucleic Acid Extraction. Genomic DNA from all prostate samples and blood samples was extracted using a commercial kit (Qiagen, Valencia, CA) after microdissection (20) and was precipitated with ethanol. Genomic DNA from cell line samples was extracted with DNAzol reagent (Invitrogen-Life Technologies, Inc., Carlsbad, CA). Total RNA was extracted by TRI Reagent (Molecular Research Center, Cincinnati, OH). RNA specimens were stored in aliquots at −80°C before use. The concentration of DNA and RNA was

Received 1/10/04; revised 6/1/04; accepted 7/6/04.

Grant support: The present study was supported by a VA Merit Review Grant/Research Enhancement Award Program and NIH Grants RO1CA101844, RO1AG21418, and T32DK07790.

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Table 1 *Clinical characteristics of prostate cancer and benign prostate hypertrophy patients*

Prostate cancer	
Total number	177
Median age (range)	69 (49–80) yrs
pT category	
pT1	0
pT2	118
pT3	56
pT4	3
GS	
≤4	33
5	33
6	31
7	47
≥8	33
Preoperative serum PSA	
<4	22
4–10	80
>10	75
Benign prostate hypertrophy	
Total number	69
Median age (range)	75 (54–87) yrs

determined by spectrophotometer, and their integrity was checked by gel electrophoresis.

cDNA Preparation and Differential Reverse Transcription-PCR (RT-PCR) of *MDR1*. RNA, 1 μg, was added to 0.5 μg of oligodeoxythymidilic acid primer (Promega, Madison, WI) and prepared to a final volume of 25 μL. The primer RNA mixture was then combined with 0.25 units of Avian myeloblastosis virus reverse transcriptase (Promega) and 0.5 units of RNase inhibitor. The reverse transcriptase reaction was then carried out at 45°C for 45 min. The cDNA was then incubated at 95°C for 5 min to inactivate the reverse transcriptase. Samples were stored at −20°C until used.

The cDNA samples, 2 μL, were diluted into 20 μL of solution containing 50 mmol/L deoxynucleoside triphosphate, 0.5 units of RedTaq polymerase and PCR reaction buffer provided by the manufacturer (Sigma, St. Louis, MO). For differential RT-PCR between *MDR1* and *G3PDH*, different primer sets of s-*G3PDH* primers and l-*G3PDH* primers were used for analysis of prostate cancer cell lines and clinical samples, respectively. For cell lines samples, l-*G3PDH*-S and l-*G3PDH*-AS primer concentrations were 50 nmol/L, whereas MDR1-RT-S and MDR1-RT-AS primer concentrations were 250 nmol/L. For clinical samples, s-*G3PDH*-S and s-*G3PDH*-AS primer concentrations were 100 nmol/L, whereas MDR1-RT-S and MDR1-RT-AS primer concentrations were 150 nmol/L. The primer sequences and RT-PCR conditions (annealing temperatures, times, and cycle numbers) are shown in Table 2. The PCR products were electrophoresed on 3% agarose gel, and the expression level of these genes was evaluated by ImageJ software.[5] The expression level of *MDR1* was quantified relative to *G3PDH* expression level and expressed as arbitrary units. For semiquantitative analysis of the amplified products, a suitable PCR cycle for MDR1 and G3PDH was determined in the way it is within the exponential phase. For this purpose, we performed RT-PCR using five different diluted specimens from the same sample (1:1, 1:2, 1:4, 1:8, and 1:16) and calculated absorbance of each band. At 32 cycles, as the logistically converted density of each band from the same sample were on the same regression line, PCR amplification is considered within the exponential phase (Fig. 4*A*).

5-Aza-2′-deoxycytidine (5-Aza-dC) Treatment. We investigated the effects of the demethylation agent (5-aza-dC) on *MDR1* gene expression in cultured prostate cancer cell lines. Prostate cancer cell lines such as DU145, DUPro, and ND-1 were treated with 5 μmol/L 5-aza-dC. The cultured cells were harvested after 4 days of 5-aza-dC treatment. Using cDNA obtained from these cell lines, the difference in expression level of *MDR1* mRNA transcripts before and after 5-aza-dC treatments was analyzed by differential RT-PCR.

Methylation Analysis. Genomic DNA from fresh tissue, 100 ng, and paraffin block DNA from transurethral resection specimens, 100 ng, were subjected to sodium bisulfite modification using CpGenome DNA Modification kit (Intergen Co., Purchase, NY). On the basis of the functional promoter sequence (21), methylation-specific (MSP) and unmethylation-specific (USP) primers were designed using MethPrimer software.[6] The location of forward and reverse primers, in which six CpG sites and two SP-1 sites are covered, and corresponding amplified regions are shown in Fig. 1. The forward and reverse primers of each MSP and USP were designed to cover the SP-1 site at their 3′-prime end because both SP-1 sites are mandatory for the functional *MDR1* promoter to be activated (22). For MSP and USP, an initial PCR product was created with universal primers (Pan-S and Pan-AS), followed by a second nested PCR with primers specific for MSP or USP (M-S, M-AS and U-S, and U-AS, respectively). In each assay, absence of DNA template served as negative control. The primer sequences and PCR conditions are shown in Table 2. The obtained MSP and USP products were analyzed on 3% agarose gel electrophoresis. The area under the curve (AUC) corresponding to each band was analyzed using the ImageJ software.[5] We used both visual and percent methylation fraction methods and found that the results are the same. At first, percent methylation of each sample was calculated using the following formula: percent methylation (%) = A(M) × 100/[A(M)+A(U)], where A(M) is AUC of MSP band and A(U) is AUC of USP band (23). We applied percent methylation of 8.5% as cutoff value, which was the average percentage of methylation fraction in 69 BPH samples. Using this criteria, MSP positive was defined as prostate cancer samples with percent methylation fraction of >8.5%, and negative was <8.5%.

Bisulfite DNA Sequencing Analysis. Bisulfite-modified DNA, 1 μL, was amplified using a pair of universal primers (Pan-S and Pan-AS) in a total volume of 20 μL of mixture. The PCR conditions are shown in Table 2. Direct bisulfite DNA sequencing of the PCR products using either forward Pan-S primer or reverse Pan-AS primer was performed according to the manufactures instruction (Applied BioSystems, Foster City, CA).

Immunostaining. Immunostaining of proliferating cell nuclear antigen (PCNA) and single-strand DNA was performed on 5-μm thick consecutive sections obtained from paraffin-embedded materials, using mouse monoclonal antibody for PCNA (clone PC-10, 1/1000 dilution; Dako, Glostrup, Denmark) and single-strand DNA (1/100 dilution; Dako, Kyoto, Japan; ref. 24), respectively. The slides were microwaved for antigen retrieval in citrate buffer [10 mM (pH 6.0)] before incubation of primary antibody. In negative controls, the primary antibody was replaced by a nonimmune serum. 3,3′-Diaminobenzidine (Sigma-Aldrich, St. Louis, MO) was used as the chromogen, and counterstaining was performed using 0.5% hematoxylin.

Statistical Analysis. Relationship between mRNA expression and methylation of the *MDR1* promoter in prostate cancer samples was analyzed with the Mann-Whitney *U* test. The relationship between clinicopathological findings and methylation status of *MDR1* was performed using χ^2 test, Mann-Whitney *U* test and logistic regression analysis. $P < 0.05$ was regarded as statistically significant.

RESULTS

Effects of 5-Aza-dC on *MDR1* Gene Expression in Prostate Cancer. At baseline, expression of the *MDR1* mRNA transcript was negative in DU145 and ND1 and was weak in DuPro prostate cancer cell lines (Fig. 2). However, after 4 days of treatment of 5-aza-dC, the expression level was significantly increased in DU145 and ND1 cell lines and moderately increased in DuPro cell lines. This data suggests that demethylation (5-aza-dC) restored expression of *MDR1* gene in prostate cancer cell lines.

Methylation Status of *MDR1* Promoter and *MDR1* mRNA Expression in Prostate Clinical Samples. Representative results of MSP and USP assays as well as RT-PCR in prostate cancer examples are shown in Fig. 3, *A* and *B*. The prostate cancer samples with positive MSP band (numbers 1, 2, 6, 7, and 8) showed only weak expression of *MDR1* mRNA transcript. Interestingly, the other prostate cancer samples with negative MSP band showed strong expression of *MDR1* mRNA transcript. No MSP bands were detectable in 10 genomic DNA samples from peripheral blood lymphocytes (Fig. 3*D*). Fig. 3*C* shows the typical bisulfite DNA sequencing in prostate cancer samples. In sample number 2, both MSP- and USP-positive bands (Fig. 3*A*) showed complete or partial methylation at the specific CpG sites. For example, at the CpG sites of −29 and −34 there was a strong "C" and no "T" signal, indicating complete CpG methylation.

[5] Internet address: http://rsb.info.nih.gov/ij.
[6] Internet address: http://itsa.ucsf.edu/~urolab/methprimer.

Table 2 *Primer sequence and PCR conditions*

	Primer sequence (5′-3′)	Product size (bp)	Annealing temperature, time (cycle)
Differential RT-PCR			Cell line; 55°C, 60 s (35)
MDR1-RT-S	GAATCTGGAGGAAGACATGACC	259	Human sample; 55°C, 30 s (38)
MDR1-RT-AS	TCCAATTTTGTCACCAATTCC		
s-G3PDH-S	GAAGGTGAAGGTCGGAGTC	226	
s-G3PDH-AS	GAAGATGGTGATGGGATTTC		
l-G3PDH-S	TCCCATCACCATCTTCCA	380	
l-G3PDH-AS	CATCACGCCACAGTTTCC		
Methylation analysis			
Universal primer			
Pan-S	GGAAGTTAGAATATTTTTTTTGGAAAT	223	48°C, 30 s (45)
Pan-AS	ACCTCTACTTCTTTAAACTTAAAAAAACC		
MSP primer			
M-S	CGAGGAATTAGTATTTAGTTAATTCGGGTCGG	95	67°C, 30 s (5)→65°C, 30 s (5)
M-AS	ACTCAACCCACGCCCCGACG		→62°C, 30 s (5) →59°C, 30 s (10)
USP primer			
U-S	TGAGGAATTAGTATTTAGTTAATTTGGGTTGG	95	
U-AS	ACTCAACCCACACCCCAACA		

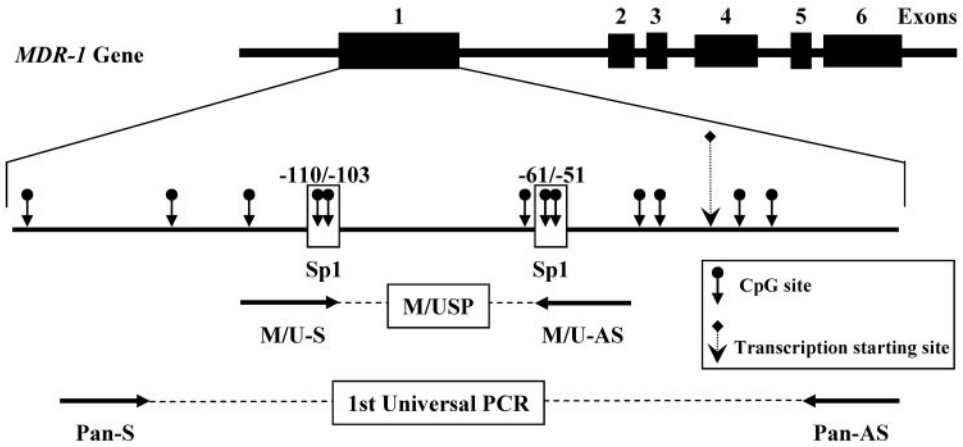

Fig. 1. Schematic representation of the location of CpG sites and primers designed within the functional promoter of *MDR1* gene. First, universal PCR was performed using the Pan-S and Pan-AS primers. These universal primers do not cover any CpG sites within the primer sequence. MSP and USP were determined by the primer sets of M-S and M-AS and U-S and U-AS, respectively, using the universal PCR product as a template. Each forward and reverse M/USP primer was designed to cover the SP-1 site in their 3′-region.

On the other hand, at the CpG sites of −51, −56, and −59, there was a modest T signal along with a stronger C peak, indicating these CpG sites were partially methylated. In the number 8 prostate cancer sample, where both MSP and USP bands were found, every CpG site was partially methylated. In the number 5 prostate cancer sample, where no MSP band was observed (Fig. 3*A*), all CpG sites of −29, −34, −51, −56, and −59 were completely unmethylated. In our series, positive MSP bands were found in 97 of 177 prostate cancer samples (54.8%). As shown in Fig. 4*B*, relative expression of *MDR1* mRNA transcript was significantly lower in the 97 prostate cancer samples with positive MSP than in the remaining 80 cases with negative MSP ($P = 0.021$).

Correlation of *MDR1* Gene Methylation with Clinicopathological Findings. Ninety-seven of 177 prostate cancer samples (54.8%) showed positive MSP bands, whereas only 8 of 69 BPH samples (11.6%) showed positive MSP bands. On the other hand, the majority of both BPH and prostate cancer samples showed USP bands, although the intensity of USP band was strong in BPH and weak in prostate cancer. The difference in the frequency of *MDR1* promoter methylation was significant between prostate cancer and BPH samples ($P < 0.001$). In the total group of prostate cancer and BPH, there was no correlation between age and methylation. The frequency of *MDR1* methylation showed a significant stepwise increase with advancing pathological stage (29% in pT2a, 56% in pT2b, 69% in pT3a, 75% in pT3b, and 100% in pT4; $P < 0.001$; Fig. 5*A*). Similarly for Gleason sum (GS), the frequency of *MDR1* methylation increased as GS increased (45% in GS < 7, 60% in GS $= 7$, and 76% in GS > 7; $P = 0.008$; Fig. 5*B*). With regard to preoperative PSA, methylation frequency was higher in PSA ≥ 10 ng/ml (66%) than in PSA < 10 ng/ml (47%; $P = 0.01$; Fig. 5*C*). As shown in Table 3, no significant association between patient age and *MDR1* methylation was found. Logistic regression analysis after age adjustment revealed that pathology (BPH *versus* prostate cancer) is a significant dependent predictor of MDR1 methylation ($P < 0.001$), where cases with MDR1 methylation are 11.5 times more likely to have prostate cancer than cases with negative methylation. Fig. 5*C* shows that there were also significant correlations between *MDR1* methylation and capsular invasion ($P < 0.001$), seminal vesicle involvement ($P = 0.007$), pN category

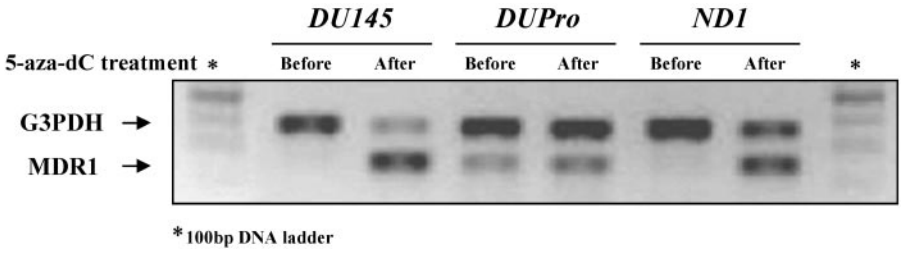

Fig. 2. *MDR1* expression evaluated by differential RT-PCR in prostate cancer cell lines. In Du145, DuPro, and ND1 cell lines, the mRNA transcript of the *MDR1* gene was significantly increased after 5-aza-dC treatment. RT-PCR condition is summarized in Table 2.

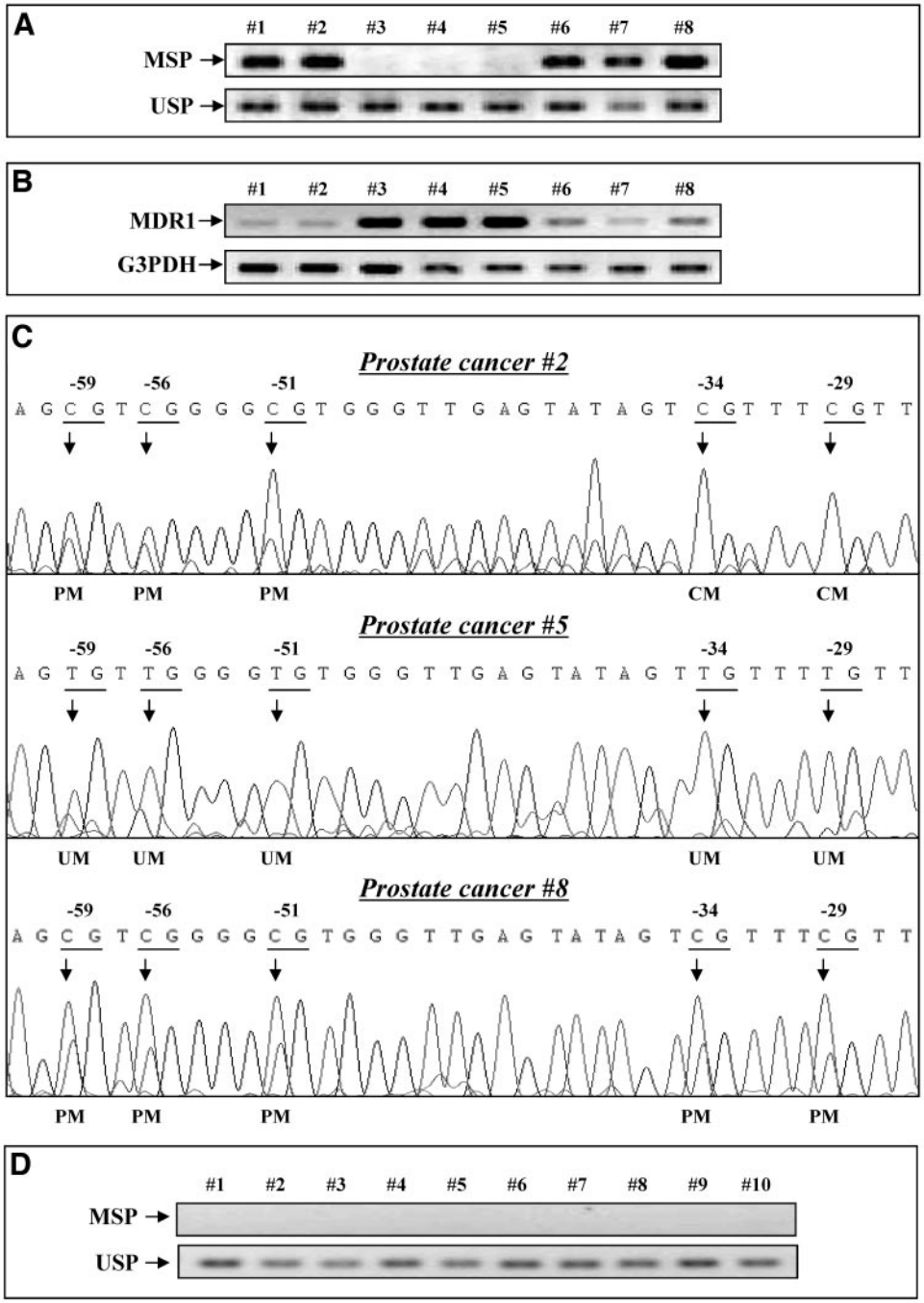

Fig. 3. Methylation status of *MDR1* promoter and *MDR1* mRNA expression in clinical samples. *A*. MSP and USP bands of eight samples are shown. *B*, differential RT-PCR of *MDR1* mRNA in the same samples in *A*. The samples with positive MSP band showed negative mRNA transcript of *MDR1*. *C*, bisulfite DNA sequencing of MSP-positive (numbers 2 and 8) and -negative (number 5) samples. In number 2, complete CpG methylation was observed at −29 and −34, whereas the CpG sites of −51, −56, and −59 were partially methylated. In number 8, every CpG site was partially methylated. In number 5, all CpG sites were unmethylated. CM, PM, and UM correspond to complete methylation, partial methylation, and unmethylation, respectively. *D*. No MSP bands were found in 10 samples of human peripheral blood lymphocytes.

($P = 0.002$), venous involvement ($P < 0.001$), lymphatic vessel involvement ($P = 0.001$), and perineural invasion (PNI) ($P = 0.002$).

Correlation of *MDR1* Gene Methylation with Cell Proliferation and Apoptosis. To investigate the correlation between *MDR1* gene methylation and increased cell proliferation and decreased cell apoptosis, PCNA-labeling index and apoptotic index determined by single-strand DNA immunostaining was calculated in each prostate cancer sample. As shown in Fig. 6*A*, the mean PCNA-labeling index was significantly higher in MSP-positive prostate cancer samples (19.9%) than in MSP-negative prostate cancer samples (8.1%; $P = 0.048$). On the other hand, no significant difference in the mean apoptotic index was observed between MSP-positive prostate cancer samples (0.60%) and MSP-negative prostate cancer samples (0.52%; Fig. 6*B*).

DISCUSSION

P-gp-mediated multidrug resistance has been extensively investigated (9–16). In prostate cancer, the expression of the *MDR1* gene is down-regulated (14–16). However, the mechanism of inactivation of the *MDR1* gene and its role in pathogenesis and progression of prostate cancer are not known. In the present study, we investigated the *MDR1* gene expression, methylation, cellular proliferation index, and apoptotic index in 177 prostate cancer and 69 BPH samples. We additionally correlated *MDR1* gene methylation with clinicopathological findings of prostate cancer.

In the present study, expression of *MDR1* mRNA transcript was negative or weak in prostate cancer cell lines. Demethylation with 5-aza-dC treatment restored expression of the *MDR1* gene, suggesting

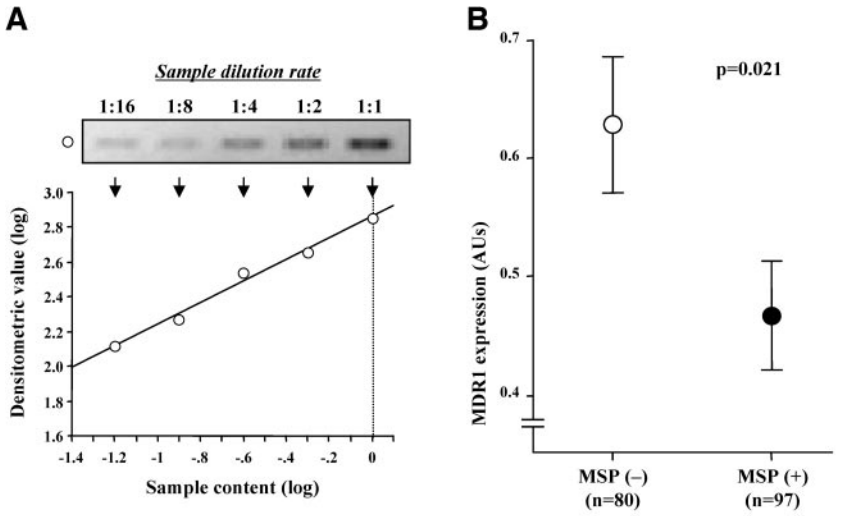

Fig. 4. Correlation of *MDR1* mRNA expression with *MDR1* promoter methylation in clinical samples. *A*, relationship between sample dilution rate (1:1, 1:2, 1:4, 1:8, and 1:16) and densitometric data after logistic conversion. The logistic conversion of band intensity with different dilution rate from the same sample was on the same regression line, indicating PCR amplification at 32 cycles being within the exponential phase. *B*, correlation of methylation status with mRNA transcript level. Samples with negative MSP bands shows significantly higher expression levels of mRNA transcript than those with MSP-positive bands.

that methylation causes inactivation of the *MDR1* gene in prostate cancer. On the basis of this result, we hypothesize that the *MDR1* promoter was affected by hypermethylation in human prostate cancer. On the basis of the functional promoter of *MDR1* gene (21), we designed the MSP and USP primers, where either forward or reverse primer sequence covered the two SP-1 sites that play an important role in regulating gene transcription (ref. 22; Fig. 1). Ninety-seven of 177 prostate cancer samples (54.8%) showed positive MSP band in which samples expression levels of *MDR1* mRNA transcript were significantly reduced in comparison with MSP-negative samples, irrespective of the coexisting USP bands (Figs. 3, *A* and *B*, and 4*B*). When dealing with surgically obtained prostate cancer samples, a major concern appears to be contaminated unmethylated normal cells in the results. Although we used microscopically dissected samples, it is impossible to avoid contamination of normal cells completely because tumor cells are intimately associated both with peripheral blood cells

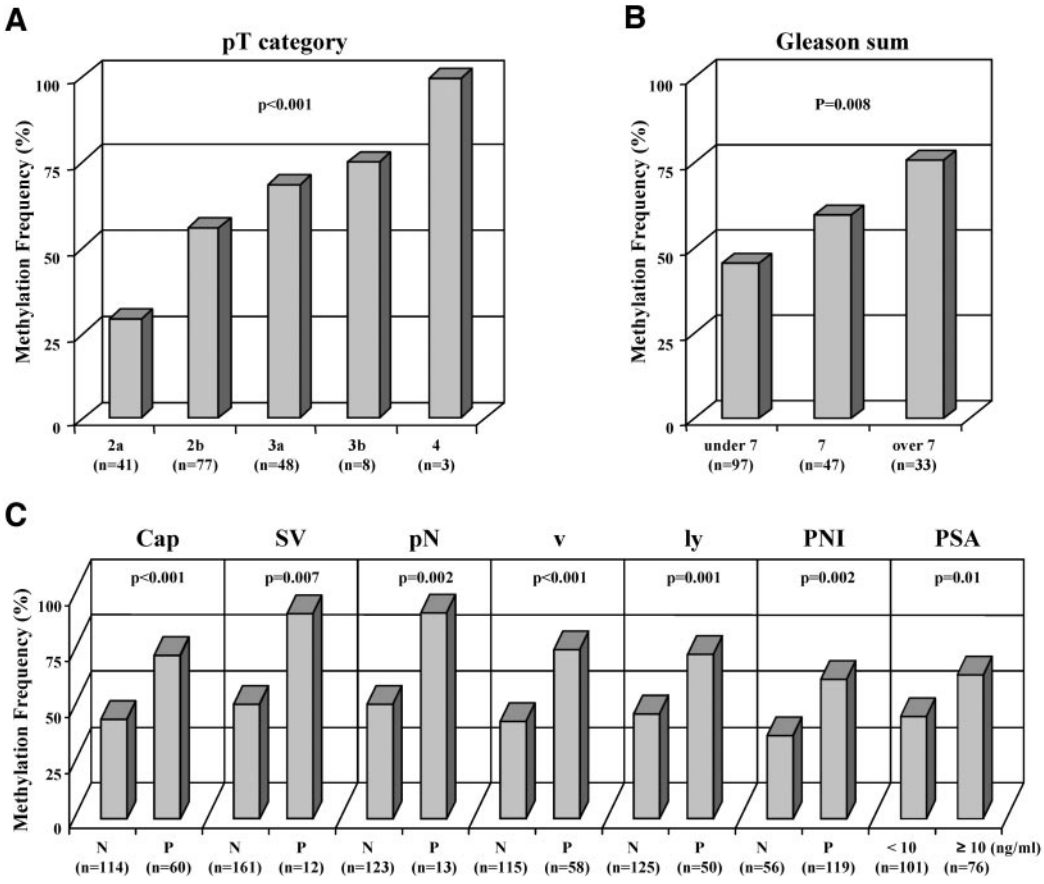

Fig. 5. Correlation of clinicopathological features with methylation frequency of *MDR1* promoter. *A*, methylation frequency of *MDR1* increased with higher stage of disease (29% in pT2a, 56% in pT2b, 69% in pT3a, 75% in pT3b, and 100% in pT4). *B*, methylation frequency of *MDR1* increased with higher Gleason sum (45% in GS <7, 60% in GS = 7, and 76% in GS >7). *C*. There was significant correlation between methylation frequency of *MDR1* and worse clinicopathological findings. For preoperative PSA, methylation frequency was higher in PSA ≥ 10 than in <10 ng/ml (47 and 66%, respectively). Cap, capsular invasion; SV, seminal vesicle involvement; pN, lymph node invasion; ly, lymphatic vessel invasion; and PNI, perineural invasion. N and P correspond to negative and positive, respectively.

Table 3 *Logistic regression analysis of MDR1 methylation in the series of PC and BPH*

	Coefficient	SE	χ^2	*P**	Hazard ratio	95% confidence interval
Variables						
Age	−0.017	0.02	0.749	0.387	0.983	0.946–1.022
Pathology†	2.224	0.405	30.126	<.0001	9.245	4.178–20.459
Age	0.037	0.025	2.280	0.131	1.038	0.989–1.089
Pathology	2.439	0.437	31.125	<0.001	11.463	4.865–27.008

* Prostate cancer *versus* BPH.
† χ^2 test.

and stromal cells such as fibroblasts and endothelial cells. The contamination of normal unmethylated cell populations could provide every CpG site with the same or very close percentage of unmethylation peaks. In the number 8 prostate cancer sample (Fig. 3*C*), where every CpG site was similar partial methylation, the coexisting USP band could represent contamination with nontumor cells. However, we used 10 genomic DNA samples from peripheral blood lymphocytes (as control), and there were no MSP bands (Fig. 3*D*). This result indicated that the methylation frequency was not affected by contamination of normal cells. Therefore, CpG hypermethylation of the *MDR1* promoter has a strong impact on gene transcription and is one of the mechanisms underlying down-regulation of P-gp expression in human prostate cancer tissues. In addition to higher frequencies of positive MSP in prostate cancer samples than in BPH samples (54.8 *versus* 11.6%), logistic regression analysis also demonstrated that prostate cancer samples were 11.5 times more likely to have methylated alleles of *MDR1* promoter than BPH samples (Table 3). The CpG hypermethylation of the glutathione S-transferase π gene (*GSTP1*) has been present in BPH (25). Similar epigenetic alteration is plausible to occur in the MDR1 promoter as well as in our series of BPH samples. In colon mucosa, two types of methylation have been postulated: age-related methylation (type A) and cancer-related methylation (type C; ref. 26). Although both prostate cancer and BPH are age-dependent disease, our results strongly suggest that CpG hypermethylation of *MDR1* promoter in human prostate is cancer-related methylation (type C) and independent of age.

Several earlier studies have explored whether aberrant expression of the *MDR1* gene might be a biomarker for the etiology and progression of various cancers (12, 13, 27, 28). Some investigators reported that overexpression of the *MDR1* gene can be an active biomarker for tumor progression in breast and colon cancers (27, 28). Other investigators reported that loss of the *MDR1* gene is associated with advanced or metastatic stage in hepatocellular carcinoma and osteosarcoma (12, 13). In prostate cancer, Yegnasubramanian *et al.* (29) demonstrated a high frequency of *MDR1* promoter hypermethylation in prostate cancer patients. In the present study, the CpG hypermethylation of *MDR1* promoter showed significant correlation with worse clinicopathological features such as higher pT and pN categories, higher GS, capsular extension, involvements of seminal vesicle, veins and lymphatic vessels, and higher preoperative PSA values. Considering that promoter CpG hypermethylation is a likely mechanism for inactivation of *MDR1* gene transcription in human prostate cancer and down-regulation of P-gp expression, *MDR1* methylation might characterize biological aggressiveness of prostate cancer. The significance of allelic methylation is not known. In this regard, recent study has shown that monoallelic methylation of p27 promoter was associated with transcriptional silencing in melanoma (30). In this study, we do not know whether promoter methylation occurred because of biallelic or monoallelic event. Additional studies are warranted to investigate the role of allelic methylation in prostate cancer.

The balance between proliferation and apoptosis of prostate cancer cells determines the net growth of prostate cancer. Active involvement of *MDR1* gene in cell proliferation has been hypothesized based on the following evidence: (*a*) an inverse correlation between Ki67 and *MDR1* expression in advanced prostate cancer (15); (*b*) high expression of ki-67 and PCNA in *mdr1a* double-knockout mice during experimental hepatocarcinogenesis (31); and (*c*) positive correlation between up-regulation of *MDR1* and enhancement in human melanoma metastasis suppressor (*KiSS-1*) activity (32). On the other hand, recent studies have shown that P-gp is associated with an apoptotic pathway *in vitro* (33–35). In the present study, we analyzed proliferation activity and apoptosis using PCNA and single-strand DNA immunostaining. In our series, the PCNA-labeling index was significantly higher in MSP-positive samples compared with negative sample (Fig. 6*A*), whereas there was no correlation of *MDR1* methylation with apoptotic index (Fig. 6*B*). These findings suggest that CpG hypermethylation of *MDR1* promoter might be involved in prostate cancer disease progression via increased cellular proliferation but less likely via an inhibition of apoptosis.

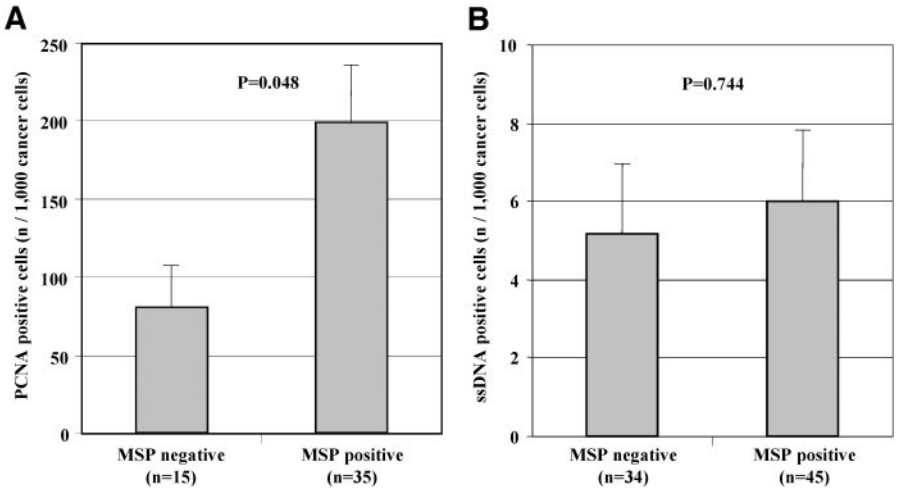

Fig. 6. Correlation of immunohistochemistry markers with methylation status of *MDR1* promoter. In the series of PC samples, *A*. The mean number of PCNA-positive cells was significantly higher for MSP-positive samples than that for MSP-negative samples (199 *versus* 81; $P = 0.048$). *B*, there was no significant difference in the mean number of single-strand DNA-positive cells between MSP-positive and -negative samples (6.0 *versus* 5.2; $P = 0.744$).

Prostate cancer patients with either Gleason scores of >8 at biopsy, PSA level of >20 ng/ml, or clinical stage of greater than T2 are considered at a higher risk of PSA failure 5 years after definite local therapy (36). In addition, no significant survival benefits have been demonstrated in prostate cancer patients who have undergone neoadjuvant hormonal therapy before radical prostatectomy (37). On the basis of these studies in locally advanced prostate cancer, both local therapy and neoadjuvant hormonal therapy are not sufficient to control and eliminate the prostate cancer cells. Recently, Oh *et al.* (3) reported that neoadjuvant chemotherapy microscopically conferred cytotoxic effects on prostate cancer cells in patients who later underwent radical prostatectomy, although each follow-up period was too short to draw any definite survival benefits. Most importantly, it is P-gp substrates such as doxorubicin, *Vinca* alkaloid, or taxanes that confer cytotoxic effects on prostate cancer cells as chemotherapeutic agents. In turn, these chemotherapeutic agents might be effective for treating high-risk prostate cancer, where P-gp is frequently downregulated through CpG hypermethylation of the *MDR1* promoter. Our results suggest that the clinical application of neoadjuvant chemotherapy using P-gp substrates in those prostate cancer patients at higher risk may be beneficial.

In conclusion, CpG hypermethylation of *MDR1* promoter is associated with disease progression in prostate cancer via increased cell proliferation activity.

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