blood plasma, urine, or ejaculate from patients with prostate cancer (7–10). Based on prior studies, it is possible that multigene methylation profiles can provide better diagnostic or prognostic values for prostate cancer (4–12). However, such studies are lacking in the literature. In the present study, we investigated whether multigene methylation analysis can be a good diagnostic and staging biomarker for prostate cancer.

The ability to predict the outcome or pathologic stage of prostate cancer is a worthwhile goal which will enable physicians to make treatment recommendations for patients with prostate cancer. Nomograms, using a combination of three variables (PSA, biopsy Gleason score, and clinical stage) are currently distributed and used clinically (13, 14). However, the utility of multigene methylation analysis as a pretreatment staging biomarker has not been reported.

We have previously shown that the methylation status of *GSTP1* or *MDR1* is a good biomarker for detecting prostate cancer and correlates with clinicopathologic features (15, 16). We hypothesize that multigene methylation analysis can be a good diagnostic and staging biomarker prior to treatment. To this end, we did methylation analysis of the *APC* gene and combined the results with the *GSTP1* and *MDR1* data from the same 170 prostate cancer and 69 benign prostatic hyperplasia patients. We used the data to calculate a methylation score (M score) that is the sum of the log of the hazard ratios (HR) for each gene, analyzed by multivariate logistic regression analysis for pathology (benign prostatic hyperplasia versus prostate cancer). We also related the M score to clinical and pathologic outcome. Using receiver operator characteristic (ROC) curve analysis to determine the optimal cutoff value for the M score, we evaluated the sensitivity and specificity of M score as a staging biomarker compared with PSA or Gleason sum. In addition, we assessed PSA failure-free probability against the clinicopathologic features of prostate cancer.

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

| | |
|---|---|
| Prostate cancer | |
| Total number | 170 |
| Median age, y (range) | 68.6 (49-80)* |
| pT category | |
| $pT_1$ | 0 |
| $pT_2$ | 111 |
| $pT_3$ | 55 |
| $pT_4$ | 4 |
| Gleason sum | |
| <7 | 88 |
| 7 | 50 |
| >7 | 32 |
| Preoperative serum PSA | |
| <4.0 | 22 |
| 4.0-9.9 | 82 |
| >10.0 | 66 |
| Benign prostate hypertrophy | |
| Total number | 69 |
| Median age, y (range) | 75 (54-87)* |

*The median age of patients with benign prostate hypertrophy is statistically higher than those with prostate cancer ($P < 0.001$).

## Materials and Methods

*Tissue samples.* A total of 170 newly diagnosed prostate cancer tissues from radical prostatectomies and 69 pathologically proven benign prostatic hyperplasia samples from transurethral resection were obtained from Shimane University Hospital (Izumo, Japan) from 1997 through 2003. Our routine diagnostic strategy for prostate cancer included serum PSA level, transrectal ultrasonography, color Doppler ultrasonography, and magnetic resonance imaging, which enabled us to detect the localization of prostate cancer before radical prostatectomy (17). The patients' background and clinicopathologic characteristics are summarized in Table 1. Each tumor was graded and staged according to the Gleason grading system (18) and the tumor-node-metastasis staging system (19). None of these patients had received androgen deprivation therapy. We used serum PSA levels after radical prostatectomy as a surrogate end-point, with a level ≥0.2 ng/mL designated as PSA failure. Forty-six patients with prostate cancer were excluded from the PSA failure-free probability study because of adjuvant hormonal therapy immediately after radical prostatectomy. PSA failure-free probability was determined as the percentage of patients without PSA failure. Follow-up ranged from 0.7 to 91.4 months, with a median of 33.9 months. Written informed consent was obtained from all patients.

*Tissue preparations.* All of the benign prostatic hyperplasia and prostate cancer samples were 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 benign prostatic hyperplasia samples, the presence of high-grade prostate intraepithelial neoplasia and cancer were ruled out by microscopic analysis.

*Nucleic acid extraction.* Genomic DNA from all prostate samples was extracted using a commercial kit (Qiagen, Valencia, CA), and precipitated with ethanol. The concentration of DNA was determined with a spectrophotometer, and its integrity was checked by gel electrophoresis.

*Methylation analysis.* Genomic DNA from all prostate samples (100 ng) was subjected to sodium bisulfite modification using a CpGenome DNA Modification Kit (Intergen Co., Purchase, NY). Based on the functional promoter sequence of *APC* (21), methylation-specific PCR (MSP) and unmethylation-specific PCR (USP) primers were designed using MethPrimer software (http://www.urogene.org/methprimer) developed in our laboratory (22). Primers used for MSP and USP analysis are as follows: universal primers, 5′-TAATTTTTTTGTTTG-TTGGGGATT-3′ (sense), 5′-ACTACACCAATACAACCACATATC-3′ (antisense); MSP primers, 5′-TATTGCGGAGTGCGGGTC-3′ (sense), 5′-TCGACGAACTCCCGACGA-3′ (antisense); USP primers, 5′-GTGT-TTTATTGTGGAGTGTGGGTT-3′ (sense), 5′-CCAATCAACAAAC-TCCCAACAA-3′ (antisense). An initial PCR product was created with universal primers, which have no CpG sites in either forward or reverse primers, followed by a second nested PCR with primers specific for MSP or USP. For semiquantitative analysis, a preliminary suitable number of PCR cycles for each MSP and USP were carried out in order to determine the linear range of the reaction. To ensure this, at least one initial PCR was done using 32 cycles each for MSP and USP. Then, a suitable PCR cycle was chosen for each sample. The annealing temperature and PCR cycles used for MSP and USP primers were 64°C and 32 cycles, respectively. The sequences of primers for *GSTP1* and *MDR1*, as well as their PCR conditions, were described previously (15, 16). In each assay, the absence of DNA template served as negative controls. The obtained MSP and USP products were analyzed by electrophoresis in 3% agarose gels and stained with ethidium bromide. With ImageJ software (http://rsb.info.nih.gov/ij), relative methylation levels (%) were calculated (15, 16, 23) by using the area under the curve corresponding to each band (MSP and USP). For methylation analysis of APC, we used 5.3% methylation as a cutoff value, which was the

**Table 2.** Multivariate logistic regression analysis of gene methylation in a series of prostate cancer and benign prostatic hyperplasias

| Variable | Log HR* | SE | $\chi^2$ | *P* | HR* | 95% CI |
|---|---|---|---|---|---|---|
| *APC* | 2.028 | 0.521 | 15.125 | <0.001 | 7.597 | 2.734-21.112 |
| *GSTP1* | 1.777 | 0.637 | 7.774 | 0.005 | 5.913 | 1.695-20.627 |
| *MDR1* | 1.178 | 0.505 | 5.450 | 0.020 | 3.247 | 1.208-8.729 |

NOTE: All data are adjusted by age.
Abbreviations: HR, hazard ratio; CI, confidence interval.
*M score is determined as the sum of log HR for each sample.

average percentage of methylation in 69 benign prostatic hyperplasia samples. Using these criteria, MSP positivity for APC was defined as those prostate cancers with a percentage of methylation of >5.3%, and negative methylation was <5.3%. The criteria for MSP positivity of *GSTP1* and *MDR1* were described previously (15, 16).

***Bisulfite DNA sequencing analysis.*** Bisulfite-modified DNA (1 μL) was amplified using a pair of universal primers in a total volume of 20 μL. Direct bisulfite DNA sequencing of the PCR products using either forward universal primer or reverse universal primer was done according to the manufacturer's instructions (Applied BioSystems, Foster City, CA).

***Statistical analysis.*** Using a previously reported analytic technique (24, 25), we calculated the M score for each sample, defined as the sum of the corresponding log HR coefficients, which were derived from multivariate logistic regression analysis of each methylated gene in the benign prostatic hyperplasia and prostate cancer samples (Table 2). The optimal sensitivity and specificity of the M score for diagnosis of prostate cancer and for staging was determined by ROC curve analysis using MedCalc Software (Mariakerke, Belgium). A pairwise comparison was employed to test for significance using the area under the ROC curve (AUC) analysis. 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. The relationship between M score and clinicopathologic findings was blindly analyzed by either the Mann-Whitney *U* test, Kruskal-Wallis test, or the Spearman rank correlation test using StatView software (SAS Institute Inc., Cary, NC). A *P* value of <0.05 was regarded as statistically significant.

## Results

***Methylation status of the APC promoter in prostate clinical samples.*** Representative results of MSP and USP assays for *APC* in prostate cancers and benign prostatic hyperplasias are shown in Fig. 1A. MSP-positive bands were present in the majority of prostate cancers, and less so in the benign prostatic hyperplasia samples. USP-positive bands were present in all of prostate cancers and benign prostatic hyperplasias. The result of the methylation study was also confirmed by bisulfite DNA sequencing. Figure 1B shows the results of a typical bisulfite DNA sequencing in a prostate cancer sample. In sample "P" (corresponding to Fig. 1A, lane "P") with both MSP and USP

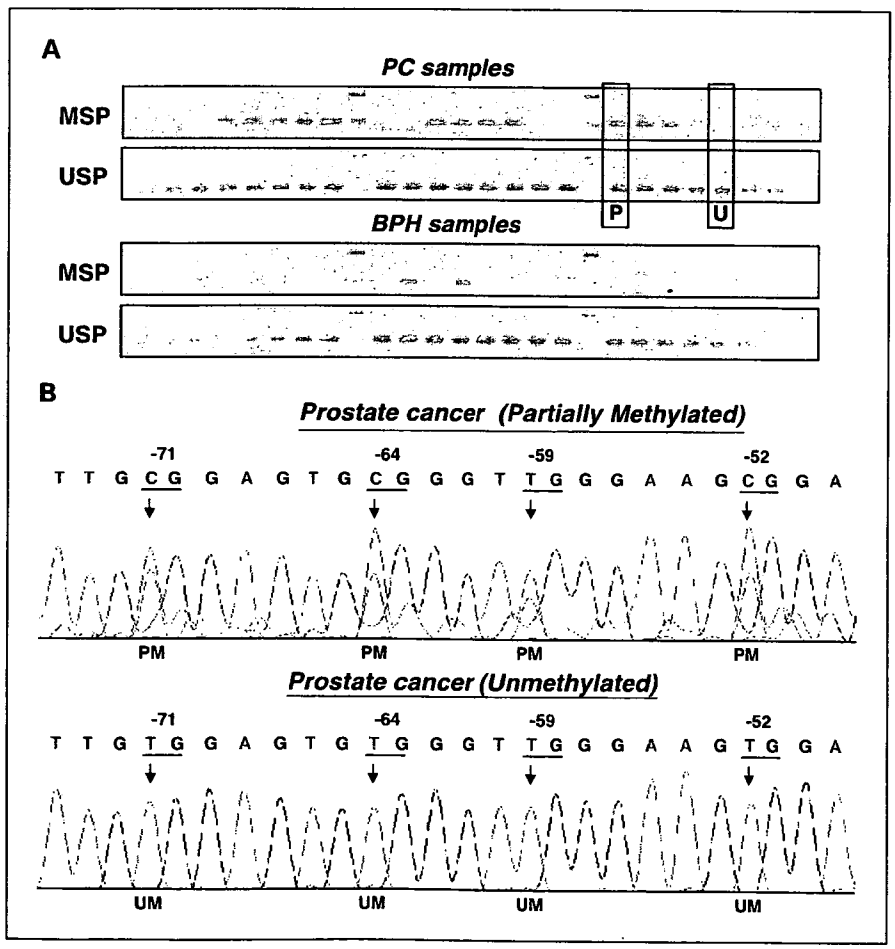


**Fig. 1.** Methylation status of the *APC* promoter in clinical samples. *A,* MSP and USP bands of 24 samples for benign prostatic hyperplasia and prostate cancer are shown. P and U, samples depicting partial methylation and unmethylation, respectively. *B,* bisulfite DNA sequencing of partially methylated (*top*), unmethylated (*bottom*) samples corresponding to lanes P and U, respectively. In partially methylated samples, there was a "T" peak along with a "C" peak at the CpG sites. In unmethylated samples, every CpG site was unmethylated. PM and UM, partial methylation and unmethylation, respectively.

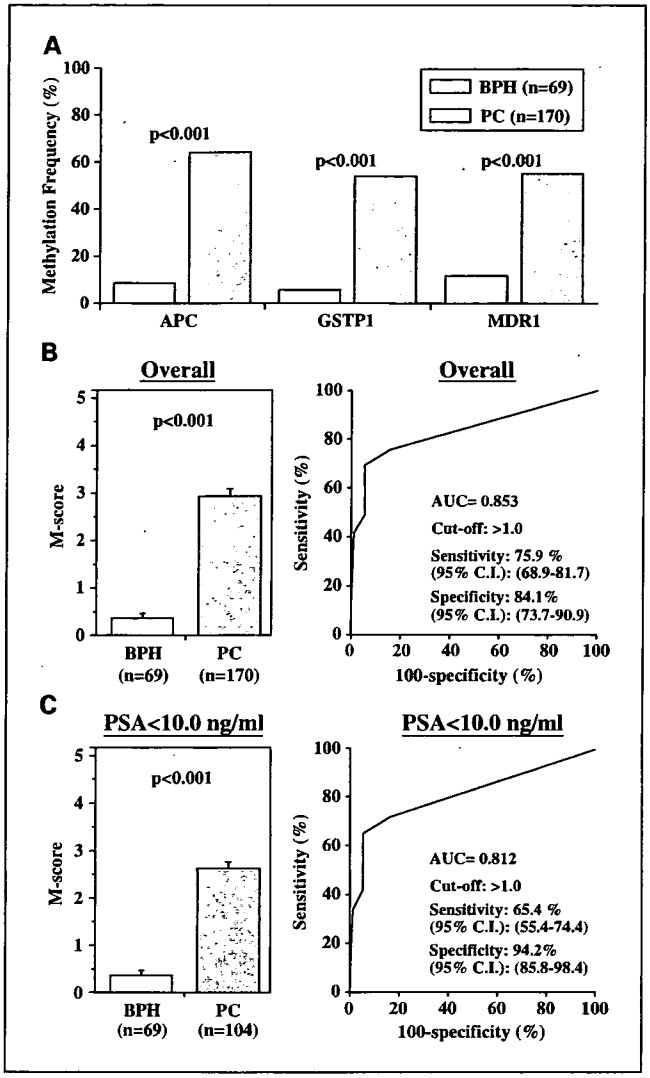


Fig. 2. Correlation of gene methylation frequency with pathology (benign prostatic hyperplasia versus prostate cancer) and methylation score (M score) as a diagnostic biomarker. *A*, the methylation frequency in prostate cancer samples was significantly higher than that in benign prostatic hyperplasia samples for *APC*, *GSTP1*, and *MDR1* genes (64.1% versus 8.7%, 54.0% versus 5.8%, and 55.3% versus 11.6%, respectively). *B*, M score, determined as the sum of the corresponding log HR for pathology (benign prostatic hyperplasia versus prostate cancer; Table 2), was also significantly higher in prostate cancer samples than in benign prostatic hyperplasia samples (*left*). *B*, ROC curve analysis determined that a cutoff value of 1.0 had optimal sensitivity (75.9%) and specificity (84.1%) for M score to distinguish prostate cancer from benign prostatic hyperplasia (*right*). *C*, M score was also significantly higher in prostate cancer samples with low or borderline PSA levels (<10.0 ng/mL) than in benign prostatic hyperplasia samples (*left*); in patients with low PSA levels the M score had high sensitivity (65.4%) and specificity (94.2%) for prostate cancer detection when 1.0 was used as a cutoff value (*right*).

bands, there was a "T" peak along with a "C" peak at the CpG sites, indicating partial methylation (Fig. 1B, top). In sample "U" (corresponding to Fig. 1A, lane "U"), where no MSP band was observed, the CpG sites were completely unmethylated (Fig. 1B, bottom).

***Evaluation of M score: multigene methylation analysis with APC, GSTP1, and MDR1 for distinguishing prostate cancer from benign prostatic hyperplasia.*** *APC* methylation analysis showed positive MSP bands in 109 of 170 (64.1%) prostate cancers and 6 of 69 (8.7%) benign prostatic hyperplasias (Fig. 2A). As we have reported previously, positive MSP bands for *GSTP1* methylation analysis were solely found in 92 of 170 (54.0%) prostate cancers, and in 4 of 69 (5.8%) benign prostatic hyperplasias, whereas that for *MDR1* methylation analysis were found in 94 of 170 (55.3%) prostate cancers, and in 8 of 69 (11.6%) benign prostatic hyperplasias (15, 16). There was a significant difference in the methylation status of each gene between the series of prostate cancer and benign prostatic hyperplasias (Fig. 2A). As shown in Table 2, multivariate logistic regression analysis revealed that *APC*, *GSTP1*, and *MDR1* methylation was a significant dependent predictor of pathology (benign prostatic hyperplasia versus prostate cancer; $P < 0.001$, $P = 0.005$, and $P = 0.020$, respectively). The individual gene HRs for pathogenesis (prostate cancer versus benign prostatic hyperplasia) were different from one another. For instance, cases with *APC* methylation are 7.597 times more likely to have prostate cancer than cases with negative methylation, whereas the HR for *MDR1* is 3.247. For all patients, the M score determined by the sum of log HR was significantly higher in prostate cancers than in benign prostatic hyperplasias (2.913 ± 0.158 and 0.357 ± 0.121, respectively; Fig. 2B, left). The optimal cutoff value of the M score for distinguishing prostate cancer from benign prostatic hyperplasia was determined using the ROC curve. The M score had a sensitivity of 75.9% and a specificity of 84.1% when 1.0 was used as a cutoff value (Fig. 2B, right). In patients with low or borderline PSA levels (<10.0 ng/mL), the M score was still significantly higher in prostate cancers than in benign prostatic hyperplasias (2.635 ± 0.200 and 0.357 ± 0.121, respectively; Fig. 2C, left). ROC curve analysis revealed that the M score had a sensitivity of 65.4% and a specificity of 94.2% when 1.0 was used as a cutoff value (Fig. 2C, right). All statistical values were age-adjusted because the mean ages were statistically different between benign prostatic hyperplasias and prostate cancers (Table 1).

***Correlation of M score with clinicopathologic findings.*** Among prostate cancers, the M score showed a significant stepwise increase with advancing pathologic stage (1.34 ± 0.26 in $pT_{2a}$, 2.92 ± 0.24 in $pT_{2b}$, 3.84 ± 0.26 in $pT_{3a}$, 4.21 ± 0.62 in $pT_{3b}$, and 4.98 ± 0 in $pT_4$; $P < 0.001$; Fig. 3A). Similarly, the M score increased as the Gleason sum increased (2.20 ± 0.23, Gleason sum <7; 3.58 ± 0.25, Gleason sum = 7; and 3.819 ± 0.29, Gleason sum >7; $P < 0.001$; Fig. 3B). With regard to preoperative PSA levels, the M score was higher in PSA >10 ng/mL (3.35 ± 0.25) than in PSA ≤10 ng/mL (2.64 ± 0.20; $P = 0.027$; Fig. 3C). Moreover, the M score was higher in advancing pathologic features as follows: in capsular invasion (Cap) [positive (3.80 ± 0.23) versus negative (2.47 ± 0.19; $P < 0.001$)], in seminal vesicle involvement [positive (4.88 ± 0.11) versus negative (2.80 ± 0.16; $P = 0.002$)], in pelvic lymph node metastasis (pN) [positive (4.60 ± 0.38) versus negative (2.57 ± 0.19; $P = 0.001$)], in venous involvement (v) [positive (3.92 ± 0.22) versus negative (2.44 ± 0.19; $P < 0.001$)], in lymphatic vessel involvement (Ly) [positive (3.77 ± 0.28) versus negative (2.61 ± 0.18; $P = 0.001$)], and in perineural invasion (PNI) [positive (3.41 ± 0.18) versus negative (1.86 ± 0.28; $P < 0.001$)] (Fig. 3C). We also observed that the M score was age-related in the total group of benign prostatic hyperplasias ($P < 0.001$) but not in the total group of prostate cancers ($P = 0.108$).

***Prognostic features.*** We analyzed PSA failure-free probability as disease-free survival. Of the clinicopathologic features considered, only Gleason sum was significantly associated with poor outcome in univariable analyses ($P = 0.022$; Fig. 4.).

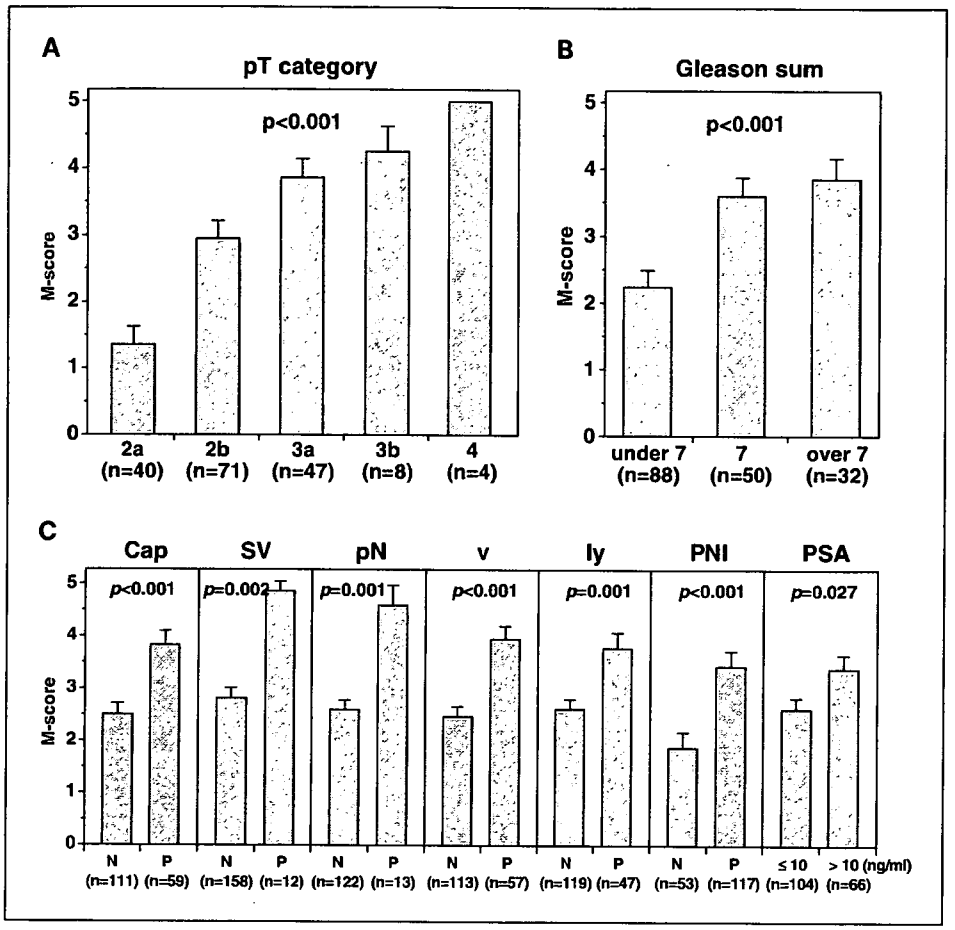


Fig. 3. Correlation of M score with clinicopathologic features. Among the overall prostate cancer patients: *A*, higher stage of disease (1.34 ± 0.26 in $pT_{2a}$, 2.92 ± 0.24 in $pT_{2b}$, 3.84 ± 0.26 in $pT_{3a}$, 4.21 ± 0.62 in $pT_{3b}$, and 4.98 ± 0 in $pT_4$). *B*, higher Gleason sum (2.20 ± 0.23, Gleason sum < 7; 3.58 ± 0.25, Gleason sum = 7; and 3.819 ± 0.29, Gleason sum >7). *C*, higher preoperative PSA levels (3.35 ± 0.25 in PSA >10 ng/mL, 2.64 ± 0.20 in PSA ≤10 ng/mL) correlated with an increased methylation score (M score). There was significant correlation between M score and worse clinicopathologic findings. Cap, capsular invasion; SV, seminal vesicle involvement; pN, lymph node invasion; ly, lymphatic vessel invasion; and PNI, perineural invasion; N and P, negative and positive, respectively.

***Evaluation of M score as a predictive biomarker for preoperative staging.*** To test the ability of the M score as a staging biomarker to distinguish between organ-confined ($\leq pT_2$) and locally advanced cancer ($\geq pT_3$), the optimal cutoff value of M score, PSA, and Gleason sum was determined using ROC curve. As shown in Fig. 5A, for all patients with prostate cancer, the M score had a sensitivity of 72.1% and a specificity of 67.8% when 4.0 is employed as a cutoff value. PSA had 69.4% and 69.5%, respectively, with a cutoff value of 9.0 ng/mL; Gleason score had 60.0% and 65.5%, respectively, with a cutoff value of 7. The corresponding AUC for each was 0.721, 0.724, and 0.687, respectively. Pairwise analysis for AUC showed no statistical difference among these three markers. Looking at the patients with PSA levels of 10 ng/mL or less, the M score had a sensitivity of 67.1% and a specificity of 85.7% with 3.3 as a cutoff value. PSA had 75.6% and 38.1%, respectively, with a cutoff value of 7.7 ng/mL; Gleason score had 66.7% and 57.1%, respectively, with a cutoff value of 7. The corresponding AUC for each was 0.780, 0.550, and 0.663, respectively. There was a significant difference between M score and PSA ($P$ = 0.010; Fig. 5B).

## Discussion

We have reported previously that the methylation status of the *GSTP1* or *MDR1* gene promoter correlate with clinicopathologic features (15, 16). In this study, we found that the M score for *APC*, *GSTP1*, and *MDR-1* genes can be used as a diagnostic biomarker for prostate cancer. This is the first study to integrate the methylation status of multigenes using the M score, which is the sum of the log HR analyzed by multivariate logistic regression analysis for pathology (benign prostatic hyperplasia versus prostate cancer). This analysis provides automatically adjusted statistical data (24), with each HR directly related to gene methylation in prostate cancer samples as compared with benign prostatic hyperplasia (methylation-negative) samples (Table 2). By adding the log HR of each gene in a multigene analysis, it is therefore possible to predict the risk of prostate cancer in individual patients. Similarly, Ray et al. employed multivariate Cox proportional hazards models for their multigene methylation analysis in medulloblastoma, and used the sum of the log HR as a risk score for each patient (25).

PSA is the most sensitive diagnostic biomarker for prostate cancer detection thus far. However, its low specificity has forced unnecessary biopsy of patients in order to exclude prostate cancer. Using various kinds of PSA analysis such as free-PSA, complexed-PSA, or total-PSA, and its combinations—% free-PSA or % complexed-PSA, many investigators have struggled to find better methods for prostate cancer detection (2, 3). However, the specificity of these tests is ~60% at best with 80% sensitivity (2, 3). To make matters worse, in patients with low or borderline PSA levels, any PSA analysis is a poor diagnostic tool for prostate cancer detection because of much lower specificity (2, 3). In the current study, there was

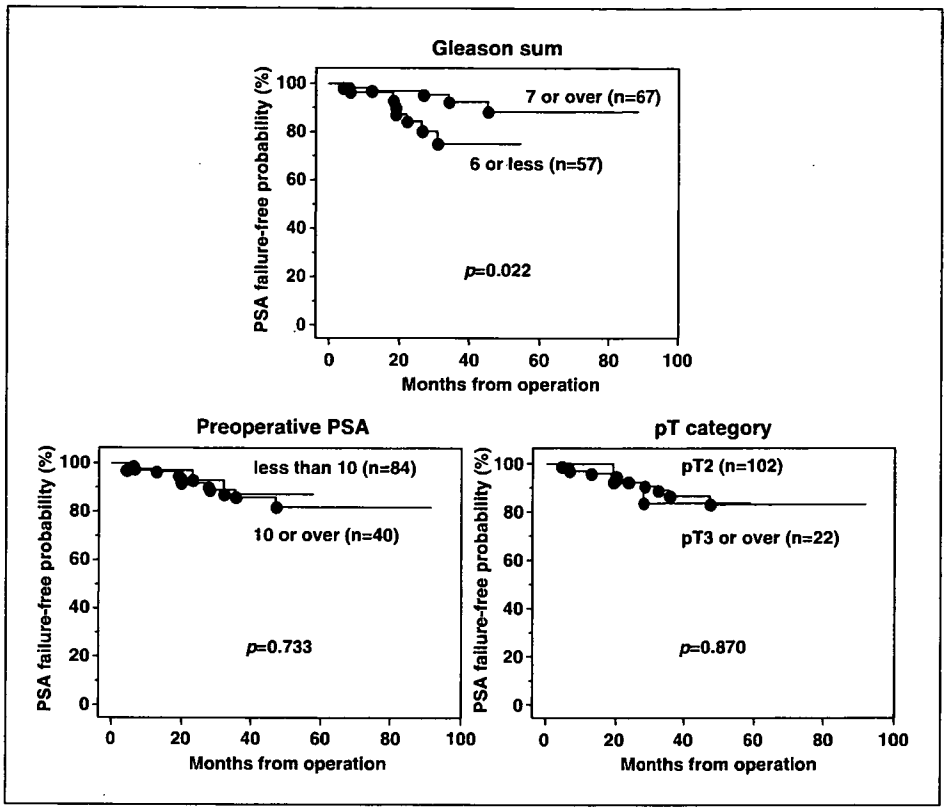


Fig. 4. Kaplan-Meier PSA failure-free survival curves of prostate cancer patients after radical prostatectomy, grouped according to the evaluated variables: Gleason sum, preoperative PSA, and pT category. Follow-up ranged from 0.7 to 91.4 months, with a median of 33.9 months. ●, censored data points.

significant difference in M score between benign prostatic hyperplasia and prostate cancer (Fig. 2B, left). For prostate cancer detection, using a cutoff value of 1.0, the M score had a 75.9% sensitivity and 84.1% specificity, which is much higher compared with that reported with PSA (Fig. 2B, right). Moreover, in patients with low or borderline PSA levels (<10.0 ng/mL), the M score had high sensitivity of 65.4% and specificity of 94.2% for prostate cancer detection when 1.0 was used as a cutoff value (Fig. 2C, right). Thus, the M score can be a very useful and improved diagnostic biomarker for prostate cancer detection, even in patients with low or borderline PSA levels. Several investigators have already shown that *GSTP1* hypermethylation can be readily detected in bodily fluids such as blood plasma, urine, or ejaculate from patients

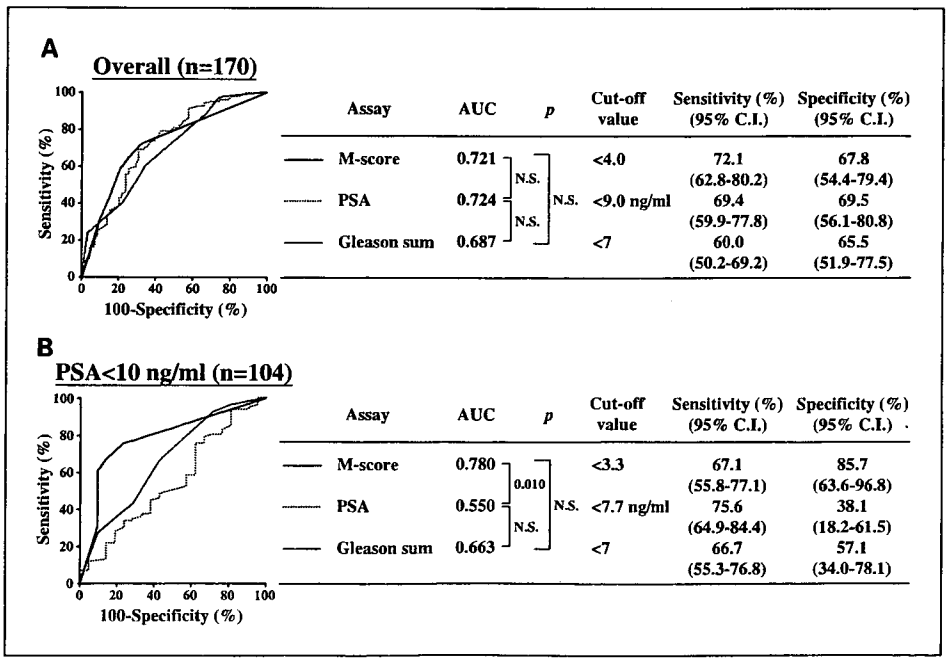


Fig. 5. Optimum sensitivity and specificity of M score, PSA, and Gleason sum for predicting pathologic stage. *A*, for all patients, ROC curve analysis was used to determine a cutoff value that had the optimum sensitivity and specificity for each variable to distinguish organ-confined ($\leq pT_2$) from locally advanced cancer ($\geq pT_3$). Pairwise comparison test showed no significant difference among M score, PSA, and Gleason sum. *B*, in patients with PSA levels of 10 ng/mL or less, there was a significant difference between M score and PSA. AUC, area under ROC curve; CI, confidence interval.

with prostate cancer (7–10). Taken together, these findings indicate that the M score is applicable for use as a diagnostic biomarker. Therefore, by using both the M score and PSA for prostate cancer screening, it may be possible to reduce the number of unnecessary biopsies, however, additional study is warranted to verify this hypothesis.

Predicting the probability of the final pathologic stage is a worthwhile goal so that physicians can make appropriate treatment recommendations for patients with prostate cancer. Nomograms using a combination of three variables (serum PSA, biopsy Gleason score and clinical stage) are already distributed and used clinically (13, 14) and have been verified in two studies (26, 27). Using ROC curve analysis, they analyzed staging probability based on the nomograms; however, the AUC reported in the two studies differ (0.787 versus 0.684). One reason for the discrepancy may be related to the fact that the clinical (tumor-node-metastasis) stage was subjectively determined by different individuals, thereby introducing a bias in the results. It would be best if pathologic staging were predicted without subjective variables. Interestingly, in this study, the M score showed significant correlation with worse clinicopathologic features such as higher pT and pN categories, higher Gleason sum, capsular extension, involvement of seminal vesicles, veins and lymphatic vessels, and higher preoperative PSA values (Fig. 3). Maruyama et al. also showed that there was significant correlation between their method for multigene methylation analysis and clinicopathologic features (12). However, in their study, the correlation with pathologic stage was less significant ($P = 0.04$) compared with our results ($P < 0.001$; Fig. 3) and no cutoff value was used in their analysis. Using ROC curve analysis, we were able to determine a cutoff value for the M score as a staging biomarker, which enabled us to distinguish organ-confined prostate cancer ($\leq pT_2$) from locally advanced prostate cancer ($\geq pT_3$). As shown in Fig. 5A, the M score has the sensitivity and specificity to serve as a good predictive staging biomarker like PSA or Gleason sum. Moreover, in patients with PSA levels of 10 ng/mL or less (Fig. 5B), the M score showed a sensitivity of 67.1% and a high specificity of 85.7% compared with corresponding PSA values of 38.1%. ROC curve analysis showed a significant difference between M score and PSA in this category. Our data also indicates that the M score can be a useful biomarker not only in distinguishing prostate cancer from benign prostatic hyperplasia, but also for predicting the final pathologic stage before medical treatment. Among patients with PSA levels of 10 ng/mL or less, the M score could predict the final pathologic stage more precisely than other biomarkers (Fig. 5B). However, a prospective study will be necessary to confirm this idea. We also analyzed PSA failure-free probability as disease-free survival. Of the clinicopathologic features considered, only Gleason sum was significantly associated with poor outcome (Fig. 4). Currently, there are no other reports demonstrating a significant correlation between multigene methylation analysis and PSA failure-free probability.

In conclusion, this is the first study to integrate the methylation status of multigenes using the M score which reflects the comprehensive methylation status of prostate tissues and is useful as a biomarker for detection and staging of prostate cancer. To elucidate the practical effect of M score in predicting prostate cancer outcomes, it will be necessary to include more genes from prostate tissue biopsy and body fluid samples in the future.

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# Smoking Influences Aberrant CpG Hypermethylation of Multiple Genes in Human Prostate Carcinoma

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**BACKGROUND.** Aberrant CpG methylation profiles of gene promoters and their correlation with advanced pathologic features have been well investigated in prostate carcinoma (PC). Several case–control and prospective studies have revealed a positive association between current smoking and PC. The authors hypothesized that smoking influences both progression and prognosis of PC through CpG hypermethylation of related genes.

**METHODS.** A total of 164 PC patients (52 current, 30 former, and 82 never smokers) and 69 benign prostatic hyperplasia (BPH) patients were examined by methylation-specific PCR (MSP) for 3 genes: adenomatous polyposis coli (*APC*), glutathione S-transferase pi (*GSTP1*), and multidrug resistance one (*MDR1*). The methylation status of representative samples was confirmed by bisulfite DNA sequencing analysis. The newly defined methylation score (M-score) of each sample is the sum of the corresponding log hazard ratio (HR) coefficients derived from multivariate logistic regression analysis for pathology (BPH vs. PC), and was related to clinical and pathologic outcome including smoking status.

**RESULTS.** The M-score was significantly higher in the current smokers than in never smokers ($P = 0.008$). Spearman rank correlation test demonstrated a significant correlation between pack-years smoked and M-score in PCs ($P = 0.039$). Significant correlation of the M-score methylation was observed with high pT category ($P < 0.001$), high Gleason sum ($P < 0.001$), high preoperative prostate-specific antigen (PSA) ($P = 0.041$), and advanced pathologic features. In addition, Gleason sum was significantly associated with PSA failure-free probability as a poor outcome ($P = 0.020$).

**CONCLUSIONS.** This is the first study to demonstrate significant correlation of the methylation status of multigenes with smoking status in PC. Smoking status may influence both progression and prognosis of PC through CpG hypermethylation of related genes. ***Cancer* 2006;106:79–86.** © *2005 American Cancer Society.*

**KEYWORDS: smoking, methylation, *APC, GSTP1, MDR1*, human prostate carcinoma, prostate cancer.**

Prostate carcinoma (PC) is one of the most common malignancies among men.[1] Smoking is strongly associated with cancers of the head and neck, esophagus, lung, and urinary bladder.[2] Several potential mechanisms whereby smoking may increase risk of PC involve male hormones or cadmium.[3–5] Aberrant methylation profiles of gene promoters and their correlation with advanced pathologic features have been well investigated in PC.[6–8] In lung cancers, some investigators have previously reported that there were significant correlations between aberrant promoter methylation of genes and smoking status of patients.[9–12] However, such studies in PC are lacking in the literature.

Several case–control studies have revealed a positive association


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between current smoking and PC.[3,13–18] Previous reports also have demonstrated an association between smoking and advanced PC.[3,17–19] Furthermore, the majority of prospective studies that used PC death as an outcome noted positive association between current smoking and PC.[4] Recent studies from our laboratory have shown that methylation analysis of the *APC, GSTP1, and MDR1* genes correlate with progression of prostate carcinoma.[20,21] When several genes are analyzed in the same samples, careful interpretation of the results is necessary because each gene or other clinical factors, including age, may influence one another.[22,23] We hypothesize that smoking influences both progression and prognosis of PC through CpG hypermethylation of related genes. To test this hypothesis, we used a methylation score (M-score) that is the sum of the log of the hazard ratios (HR) for each gene, analyzed by multivariate logistic regression analysis for pathology (benign prostatic hyperplasia [BPH] vs. PC). We also related the M-score to clinical and pathologic outcome, including smoking status. In addition, we assessed prostate specific antigen (PSA) failure-free probability against the clinicopathologic features of PC.

TABLE 1
**Clinicopathologic Characteristics of Prostate Carcinoma according to Smoking Status**

| | | Smoking | | | |
|---|---|---|---|---|---|
| | Overall | Current smokers | Former smokers | Never smokers | *P* value |
| Total no. | 164 | 52 | 30 | 82 | 0.154 |
| Median age (range) | 69 (49–87) | 68 (49–80) | 71 (62–78) | 69 (51–80) | 0.154 |
| Smoke exposure | | | | | |
| Pack-yrs smoked[a] | 40 ± 26 | 42 ± 24 | 37 ± 30 | — | |
| Yrs smoked | 40 ± 11 | 44 ± 8 | 34 ± 13 | — | |
| Starting age | 23 ± 8 | 23 ± 7 | 23 ± 10 | — | |
| pT category | | | | | |
| pT1 | 0 | 0 | 0 | 0 | 0.590 |
| pT2 | 108 | 31 | 21 | 56 | |
| pT3 | 52 | 20 | 9 | 23 | |
| pT4 | 4 | 1 | 0 | 3 | |
| Gleason sum | | | | | |
| < 7 | 82 | 23 | 19 | 40 | 0.495 |
| 7 | 53 | 20 | 7 | 26 | |
| > 7 | 29 | 9 | 4 | 16 | |
| Preoperative serum PSA | | | | | |
| < 4 | 22 | 4 | 6 | 12 | 0.611 |
| ≥ 4, < 10 | 77 | 26 | 13 | 38 | 0.611 |
| ≥ 10 | 65 | 22 | 11 | 32 | |

PSA: prostate - specific antigen.
There are no significance differences in clinical backgrounds such as age, pT category, Gleason sum, and preoperative PSA among smoking statuses.
[a] Pack-years smoked is years smoked × cigarettes per day/20.

## MATERIALS AND METHODS

### Tissue Samples

A total of 164 newly diagnosed PC tissues from radical prostatectomies and 69 pathologically proven BPH samples from transurethral resection (TUR-P) were obtained from Shimane University Hospital (Izumo, Japan). The patients' clinicopathologic characteristics including their smoking status are summarized in Table 1. Each tumor was graded and staged according to the Gleason grading system and the TNM staging system.[24,25] Current smokers were defined as those who smoked within 12 months of tumor development. Former smokers were those who had quit smoking more than 12 months before tumor development. None of these patients had received androgen deprivation therapy before radical prostatectomy. We used serum PSA levels after radical prostatectomy as a surrogate end-point, with a level equal to or above 0.2 ng/mL designated as PSA failure. Forty-five patients with PC were excluded from the PSA failure-free probability study because of adjuvant hormonal therapy immediately after radical prostatectomy. PSA failure-free probability was determined as the percentage of patients without PSA failure. Follow-up ranged from 0.7 to 91.4 months, with a median of 33.9 months.

### Tissue Preparations

All samples were fixed in 10% buffered formalin (pH 7.0) and embedded in paraffin wax. For histologic evaluation, 5 $\mu$m-thick sections were used for hematoxylin and eosin (H & E) staining. All samples were microscopically dissected and analyzed for methylation.[26] In BPH samples, the presence of high-grade prostate intraepithelial neoplasia (PIN) and carcinoma were ruled out by microscopic analysis.

#### *Nucleic acid extraction*

Genomic DNA from all prostate samples was extracted using a commercial kit (Qiagene, Valencia, CA), and precipitated with ethanol. The concentration of DNA was determined with a spectrophotometer, and its integrity was checked by gel electrophoresis.

### Methylation Analysis

Genomic DNA from all prostate samples (100 ng) was subjected to sodium bisulfite modification using a CpGenome DNA Modification Kit (Intergen Co., Purchase, NY). Based on the functional promoter sequence of *APC*,[27] methylation-specific polymerase chain reaction (MSP) and unmethylation-specific polymerase chain reaction (USP) primers were designed by using MethPrimer software (http://itsa.ucsf.

TABLE 2
Multivariate Logistic Regression Analysis of Gene Methylations in the Series of PC and BPH

| Variable | Coefficient | SE | Chi-square | P[a] | HR[b] | 95% CI[c] | log HR[d] |
|---|---|---|---|---|---|---|---|
| *APC* | 2.056 | 0.525 | 15.338 | < 0.001 | 7.813 | 2.792–21.859 | 2.056 (0.067) |
| *GSTP1* | 1.773 | 0.644 | 7.586 | 0.006 | 5.891 | 1.667–20.814 | 1.773 (0.057) |
| *MDR1* | 1.151 | 0.514 | 5.009 | 0.025 | 3.162 | 1.154–8.664 | 1.151 (0.037) |

All data are adjusted by age. PC: prostate carcinoma; BPH: benign prostatic hyperplasia.
[a] *APC, GSTP1,* and *MDR1* methylation was a significant dependent predictor of pathology.
[b] Individual gene hazard ratios for pathogenesis were different from one another.
[c] 95% CI: 95% confidence interval.
[d] M-score was determined as the sum of the corresponding log hazard ratio for pathology.

edu/~urolab/methprimer) developed in our laboratory.[28] Primers used for MSP and USP analysis are as follows: universal primers: 5′-TAATTTTTTTGTTTGTTGGGGATT-3′ (sense), 5′-ACTACACCAATACAACCACATATC-3′ (antisense); MSP primers: 5′-TATTGCGGAGTGCGGGTC-3′ (sense), 5′-TCGACGAACTCCCGACGA-3′ (antisense); USP primers: 5′-GTGTTTTATTGTGGAGTGTGGGTT-3′ (sense), 5′-CCAATCAACAAACTCCCAACAA-3′ (antisense). An initial polymerase chain reaction (PCR) product was amplified with universal primers, which have no CpG sites in either forward or reverse primers, followed by a second nested PCR with primers specific for MSP or USP. For semiquantitative analysis, a preliminary suitable number of PCR cycles for each MSP and USP were carried out to determine the linear range of the reaction. To ensure this, at least one initial PCR was performed using 32 cycles each for MSP and USP. Then, a suitable PCR cycle was chosen for each sample. The annealing temperature and PCR cycles used for MSP and USP primers was 64 °C and 32 cycles. The sequences of primers for *GSTP1* and *MDR1* and their PCR conditions have been described previously.[20,21] In each assay, absence of DNA template served as negative control. The obtained MSP and USP products were analyzed by electrophoresis in 3% agarose gels and stained with ethidium bromide. With ImageJ software (http://rsb.info.nih.gov/ij), relative methylation levels (%) were calculated by using the area under the curve corresponding to each band (MSP and USP).[20,21] For methylation analysis of *APC*, we used 5.3 % methylation as a cut-off value, which was the average percentage of methylation in 69 BPH samples. Using these criteria, MSP positivity for *APC* was defined as those PCs with a percentage of methylation of more than 5.3%, and negative methylation was less than 5.3%. The criteria for MSP positivity of *GSTP1* and *MDR1* were described previously.[20,21]

### Bisulfite DNA Sequencing Analysis

Bisulfite-modified DNA (1 $\mu$L) was amplified using a pair of universal primers in a total volume of 20 $\mu$L. Direct bisulfite DNA sequencing of the PCR products using either forward universal primer or reverse universal primer was performed according to the manufacturer's instructions (Applied BioSystems, Foster City, CA).

### Statistical Analysis

To compare patients' background or methylation frequency for each gene, chi-square test was employed. Using a previously reported analytical technique,[23] we calculated the M-score for each sample, defined as the sum of the corresponding log hazard ratio (HR) coefficients, which were derived from multivariate logistic regression analysis of each methylated gene in the BPH and PC samples (Table 2). For each clinicopathologic finding, the association with PSA failure-free probability was determined using Kaplan–Meier curves with a log-rank analysis. The relation between M-score and clinicopathologic findings, except smoking status, was analyzed by either the Mann–Whitney *U* test, Kruskal–Wallis test, or Spearman rank correlation test. The relation between M-score and smoking status was analyzed by the Bonferroni-adjusted Mann–Whiteney *U* test. For this comparison test among the three groups of smoking statuses, the nonadjusted statistical levels of significance of $P < 0.05$ corresponds to a Bonferroni-adjusted statistical significance of $P < 0.0167$.

## RESULTS

### Methylation Status of the *APC* Promoter in Clinical Samples

Representative results of MSP and USP assays for *APC* in PCs and BPHs are shown in Figure 1A. MSP-positive bands were present in the majority of PCs, and less so in BPH samples. The result of the methylation study

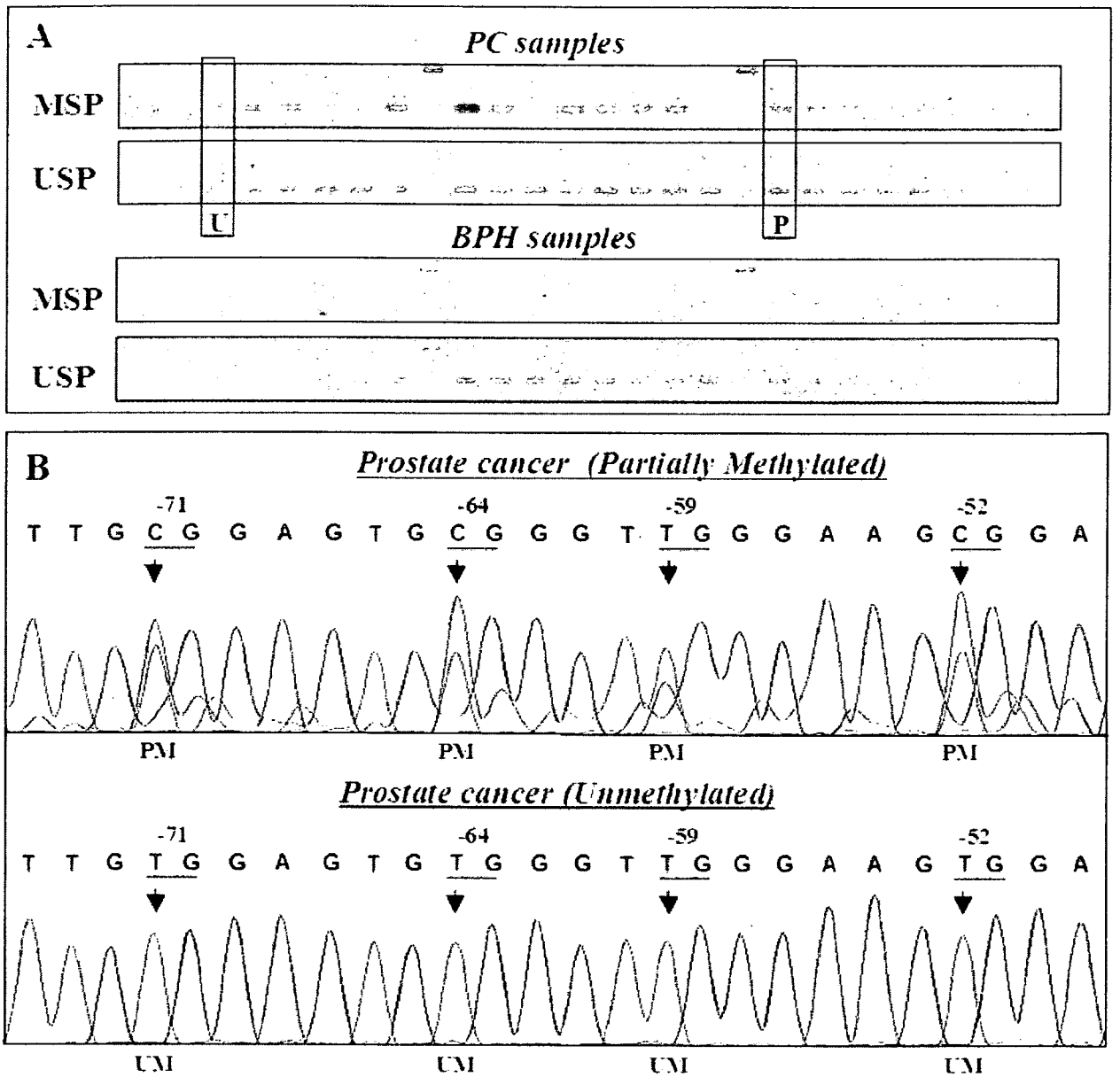


**FIGURE 1.** Methylation status of the *APC* promoter in clinical samples. (A) MSP and USP bands for 24 samples of BPH and PC are shown. Lanes 'P' and 'U' represent samples depicting partial methylation and unmethylion, respectively. (B) Bisulfite DNA sequencing of partially methylated (top), unmethylated (bottom) samples correspond to lanes 'P' and 'U', respectively. In partially methylated samples, there was a "T" peak along with a "C" peak at the CpG sites. In unmethylated samples, every CpG site was unmethylated. PM and UM correspond to partial methylation and unmethylation, respectively.

was also confirmed by bisulfite DNA sequencing. Figure 1B shows results of a typical bisulfite DNA sequencing in a PC sample. In sample 'P' (corresponding to Fig. 1A, lane 'P') with both MSP and USP bands, there was a "T" peak along with a "C" peak at the CpG sites, indicating partial-methylation (Fig. 1B, top). In sample 'U' (corresponding to Fig. 1A, lane 'U'), where no MSP band was observed, the CpG sites were completely unmethylated (Fig. 1B, bottom).

### Evaluation of M-Score; Multigene Methylation Analysis with *APC*, *GSTP1*, and *MDR1* to Distinguish PC from BPH

*APC* methylation analysis showed positive MSP bands in 104 of 164 (63.4%) PCs and 6 of 69 (8.7%) BPHs (Fig. 2). As we have reported previously, positive MSP bands for *GSTP1* methylation analysis were found solely in 87 of 164 (53.0%) PCs and in 4 of 69 (5.8%) BPHs, whereas, for *MDR1* methylation analysis, positive MSP bands were found in 89 of 164 (48.7%) PCs

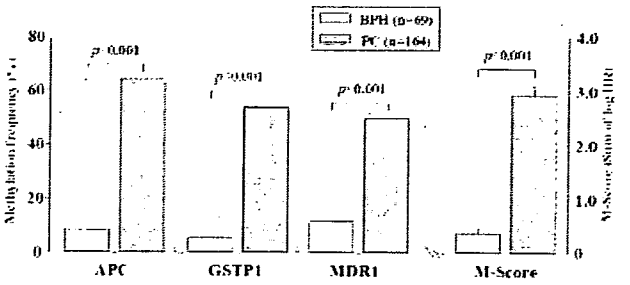


**FIGURE 2.** Correlation of gene methylation frequency with pathology (BPH vs. PC) and methylation score (M-score). The methylation frequency in PC samples was significantly higher than that in BPH samples for *APC*, *GSTP1* and *MDR1* genes (64.1% vs. 8.7%, 54.0% vs. 5.8%, and 55.3% vs. 11.6%, respectively). Right side: M-score, determined as the sum of the corresponding log hazard ratio for pathology (BPH vs. PC) (Table 2), was also significantly higher in PC samples than in BPH samples.

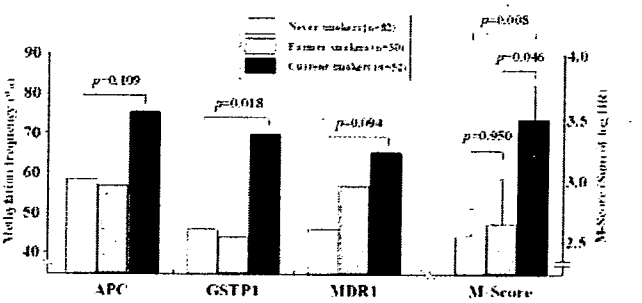


**FIGURE 3.** Correlation of methylation frequency with smoking status and methylation score (M-score). The methylation frequency in current smokers was significantly higher than that in former or never smokers for *GSTP1* (69.2%, 43.3%, and 46.3%, respectively). There was no significant difference in *APC* or *MDR1* methylation frequency among smokers (75.0%, 56.7%, and 58.5%, respectively for *APC*; 65.4%, 56.7%, and 46.3%, respectively for *MDR1*). Right side: When methylation data was combined (M-score), there was a significant difference in M-score between current and never smokers (3.52 ± 0.27 vs. 2.56 ± 0.23, $P = 0.008$ by Bonferroni-adjusted test). However, there was only a trend to significance in M-score between current and former smokers (3.52 ± 0.27 vs. 2.59 ± 0.37, $P = 0.046$ by Bonferroni-adjusted test).

and in 8 of 69 (11.6%) BPHs.[20,21] A significant difference in the methylation status of each gene was found between the series of PCs and BPHs (Fig. 2). As shown in Table 2, multivariate logistic regression analysis revealed that *APC*, *GSTP1*, and *MDR1* methylation was a significant dependent predictor of pathology (BPH vs. PC) ($P < 0.001$, $P = 0.006$, and $P = 0.025$, respectively). The individual gene hazard ratios for pathogenesis (PC vs. BPH) were different from one another. For instance, cases with *APC* methylation are 7.813 times more likely to have PC than those with negative methylation, whereas the HR for *MDR1* is 3.162. The M-score determined by the sum of log HR was significantly higher in PC than in BPH (Fig. 2). All statistics were age adjusted to eliminate potential influence caused by age.

### Correlation between Aberrant Methylation and Smoking Status

Looking at PC samples by smoking status, positive MSP bands for *APC*, *GSTP1*, and *MDR1* methylation analysis were found: in 48 (58.5%), 38 (46.3%), and 38 (46.3%) of never smokers,[82] respectively; in 17 (56.7%), 13 (43.3%), and 17 (56.7%) of former smokers,[30] respectively; and in 39 (75.0%), 36 (69.2%), and 34 (65.4%) of current smokers,[52] respectively (Fig. 3). There was significant difference between smoking status in *GSTP1* methylation status ($P = 0.018$), whereas no significant difference was found in *APC* or *MDR1* methylation status ($P = 0.109$ and $P = 0.094$, respectively) (Fig. 3). When we employed the M-score that combined the methylation analysis for three genes, the difference in M-score between current and never smokers was statistically significant ($P = 0.008$) (Fig. 3, right). Conversely, the difference in M-score was not significant: between current and former smokers ($P = 0.046$) or between former and never smokers ($P = 0.950$) (Fig. 3, right). Spearman rank correlation test revealed significant correlation between pack-years smoked and M-score in PCs ($P = 0.039$). There were no relations between methylation status and other smoking variables, such as duration of smoking and age at starting smoking.

### Correlation of M-Score with Clinicopathologic Findings

Among PCs, the M-score showed a significant stepwise increase with advancing pathologic stage (1.34 ± 0.26 in pT2a, 2.97 ± 0.24 in pT2b, 3.64 ± 0.29 in pT3a, 4.07 ± 0.56 in pT3b, and 4.98 ± 0 in pT4) ($P < 0.001$) (Fig. 4A). Similarly the M-score increased as the Gleason sum (GS) increased (2.20 ± 0.23 in GS < 7, 3.44 ± 0.26 in GS = 7, and 3.74 ± 0.31 in GS > 7) ($P < 0.001$) (Fig. 4B). For preoperative PSA levels, the M-score was higher in PSA > 10 ng/mL (3.27 ± 0.26) than in PSA ≤10 ng/mL (2.61 ± 0.20) ($P = 0.041$) (Fig. 4C). Moreover, the M-score was higher in advancing pathologic features as follows: in capsular invasion (Cap) (positive [3.65 ± 0.25] vs. negative [2.48 ± 0.20] [$P < 0.001$]), in seminal vesicle involvement (SV) (positive [4.71 ± 0.19] vs. negative [2.74 ± 0.17] [$P = 0.002$]), in pelvic lymph lymph node metastasis (pN) (positive [4.48 ± 0.37] vs. negative [2.55 ± 0.19] [$P = 0.002$]), in venous involvement (v) (positive [3.79 ± 0.24] vs. negative [2.44 ± 0.20] [$P < 0.001$]), in lymphatic vessel involvement (ly) (positive [3.70

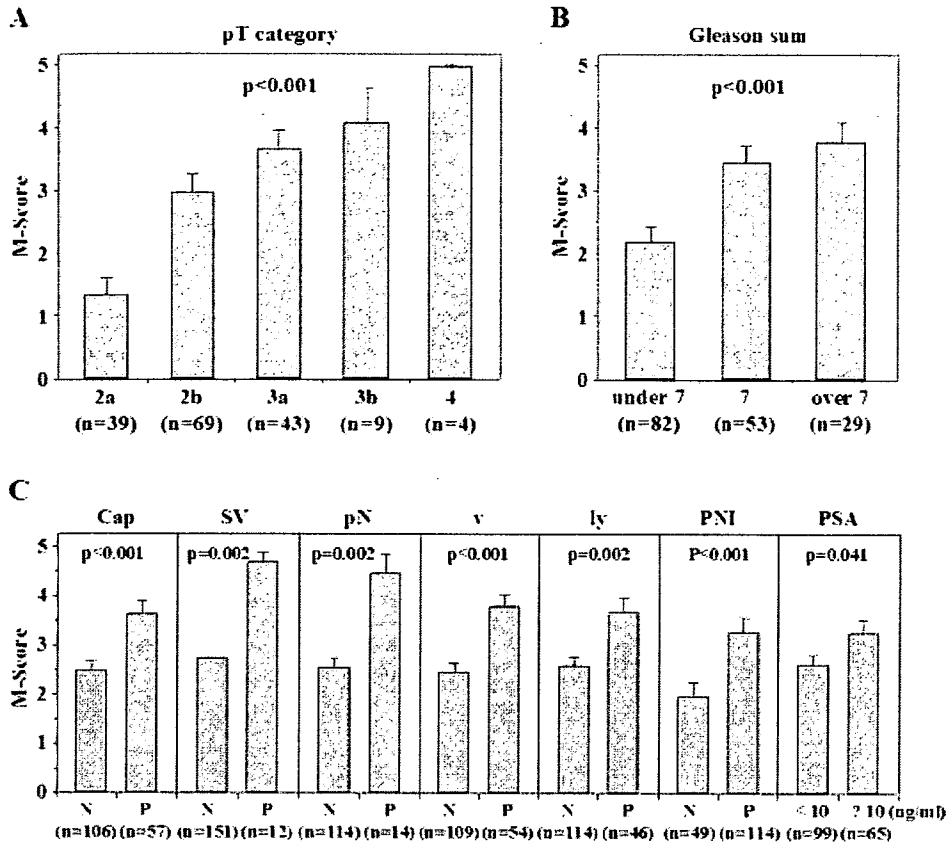


**FIGURE 4.** Correlation of M-score with clinicopathologic features. Among the overall PC patients: (A) Higher stage of disease (1.34 ± 0.26 in pT2a, 2.92 ± 0.24 in pT2b, 3.84 ± 0.26 in pT3a, 4.21 ± 0.62 in pT3b, and 4.98 ± 0 in pT4); (B) Higher Gleason sum (2.20 ± 0.23 in GS <7, 3.58 ± 0.25 in GS = 7, and 3.819 ± 0.29 in GS > 7; and (C) Higher preoperatve PSA levels (3.35 ± 0.25 in PSA > 10 ng/mL, 2.64 ± 0.20 in PSA ≤10 ng/mL) correlated with an increased methylation score (M-score). There was significant correlation between M-score and worse clinicopathologic findings. Cap: capsular invasion; SV: seminal vesicle involvement; pN: lymph lymph node invasion; ly: lymphatic vessel invasion; and PNI: perineural invasion. N and P correspond to negative and positive, respectively.

± 0.29] vs. negative [2.57 ± 0.19] [$P = 0.002$]), and in perineural invasion (PNI) (positive [3.29 ± 0.18] vs. negative [1.95 ± 0.29] [$P < 0.001$]) (Fig. 4C). We also observed that the M-score was age related in the total group of BPHs ($P = 0.001$) but not in the total group of PCs ($P = 0.067$).

### Prognostic Features

We analyzed PSA failure-free probability as disease-free survival. Of the clinicopathologic features considered, only Gleason sum was significantly associated with poor outcome in univariate analyses ($P = 0.020$) (Fig. 5).

## DISCUSSION

Our results indicate that the M-score was significantly higher in current smokers than in never smokers in PCs (Fig. 3, right). Spearman rank correlation test also revealed significant correlation between pack-years smoked and M-score in PCs ($P = 0.039$). Moreover, there was significant correlation of M-score with advanced pathologic features such as pT category and Gleason sum (Fig. 4). These results suggest that smoking status may influence tumor progression through CpG hypermethylation of related genes dose-dependently. Hickey et al. reviewed 23 prospective cohort studies, 5 nested case–control studies, 1 retrospective cohort study, and 36 case–control studies addressing smoking and PC.[4] Although most of the prospective cohort studies and all of the nested case–control studies that used incident PC as the outcome found no association between current smoking and PC, 33% of 15 population-based case–control studies showed a significant association between smoking and PC risk. They concluded these conflicting results depend on the research design used and how well the study controlled for possible confounding factors. Furthermore, the majority of prospective studies (62% of 13) that used PC death as the outcome noted a positive association between current smoking and PC, supporting our findings where current smoking and advanced PC are associated through CpG hypermethylation of related genes. Therefore, the present study elucidates novel mechanisms whereby smoking may increase PC risk.

Interestingly in lung cancers, some investigators have previously reported that starting smoking during adolescence was the only significant parameter associated with *RASSF1A* gene methylation, and no association was observed with pack-years smoked.[11,12] In

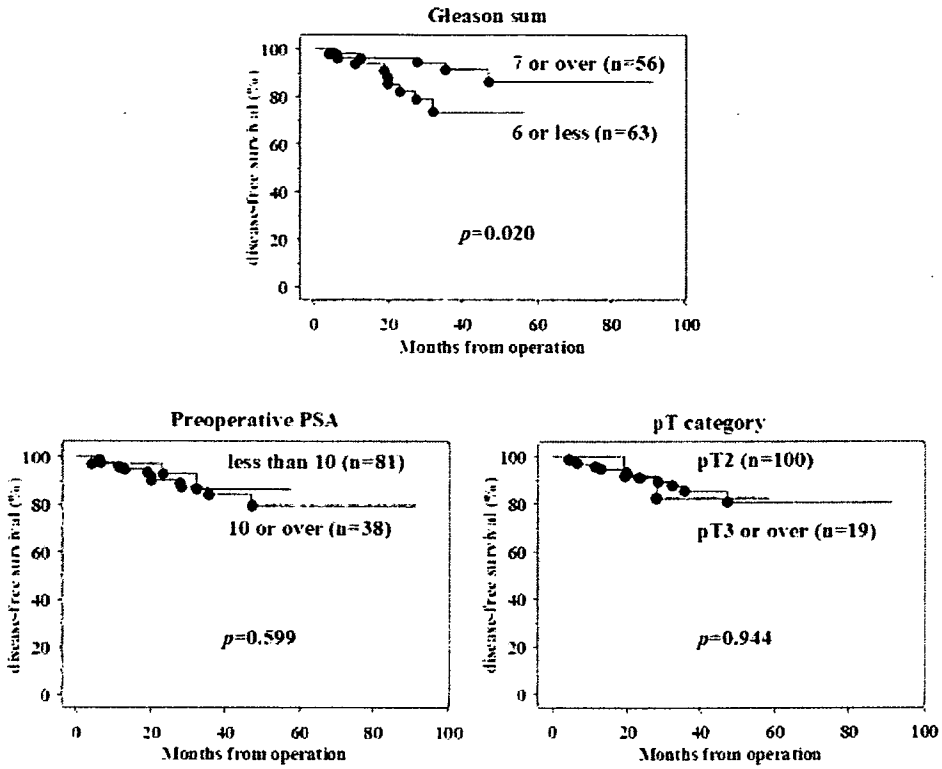


**FIGURE 5.** Kaplan–Meier PSA failure-free survival curves of PC patients after radical prostatectomy are grouped according to evaluated variables: Gleason sum, preoperative PSA level, and pT category. Follow-up ranged from 0.7 to 91.4 months, with a median of 33.9 months. Circles represent censored data points.

contrast, our result indicated that pack-years smoked was the only parameter associated with methylation status in PCs. Also, methylation status in PC was influenced by smoke exposure in a dose-dependent manner. These findings suggest that methylation induced by smoke exposure may depend on tumor type or gene and that it may be one of the reasons why the mean age of patients with lung carcinoma is younger than that of patients with PC.

We also analyzed PSA failure-free probability as disease-free survival. Of the clinicopathologic features considered, only Gleason sum was significantly associated with poor outcome (Fig. 5). The significant association of the M-score with Gleason sum (Fig. 4) suggests that smoking may influence PC prognosis through CpG hypermethylation of these genes. However, our median follow-up time was probably too short for thorough analysis. We also found there was a trend toward a decreased M-score among former smokers compared with current smokers ($P = 0.046$ by Bonferroni-adjusted test) (Fig. 3, right), suggesting that demethylation of these genes may be occurring in patients who had quit smoking for more than 12 months. Therefore, patients could decrease their risk for advanced PC by becoming nonsmokers.

Other investigators have used multigene methylation analysis in their studies of various cancers.[7,8,10] However, their methods of multigene methylation analysis are the sum of the number of genes methylated. Studies have shown that when multigenes are analyzed in the same samples, interpretation of results should be carefully considered because each gene or other clinical factors, including age, may influence one another.[22,23] In the current study, we attempted to integrate the methylation status of multigenes by using a M-score that is the sum of the log HR analyzed by multivariate logistic regression analysis for pathology (BPH vs. PC). This analysis provides automatically adjusted statistical data,[22] with each HR directly related to gene methylation in PC samples compared with BPH (methylation-negative) samples (Table 2). By adding the log HR of each gene in a multigene analysis, it is therefore possible to predict the risk of PC in individual patients. Similarly, Ray et al. employed multivariate Cox proportional hazards models for their multigene methylation analysis in medulloblastoma, and they used the sum of the log HR as a risk score for each patient.[23] In our study, we found no significant correlation between smoking status and methylation frequency of the *APC* or *MDR1* gene individually. However, by employing a combined analysis (M-score) of the methylation status of the three genes used in this study, differences in methylation status between categories of smokers became appar-

ent (Fig. 3, right). Furthermore, among PCs, the M-score showed a significant stepwise increase with advancing PT category, increasing Gleason score, higher PSA levels, and advancing pathologic features (Fig. 4). Thus, when examining the methylation status of multigenes in PC, the M-score is a reliable, superior, analytical tool for diagnosis and outcome prediction.

In conclusion, this is the first study to demonstrate significant correlation of methylation status of multigenes with smoking status in PC. Smoking status may influence both progression and prognosis of PC through CpG hypermethylation of related genes.

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# Percentage of positive biopsy cores, preoperative prostate-specific antigen (PSA) level, pT and Gleason score as predictors of PSA recurrence after radical prostatectomy: a multi-institutional outcome study in Japan

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## OBJECTIVE

To evaluate the clinical outcome of radical prostatectomy (RP) in Japan, by retrospectively analysing the clinicopathological data in patients with clinical T1-T2 prostate cancer treated by RP, as there can be prostate-specific antigen (PSA) recurrence after RP in substantially many patients, and its character can differ according to ethnic group and/or country.

## PATIENTS AND METHODS

We reviewed 1192 patients who had a RP from 1993 to 2002 with no neoadjuvant/adjuvant therapy and whose PSA level after RP decreased at least once to undetectable levels (<0.2 ng/mL). PSA recurrence was defined as ≥0.20 ng/mL. The patient data were collected from the Urological Oncology Study Group, a subgroup of Japan Clinical Oncology Group.

## RESULTS

The patients' median (range) age was 67 (47–83) years and their PSA level before RP was 8.7 (1.0–153) ng/mL. During the median follow-up of 45.6 months, 302 of the 1192 patients (25.3%) developed PSA recurrence. The median time to recurrence was 369 (61–2128) days after RP. A log-rank test showed that five significant clinicopathological factors were associated with PSA recurrence after RP: The percentage of prostate needle-biopsy cores with cancer, the biopsy Gleason score, PSA level before RP, pathological stage, and the Gleason score of the RP specimen ($P < 0.001$ for all). In multivariate analyses, the percentage of positive biopsy cores, PSA level before RP, pT and the Gleason score of the RP specimen were all independent significant predictors of PSA recurrence after RP in Japanese men.

## CONCLUSIONS

The frequency of PSA recurrence after RP was 25.3% in Japan and the percentage of positive biopsy cores, PSA level before RP, pT and the Gleason score of the RP specimen were independent significant factors for PSA recurrence.

## KEYWORDS

prostate cancer, PSA recurrence, radical prostatectomy

## INTRODUCTION

Prostate cancer is one of the most common malignancies among men in western countries [1]. Radical prostatectomy (RP) has been established as one of the standard management options for localized prostate cancer [2], but the outcome of RP might differ among countries due to racial, economic and medical factors. Very few outcome studies have so far been reported on RP in Japan, and therefore a large-scale investigation was planned to reveal the characteristics of PSA recurrence after RP in Japan, associated with a prospective randomized controlled trial after RP [3].

## PATIENTS AND METHODS

We reviewed 1192 patients who had a RP from 1993 to 2002 with no neoadjuvant/adjuvant therapy and whose PSA level after RP decreased at least once to undetectable levels (<0.2 ng/mL), suggesting that the surgical resection was complete biochemically. All the patients enrolled were Japanese and the RP was done at 37 specialized institutes belonging to Urologic Oncology Study Group in the Japan Clinical Oncology Group (Appendix). In this group, a randomized controlled trial was started to evaluate radiotherapy and endocrine therapy for PSA failure after RP [3]. Apart from this study, the patients' data were collected and analysed to design an appropriate randomized trial. PSA recurrence was defined as a PSA level of ≥0.2 ng/mL after decreasing to an undetectable level (<0.2 ng/mL) after RP. Information was recorded on patient age, the positive number/total number of biopsy specimens, biopsy Gleason score (GS), clinical stage, PSA level before RP, the date of RP, the operative procedure (open or laparoscopic), pT, pN, GS of the RP specimen, PSA recurrence or not, the date of PSA recurrence, the timing and type of secondary cancer treatment after PSA recurrence, clinical recurrence or not, the latest follow-up date and the date of death. The margin status of the RP specimen, i.e. the external wedge, was not investigated in this

*TABLE 1 Descriptive characteristics of the 1192 patients who had a RP*

| Characteristic | Value |
|---|---|
| Median (range): | |
| Age at RP, years | 67 (48–83) |
| PSA before RP, ng/mL | 8.7 (0.8–153) |
| Biopsy GS | |
| Median GS | 6 |
| N (%): | |
| 4 | 234 (19.6) |
| 5–6 | 441 (37.0) |
| 7 | 260 (21.8) |
| 8–10 | 127 (10.7) |
| unknown | 130 (10.9) |
| Clinical stage | |
| T1 | 27 (2.3) |
| T1c | 626 (52.5) |
| T2a | 378 (31.7) |
| T2b | 149 (12.5) |
| unknown | 12 (1.0) |
| Pathological GS score of RP specimen | |
| Median GS | 7 |
| N (%): | |
| 4 | 81 (6.3) |
| 5–6 | 449 (37.7) |
| 7 | 478 (40.1) |
| 8–10 | 128 (10.7) |
| unknown | 56 (4.7) |
| pT stage, n (%) | |
| T0 | 6 (0.5) |
| T2 | 778 (65.3) |
| T3 | 389 (32.6) |
| T4 | 2 (0.2) |
| TX | 17 (1.4) |
| Positive lymph nodes, n (%) | 21 (1.8) |

*TABLE 2 Results from the univariate analysis of clinicopathological factors*

| Variable | P |
|---|---|
| Biopsy GS | <0.001 |
| PSA level before RP | <0.001 |
| pT stage | <0.001 |
| GS of RP specimen | <0.001 |
| Percentage of positive biopsy cores | <0.001 |
| Age | 0.350 |
| pN | 0.960 |
| RP method (laparoscopic or open) | 0.567 |

study because not all the institutes prepared whole mounts of the RP specimens. The results were assessed statistically using the Kaplan–Meier method, univariate log-rank tests and a multivariate analysis using Cox's proportional hazards.

*TABLE 3 Results from the multivariate survival analysis with the Cox proportional hazards model, based on the biopsy GS, PSA level before RP, pT stage, RP specimen GS, percentage of positive biopsy cores and biochemical recurrence*

| Variable | Hazard ratio (95% CI) | P |
|---|---|---|
| Biopsy GS (≤6 vs ≥7) | – | 0.1301 |
| PSA level before RP (< vs ≥10) | 1.84 (1.40–2.40) | <0.001 |
| pT (≤2 vs ≥3) | 1.77 (1.35–2.32) | <0.001 |
| GS of RP (≤7 vs ≥8) | 1.81 (1.29–2.53) | 0.001 |
| % +ve biopsy cores (< vs ≥60) | 2.05 (1.46–2.86) | <0.001 |

## RESULTS

In all, 1192 patients were included in the analysis; their characteristics are shown in Table 1; comparing the clinical stage and RP pT stage suggested that almost a third of patients were understaged before RP (Table 1). After a median (range) follow-up of 45.6 (1.8–132.6) months, 302 patients (25.3%) had a PSA recurrence; the median time to PSA recurrence after RP was 369 (177–3977) days.

The log-rank test showed that five significant clinicopathological factors were associated with PSA recurrence after RP, i.e. biopsy GS (threshold 7, Fig. 1a), the PSA level before RP (10 ng/mL, Fig. 1b), the pathological stage (pT3, Fig. 1c), the GS of the RP specimen (8, Fig. 1d) and the percentage of positive biopsy cores (60%, Fig. 1e; all $P < 0.001$). In a univariate analysis, these five factors were also significant prognostic variables for PSA recurrence after RP in Japan (Table 2). However, age, pN, RP method (laparoscopic or open surgery) were not significantly associated with PSA recurrence (Table 2). A multivariate survival analysis with Cox's proportional regression indicated that the PSA level before RP, pathological stage, GS of the RP specimen, and the percentage of positive biopsy cores were all powerful independent predictors of PSA recurrence (Table 3). The biopsy GS was a significant factor on the univariate analysis but not on the multivariate analysis (Table 2).

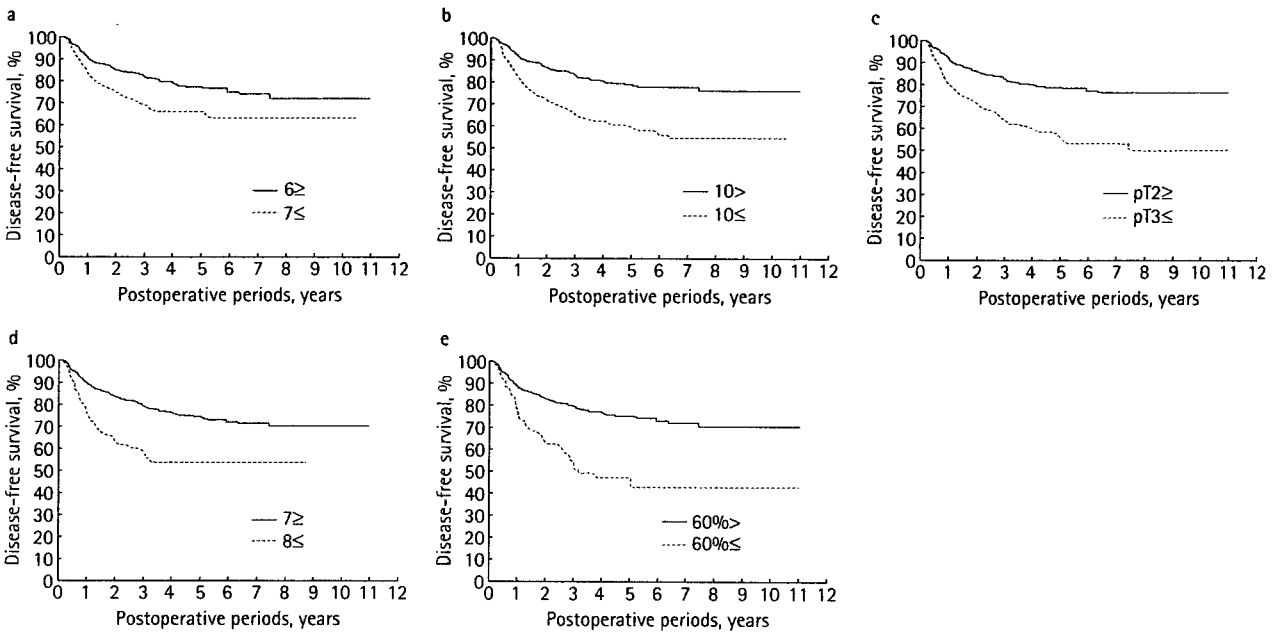
The year that the RP was done might influence the outcome, because operative skill can improve with time and the indication for RP might change depending on the year. Therefore, PSA failure-free survival rate (Kaplan–Meier) in each year was calculated, but there was no significant difference (data not shown). In addition, we did not investigate the number of patients who were lost to follow-up, but if considering patients who did not visit the hospital for ≥1 year as lost to follow-up, there were 20 (22.2%) in 1993–95, 81 (27.0%) in 1996–98 and 92 (11.5%) in 1999–2002. However, for the survival outcome, only seven patients died from cancer-related causes (data not shown), thus suggesting that the survival outcome was quite good with this treatment.

## DISCUSSION

Patients enrolled in the present study represent the total experience of many surgeons from various geographical locations, and of different pathologists and their techniques, in Japan. Indeed, this study was retrospective and multi-institutional, and therefore the data described do not necessarily represent the real data on RP in Japan. Furthermore, the pathological results of the biopsy and RP specimen were not derived from central pathologists, and therefore might contain errors in GS and pT diagnosis. However, we think that this study reflects the Japanese urological community as a whole, avoiding any institutional bias and not favouring any one technique.

Pathological and epidemiological data suggest that racial variation exists for the clinically diagnosed form of prostate cancer, i.e. it is highest in African-Americans, next highest in Caucasians and lowest in Asians [4], thus suggesting that the bioactivity of prostate cancer is different for each ethnic group. However, very few outcome results of large-scale studies have been reported on RP in Japan, and thus the present study was planned to clarify the outcome of Japanese patients treated by RP. An important point of

*FIG. 1. Actuarial 10-year Kaplan–Meier estimates of biochemical recurrence rates in patients who had RP, stratified according to: **a**, biopsy GS; the PSA RFS at 5 years was 77.3% in those with GS ≤6 and 66.2% for GS ≥7. **b**, the PSA level before RP; the PSA RFS at 5 years was 79.2% for PSA levels of value <10 ng/mL and 60.1% for ≥10 ng/mL. **c**, pT stage; the PSA RFS at 5 years was 78.6% for ≤pT2 and 56.3% for ≥pT3. **d**, GS of the RP specimen; the PSA RFS at 5 years was 74.5% for Gleason ≤7 and 53.9% for Gleason ≥8. **e**, the percentage of positive biopsy cores; the PSA RFS at 5 years was 75.8% for <60% and 48.0% for ≥60%. Overall log-rank P < 0.001 in all plots. All log-rank P < 0.001.*




the present study was that we limited the patients to those whose PSA level after RP decreased to undetectable levels with no neoadjuvant/adjuvant therapy. Using this 'pure' characterized database of 1192 patients treated with RP at 37 specialized institutes in Japan, we can thus also compare the racial differences among the clinical and pathological variables and PSA recurrence with those of Caucasian and American-Africans. Pound *et al.* [5] reported a PSA increase in 16% of men (315/1997) treated by RP with no neoadjuvant/adjuvant therapy at a median follow-up of 5.3 years. Others reported that ≈35% of all men had a PSA increase within 10 years of RP [6–9]. In the present study, there was a PSA increase in 25.3% of 1192 men, thus suggesting PSA recurrence after RP to be closely similar in Japan, or even a little higher, considering the shorter follow-up. Indeed, the PSA recurrence rate within 2 years was 6.8% in the study of Pound *et al.* [5], while it was 18.4% in the present study, indicating an earlier PSA increase after RP in Japan. The reason for this was not clear, but as a potential cause, the frequency of PSA assay after RP could affect it. In Japan, serum PSA is assayed every 1–3 months after RP for several years, while it is assessed every 6–12 months in the USA [6–9]. The time to PSA recurrence after surgery should be shorter if the serum PSA value is assayed more frequently. The PSA level before RP, GS, T category and margin status reportedly provide more clinically relevant stratification of the PSA outcome with T1-T2 disease [10–12]. In the present study, significant clinicopathological factors were similarly assessed by the log-rank test and the biopsy GS, PSA level before RP, pathological stage and the GS of the RP specimen were all strongly associated with PSA recurrence after RP. There were only 21 men with pN1 disease (1.8%), which resulted in an insignificant statistical result. For the PSA level before RP, Partin *et al.* [13] reported that 64%, 50%, 35% and 16% of patients with a serum PSA level of <4, 4–10, 10–20 and >20 ng/mL, respectively, had pathologically organ-confined disease. As a result, patients with a serum PSA level of 10–20 ng/mL are at intermediate risk of PSA recurrence, while those with a serum PSA level of >20 ng/mL represent a high-risk population for developing PSA recurrence after RP [13]. Regarding the importance of the pathological stage and surgical margin status, Khan *et al.* [14] constructed a nomogram that was simple to use and divided the probability of long-term PSA recurrence-free survival (RFS) into four groups according to the RP GS, pathological stage, and surgical margin status, i.e. excellent, good, moderate and low. The PSA RFS at 10 years was 95%, 72%, 41% and 13%, respectively [14]. These data suggest that the factors associated with PSA recurrence after RP were similar in Japanese patients, and the character and bioactivity closely matched that in Western countries. In addition, there was extraprostatic extension (i.e. ≥pT3) in 391 patients (32.8%), suggesting that the understaging of clinical stage in Japan was similar to that in the USA. In the present study, 52.5% of all the cases were clinical stage T1c (Table 1). At present, a greater percentage of men might have T1c disease, but in the present study patients had RP between 1993 and 2002. In the USA, the proportion of T1c was 48.3% of the 2417 cases in 1988–2002 [15] and 63% of the 5079 cases in 1994–2000 [16]. Therefore, there were no significant differences in the staging of the patients between those in Japan and in the USA, which probably did not affect the study outcome.

For the percentage of positive biopsy cores, several previous studies developed tables for

predicting the PSA recurrence risk using biopsy tumour volume measurements [17–19]. D'Amico *et al.* [17] developed a nomogram to predict PSA recurrence using the total percentage of cores that were positive, whereas Nelson *et al.* [18] used the greatest percentage of a biopsy core involved by cancer. Freedland *et al.* [19] reported that the percentage of cores positive from the dominant side of the prostate was a better predictor of PSA recurrence than the total percentage of positive cores. The present multivariate analysis identified that the percentage of positive biopsy cores was the most significant independent factor for PSA recurrence (hazard ratio of 2.05 at a threshold of 60%; Table 3). Although the difference was significant even when the threshold was set at 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90%, it was most significant when the patients were divided at 60% (data not shown). A prostate biopsy is one of the essential methods to diagnose prostate cancer, and this is often and most easily quantified by determining the percentage of the biopsy cores with cancer. The percentage of the cores, with the PSA level, pT and GS, provided significant risk stratification for PSA failure after RP.

However, the median GS was 6 in biopsy specimens but 7 in the RP specimen (Table 1), indicating that the GS tends to be lower by one grade at biopsy. A multivariate analysis with Cox's proportional regression indicated the GS of the RP specimen, but not of the biopsy, was significantly associated with PSA recurrence, which clearly indicates that the GS of the RP specimen is a reliable biomarker of PSA recurrence.

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## CONFLICT OF INTEREST

None declared.

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Abbreviations: **GS**, Gleason score; **RP**, radical prostatectomy; **RFS**, recurrence-free survival.

## APPENDIX

Participating Institutions (Urologic Oncology Study Group in the Japan Clinical Oncology Group): Hokkaido University, Sapporo Medical University, Tohoku University, Miyagi Cancer Center, Akita University, Tsukuba University, Tochigi Cancer Center, Gunma University, Chiba Cancer Center, Chiba University, National Defense Medical College, National Cancer Center Hospital, Tokyo Women's Medical School, Keio University, The Jikei University, Nippon Medical School, Kitasato University, Niigata Cancer Center Hospital, Niigata university, Yamanashi University, Shinshu University, Hamamatsu Medical School, Shizuoka Cancer Center, Nagoya University, Mie University, Kyoto University, Osaka Medical Center for Cancer and Cardiovascular Diseases, Kobe University, Nara Medical University, Shimane University, Kurashiki Central Hospital, Okayama University, Kagawa Medical University, National Shikoku Cancer Center, Kyushu University, Kurume University and Kagoshima University.

# The Case for Androgen Deprivation as Primary Therapy for Early Stage Disease: Results From J-CaP and CaPSURE™

**Hideyuki Akaza,* Shiro Hinotsu, Michiyuki Usami, Osamu Ogawa, Susumu Kagawa, Tadaichi Kitamura, Taiji Tsukamoto, Seiji Naito, Yoshihiko Hirao, Masaru Murai, Hidetoshi Yamanaka and Mikio Namiki**

*From the J-CaP Study Group, Japan*

**Purpose:** We analyzed the outcome of primary androgen depletion therapy, which has gained more attention as a potential therapeutic option in patients with localized or locally advanced prostate cancer as it has been increasingly implemented despite limited data on its therapeutic impact in Japan and the United States.

**Materials and Methods:** We analyzed data from CaPSURE™ and the Japanese Prostate Cancer study.

**Results:** In Japan primary androgen depletion therapy has long been the treatment of choice for localized and locally advanced prostate cancer. Based on CaPSURE™ data the frequency of primary androgen depletion therapy being chosen to treat localized and locally advanced disease is also increasing in clinical practice in the United States. A study of the outcomes of endocrine therapy is currently being performed in Japan by the Japanese Prostate Cancer Study Group.

**Conclusions:** It is important to obtain such information about the role of primary androgen depletion therapy for localized and locally advanced prostate cancer from studies of natural history and clinical trials. It is also important to update practical treatment guidelines.

*Key Words: prostate, prostatic neoplasms, androgen antagonists, Japan, natural history*

To date no published guidelines for prostate cancer treatment have included a recommendation for PADT for LPC or LAPC.[1–3] However, in clinical practice PADT has often been selected for nonmetastatic prostate cancer in Japan[4] and the United States.[5] Why is there such a gap between the guidelines and clinical practice in terms of treatment methods? Generally guidelines are based on medical evidence. However, there is a paucity of medical evidence regarding PADT for LPC and LAPC, which may be interpreted as indicating that there is no adequate evidence that PADT is effective for LPC or LAPC. If so, why has PADT been ignored in the guidelines? Another question to be asked is why there have been few clinical studies performed to date to examine the validity of PADT.

## SURVEYS OF THE JAPANESE SOCIETY OF UROLOGY AND CAPSURE™

The Japanese Society of Urology survey, which was started in 2001 and performed at 173 facilities, enrolled 4,529 patients newly diagnosed with prostate cancer during 2000.[4] Prostate cancer clinical T stage was T1c, T2a, T2b, T3a, T3b and T4 in 20.3%, 21.8%, 17.3%, 15.8%, 11% and 8% of the subjects, respectively. It is worth noting that PADT was often selected in patients with every stage of disease. Table 1 shows the initial therapies given in cases of stages T1c through T3b. This trend in Japan differed from that in the United States and it has remained unchanged for many years.[6]

Table 2 lists the results of the CaPSURE™ survey. It shows the increase in the incidence of patients treated with PADT from 1989 to 2000, during which time the percent assigned to the high, intermediate and low risk groups increased from 32.8% to 48.2%, 8.9% to 19.7% and 4.6% to 14.2%, respectively.[4] Additionally, the CaPSURE™ survey revealed that the percent of patients who received neoadjuvant hormone therapy before external beam radiotherapy increased sharply from 9.8% to 74.6%. The sharp increase in the application of neoadjuvant ADT was probably attributable to the increased awareness of this therapy after the publication of the results of certain clinical studies.[7–9] However, how can we explain the high percent of patients treated with PADT in Japan and the United States?

## J-CAP SURVEILLANCE

Recently several reports have been published of the results of clinical studies of the effectiveness of PADT for LPC or LAPC.[10,11] The results of these studies provide the motivation to perform further clinical studies comparing PADT and radical prostatectomy, PADT and watchful waiting or studies involving active surveillance. In such new studies the obvious end point of comparison would be the survival rate, for which precise analysis of the cause of death will be important. Although the patient age at which prostate cancer develops is often advanced, it is plausible that patients enrolled in clinical studies are often more elderly than the general patient population, which is why studies of less aggressive treatment modalities, such as watchful waiting

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