Multigene Methylation Analysis for Detection and Staging of Prostate Cancer

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Abstract Purpose: Aberrant gene promoter methylation profiles have been well-studied in human prostate cancer. Therefore, we rationalize that multigene methylation analysis could be useful as a diagnostic biomarker. We hypothesize that a new method of multigene methylation analysis could be a good diagnostic and staging biomarker for prostate cancer.

Experimental Design: To test our hypothesis, prostate cancer samples (170) and benign prostatic hyperplasia samples (69) were examined by methylation-specific PCR for three genes: adenomatous polyposis coli (APC), glutathione S-transferase pi (GSTP1), and multidrug resistance 1 (MDR1). The methylation status of representative samples was confirmed by bisulfite DNA sequencing analysis. We further investigated whether methylation score (M score) can be used as a diagnostic and staging biomarker for prostate cancer. The M score of each sample was calculated as the sum of the corresponding log hazard ratio coefficients derived from multivariate logistic regression analysis of methylation status of various genes for benign prostatic hyperplasia and prostate cancer. The optimal sensitivity and specificity of the M score for diagnosis and staging of prostate cancer was determined by receiver-operator characteristic (ROC) curve analysis. A pairwise comparison was employed to test for significance using the area under the ROC curve analysis. For each clinicopathologic finding, the association with prostate-specific antigen (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 analyzed by either the Mann-Whitney U test, Kruskal-Wallis test, or the Spearman rank correlation test.

Results: The frequency of positive methylation-specific PCR bands for APC, GSTP1, and MDR1 genes in prostate cancer samples was 64.1%, 54.0%, and 55.3%, respectively. In benign prostatic hyperplasia samples, it was 8.7%, 5.8%, and 11.6%, respectively. There was a significant correlation of M score with high pT category ($P < 0.001$), high Gleason sum ($P < 0.001$), high preoperative PSA ($P = 0.027$), and advanced pathologic features. For all patients, the M score had a sensitivity of 75.9% and a specificity of 84.1% as a diagnostic biomarker using a cutoff value of 1.0. In patients with low or borderline PSA levels ($<10.0$ ng/mL), the M score was significantly higher in prostate cancers than in benign prostatic hyperplasias (2.635 ± 0.200 and 0.357 ± 0.121, respectively). 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. For all patients, M score can distinguish organ-confined ($\leq pT2$) from locally advanced cancer ($pT3$) with a sensitivity of 72.1% and a specificity of 67.8%. Moreover, considering patients with PSA levels of ($<10$ ng/mL, the M score has a sensitivity of 67.1% and a specificity of 85.7%. The ROC curve analysis showed a significant difference between M score and PSA ($P = 0.010$).

Conclusions: This is the first report demonstrating that M score is a new method for multigene methylation analysis that can serve as a good diagnostic and staging biomarker for prostate cancer.
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.

**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 endpoint, 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'-TAATTTTTTTCCTGTTGTGCGGATT-3' (sense), 5'-ACTACCAACCAGGAACTATC-3' (antisense); MSP primers, 5'-TATCTGGGAGTGGCGGTGTC-3' (sense), 5'-TCAGAAGATGCCACACGAC-3' (antisense); USP primers, 5'-CTGTGTTTATGTTGAGTGGATTGTT-3' (sense), 5'-GCCAACAGAAATCTCAGGCCAC-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 1. Clinical characteristics of prostate cancer and benign prostate hyperplasia patients

<table>
<thead>
<tr>
<th></th>
<th>Prostate cancer</th>
<th>Benign prostate hyperplasia</th>
</tr>
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<tbody>
<tr>
<td>Total number</td>
<td>170</td>
<td>69</td>
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<tr>
<td>Median age, y (range)</td>
<td>68.6 (49-80)*</td>
<td>75 (54-87)*</td>
</tr>
<tr>
<td>pT category</td>
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</tr>
<tr>
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<td>0</td>
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<td>pT4</td>
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<td></td>
</tr>
<tr>
<td>&gt;7</td>
<td>50</td>
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<td>Preoperative serum PSA</td>
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<td>&lt;4.0</td>
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<td>4.0-9.9</td>
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<tr>
<td>&gt;10.0</td>
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<td>Benign prostate hyperplasty</td>
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<td></td>
</tr>
<tr>
<td>Total number</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>Median age, y (range)</td>
<td>75 (54-87)*</td>
<td></td>
</tr>
</tbody>
</table>

*The median age of patients with benign prostate hyperplasia is statistically higher than those with prostate cancer (P < 0.001).*
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 \textit{GSTP1} and \textit{MDR1} were described previously (15, 16).

\textbf{Bisulfite DNA sequencing analysis.} Bisulfite-modified DNA (1 \textmu L) was amplified using a pair of universal primers in a total volume of 20 \textmu L. 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).

\textbf{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 \textit{U} test, Kruskal-Wallis test, or the Spearman rank correlation test using StatView software (SAS Institute Inc., Cary, NC). A \textit{P} value of <0.05 was regarded as statistically significant.

\textbf{Results}

\textbf{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

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Log HR*</th>
<th>SE</th>
<th>( \chi^2 )</th>
<th>( P )</th>
<th>HR*</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
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<td>APC</td>
<td>2.028</td>
<td>0.521</td>
<td>15.125</td>
<td>&lt;0.001</td>
<td>7.597</td>
<td>2.734-21.112</td>
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<tr>
<td>GSTP1</td>
<td>1.777</td>
<td>0.637</td>
<td>7.774</td>
<td>0.005</td>
<td>5.913</td>
<td>1.695-20.627</td>
</tr>
<tr>
<td>MDR1</td>
<td>1.178</td>
<td>0.505</td>
<td>5.450</td>
<td>0.020</td>
<td>3.247</td>
<td>1.208-8.729</td>
</tr>
</tbody>
</table>

\textit{NOTE:} All data are adjusted by age.

\textit{Abbreviations:} HR, hazard ratio; CI, confidence interval.

\*M score is determined as the sum of log HR for each sample.
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 \( \pm \) 0.158 and 0.357 \( \pm \) 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 \( \pm \) 0.200 and 0.357 \( \pm \) 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 \( \pm \) 0.26 in \( pT2a \), 2.92 \( \pm \) 0.24 in \( pT2b \), 3.84 \( \pm \) 0.26 in \( pT3a \), 4.21 \( \pm \) 0.62 in \( pT3b \), and 4.98 \( \pm \) 0 in \( pT4 \); \( P < 0.001 \); Fig. 3A). Similarly, the M score increased as the Gleason sum increased (2.20 \( \pm \) 0.23, Gleason sum \( <7 \); 3.58 \( \pm \) 0.25, Gleason sum = 7; and 3.819 \( \pm \) 0.29, Gleason sum \( \geq 8 \); \( P < 0.001 \); Fig. 3B). With regard to preoperative PSA levels, the M score was higher in PSA >10 ng/mL (3.35 \( \pm \) 0.121, respectively; Fig. 2B, left). ROC curve analysis revealed that the M score was higher in advancing pathologic features as follows: in capsular invasion (Cap) [positive (3.80 \( \pm \) 0.23) versus negative (2.47 \( \pm \) 0.19; \( P < 0.001 \)], in seminal vesicle involvement [positive (4.88 \( \pm \) 0.11) versus negative (2.80 \( \pm \) 0.16; \( P = 0.002 \)], in pelvic lymph node metastasis (pN) [positive (4.60 \( \pm \) 0.38) versus negative (2.57 \( \pm \) 0.19; \( P < 0.001 \)], in venous involvement (v) [positive (3.92 \( \pm \) 0.22) versus negative (2.44 \( \pm \) 0.19; \( P < 0.001 \)], and in perineural invasion (PNI) [positive (3.41 \( \pm \) 0.18) versus negative (1.86 \( \pm \) 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).
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 (≤pT2) and locally advanced cancer (≥pT3), 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
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. 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.

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 (≤pT3) from locally advanced cancer (>pT3). 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 (≤pT2) from locally advanced prostate cancer (>pT2). 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 multigens 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.

References
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