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Hypermethylation of Genes for Diagnosis and Risk Stratification of Prostate Cancer

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Abstract

To identify the relevant CpG sites as molecular markers, for the diagnosis and to distinguish the indolent and aggressive prostate tumors, we have determined the methylation status of 8 genes, including FLNC, EFS, ECRG4, RARB2, PITX2, GSTP1, PDLIM4, and KCNMA1 in 32 nonrecurrent, 32 recurrent primary prostate tumors, and 32 benign prostate tissues using EpiTYPER technology. Specific CpG site hypermethylation of RARB2 and GSTP1 CpG sites were useful for diagnosis of prostate cancer. Furthermore, CpG site hypermethylation of genes FLNC, EFS, ECRG4, PITX2, PDLIM4, and KCNMA1 were associated with prediction of biochemical, local, and systemic recurrence of prostate cancer.

Keywords

CpG site methylation; Mass spectrometry assay; Prostate cancer; Recurrence prediction; Molecular markers

INTRODUCTION

Biochemical (prostate-specific antigen) recurrence of prostate cancer after radical prostatectomy remains a major problem (1). Those who experience early PSA recurrence, particularly within the first 2 years of surgery, are prone to develop metastatic lesions and are likely to succumb to their prostate cancer (2,3). Several clinicopathologic scoring systems have been developed to identify the patients at greatest risk of recurrence after surgery, including the weighted risk of recurrence, determined by the lymph node status, seminal vesicle status, surgical margin status, and postoperative Gleason score and the Kattan nomograms (4–7). These factors are helpful but far from perfect due to significant clinical heterogeneity of the disease. However, many patients defined as being at low risk still develop cancer recurrence (8–12). Clearly, new biological markers are urgently needed to predict more accurately the risk of relapse.

Gene silencing due to DNA methylation of promoter CpG islands (CGIs) is one of the major mechanisms of tumor-suppressor gene inactivation, along with mutations and loss of heterozygosity (13). DNA methylation plays an important role in cancer control and provides

potential molecular markers to assess cancer risk, improve cancer detection, monitoring of cancer prognosis, and predict therapy responses (14,15). Reversal of tumor-associated silencing of tumor suppressor genes is increasingly being targeted for cancer treatment and prevention strategies (16–19). Integration of the methylation status of multiple genes as methylation score was reported to serve as a potential diagnostic and staging biomarker for prostate cancer (20). Detection of altered methylation has a vast diagnostic and prognostic potential but requires a comprehensive characterization of the methylation patterns in tumor and normal tissues to determine which hypermethylation events are disease specific. However, such studies are limited in the literature. The ability to predict the outcome or recurrence of prostate cancer is a worthwhile goal that will enable physicians to tailor treatment recommendations for patients.

Promoter regions of several genes have been found to be hypermethylated in prostate cancer using conventional methylation-specific PCR (MSP) method (20–22). The method only allows assessment of the presence or absence of methylation in a limited region of about 100–150 bases and the MSP product is generated only when the entire amplicon is methylated. Consequently, this will not identify the regions with methylation variations in the amplicon. Other methods, such as semiquantitative real-time PCR and methylation microarrays, are limited to a restricted number of CpG sites for analysis. Here, we analyzed the methylation profiles in 64 prostate cancer tissues from patients with and without disease recurrence after radical prostatectomy and in 32 benign prostate tissues obtained after radical prostatectomy by a high-throughput Mass-CLEAVE/MassARRAY method (23). This method of methylation analysis by base-specific cleavage in combination with matrix assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF-MS) detection has enabled us to quantitatively measure the individual CpG unit methylation in more than 500 bases, multiplexing with 20 ng of DNA and at a detection limit approximately 5% methylated DNA (23). In this method, bisulfite-modified DNA is PCR amplified followed by *in vitro* transcription to generate single-stranded RNA molecule. The RNA strand is then cleaved base specifically and the resulting cleavage products were analyzed by MALDI-TOF MS. This is a rapid and cost-effective quantitative analysis of methylation at individual CpG sites.

We evaluated the promoter CpG unit methylation status of 8 genes. This includes a total of 2,900 base pair regions containing 238 CpG sites within 136 CpG units of the genes filamin C (FLNC), embryonal Fyn-associated substrate (EFS), esophageal cancer-related gene 4 protein (ECRG4), retinoic acid receptor beta 2 (RARβ2), paired-like homeodomain transcription factor 2 (PITX2), glutathione s-transferase pi (GSTP1), PDZ and LIM domain 4 (PDLIM4), and potassium large conductance calcium-activated channel, subfamily m, alpha member 1 (KCNMA1). We identified the specific CpG sites methylation for diagnosis and progression of prostate cancer. We assessed the correlations of methylation score for diagnosis of prostate cancer (M_{dp}) and methylation score for recurrence of prostate cancer (M_{rp}) with the clinical and histopathological outcome.

MATERIALS AND METHODS

Prostate tissue samples

Surgically resected prostate cancer tissue specimens were obtained from patients who had undergone radical prostatectomy from 1999 to 2003 without prior therapy for prostate cancer at Mayo Clinic with the Institutional Review Board approval (22). Fresh frozen prostate cancer specimens were trimmed to obtain tissue sections containing >70% tumor nuclei. We used organ-confined prostate cancer tissues of patients who were free of disease for >5 years after prostatectomy ($n = 32$ nonrecurrent) and primary prostate cancer tissues of patients who had recurrence of the disease within 5 years of prostatectomy ($n = 32$), which included PSA recurrences ($n = 10$), local recurrences ($n = 10$), and systemic progressions cases ($n = 12$) (Table

1). Separately collected nonmalignant benign prostatic hyperplasia tissues ($n = 32$) obtained after suprapubic prostatectomy were used as controls. Nonrecurrence cases were defined as the prostate cancer patients without any recurrence after radical prostatectomy for >5 years of follow-up, with serum PSA levels <0.2 ng/mL. PSA recurrence was defined as the first postprostatectomy serum PSA value 0.2 ng/mL or above, followed by a value higher than the first (24–27) and excluding any PSA within the first 30 days of surgery. Local recurrence was defined as cancer demonstrated on biopsy of the prostatic bed or the receipt of salvage radiation therapy to the prostatic bed without evidence of systemic recurrence. Systemic progression involved demonstrable metastatic deposits on radionuclide bone scan or on biopsies other than biopsy of the prostatic bed (28).

DNA isolation and bisulfite conversion

DNA was isolated from 10 tissue sections of 20- μ m thickness using the ZR Genomic DNA II kit (Zymo Research Corp., Orange, CA, USA). Bisulfite treatment of genomic DNA was performed with a commercial kit from Zymo that combines bisulfite conversion and DNA cleanup (23). The converted DNA was measured using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA).

Selection of genomic targets for analysis

From our previous gene expression profiling of prostate cancer tissues (21,22), we selected the genes FLNC, EFS, ECRG4, and KCNMA1 for methylation analysis primarily because they were most significantly under expressed in organ-confined and metastatic tumors (study name, Vanaja Prostate; www.oncomine.org) and also for the high frequency of CG content in the predicted promoter regions with CpG islands (e.g., <http://www.cpgislands.com>) (29,30) (%GC \Rightarrow 70 and Obs/ExpCpG \Rightarrow 0.75). In addition, four genes GSTP1 (31), PDLIM4 (21), RARB2 (32), and PITX2 (33) were selected from the literature reported to be hypermethylated in prostate cancer. Methylation primers were designed using the MethPrimer software (<http://www.ucsf.edu/urogene.org/methprimer>) (34). Primers were selected from the CpG island regions with homogenous CpG site methylation patterns in the amplicon sequence. The target region of the genes used for methylation analysis (Table 2) and the primer sequences used for PCR amplification were shown (Table 3). One of the two primers in the PCR amplification of the target regions is tagged with a T7 promoter sequence: cagtaatacgactcactatagggagaaggct. This includes ggg transcription start and an 8-bp insert (agaaggct) on the 5' end.

PCR and in vitro transcription

The PCR reactions were carried out in a total volume of 5 μ L using 1 pmol of each primer, 40 μ M dNTP, 0.1 U HotStar Taq DNA polymerase (Qiagen Inc., Valencia, CA), 1.5 mM MgCl₂, and 5 \times PCR buffer (final concentration 1 \times). The reaction mix was preactivated for 15 min at 95°C. The reactions were amplified in 45 cycles of 95°C for 20s, 62°C for 30s, and 72°C for 30s followed by 72°C for 3 min. Unincorporated dNTPs were dephosphorylated by adding 1.7 μ L DNase free water and 0.3 U Shrimp Alkaline Phosphatase (SAP) (Sequenom, Inc., San Diego, CA, USA). The reaction was incubated at 37°C for 20 min and SAP was then heat inactivated for 10 min at 85°C. Typically, 2 μ L of the PCR reaction were directly used as template in a 6.5 μ L combined transcription-cleavage reaction. Twenty units of T7 R&DNA polymerase (Epicentre, Madison, WI, USA) were used to incorporate either dCTP or dTTP in the transcripts. Ribonucleotides at 1 mM and the dNTP substrate at 2.5 mM were used. RNase A (Sequenom) was included to cleave the *in vitro* transcript. The mixture was then further diluted with water to a final volume of 27 μ L. Conditioning of the phosphate backbone prior to MALDI-TOF MS was achieved by the addition of 6 mg CLEAN resin (Sequenom).

Mass spectrometry measurements

The cleavage reaction samples (15 nL) were dispensed onto silicon chips preloaded with matrix (SpectroCHIP, Sequenom). Mass spectra were collected using a Mass ARRAY mass spectrometer (Sequenom). Spectra were analyzed using proprietary peak picking and spectra interpretation tools (EpiTYPER, Sequenom). For analysis of DNA methylation, we examined the methylation-dependent C/T sequence changes introduced by bisulfite treatment. Those C/T changes are reflected as G/A changes on the reverse strand and hence result in a mass difference of 16 kDa for each CpG site enclosed in the cleavage products generated from the RNA transcript. The mass signals representing nonmethylated DNA and those representing methylated DNA built signal pairs, which are representative for the CpG sites within the analyzed sequence substring. The intensities of the peaks were compared, and the relative amount of methylated DNA was calculated from this ratio. The method yields quantitative results for each of these sequence defined analytic units referred as CpG unit, which contain either one individual CpG site or an aggregate of subsequent CpG sites.

Statistical analysis

The quantitative methylation of each CpG unit measured by the MALDI-TOF-MS assay was filtered to exclude poor-quality and nonvariable data. For the prediction analysis, only the CpG units with values in at least 75% of the 96 samples were selected. This resulted in 101 CpG units from 8 genes. We calculated methylation score for diagnosis of prostate cancer (Mdp score) accounting for the hypermethylation in tumor samples compared with benign tissues and methylation score for recurrence of prostate cancer (Mrp score) separately for PSA recurrence, local recurrence, and systemic recurrence versus nonrecurrence cases, as well as all the recurrence groups compared with nonrecurrence group, as a prediction from a diagonal linear discriminant analysis (DLDA) using combinations of the CpG unit methylation of various genes (35–37). The methylation scores Mdp and Mrp for each sample were defined as the difference of the distance for each class of the methylation value for each CpG weighted by the class average CpG methylation and further divided by the class methylation variance to half the class average CpG unit methylation. The classes were defined as the sum of the corresponding log hazard ratio coefficients, which were derived from multivariate logistic regression analysis of each methylated gene in the benign versus prostate cancer samples and tumor nonrecurrence versus recurrence groups.

Methylation scores of Mdp and Mrp were based on training and test sets of the samples. For the Mdp score calculation, we used CpG feature selection based on the order of p values obtained from paired t -tests comparing tumors versus benign tissues. Both the benign and the tumor samples were randomly divided into two equal groups. Group-1 samples were then used as the prediction training set and group-2 samples were used as the test set to obtain the area under the curve (AUC) performance of the group-2 tumor samples. For the Mrp score calculation, we used CpG feature selection based on the unequal-variance t -tests for the difference in the methylation values of nonrecurrence tumors compared with the recurrence tumor group. DLDA models were built using respective training sets for all increasing combinations of the ranked CpG units beginning with the top two most significant CpG units and increasing until all the CpG units were included in the model. The numbers of CpG units to include in the final Mdp and Mrp score models were determined by the first local maximum AUC value with at least 5 CpG units and at most 20 CpG units. The final Mdp and Mrp scores were the DLDA-predicted values using the respective number of CpG units indicated as the optimal from the test set and using all the samples. The number followed by the CpG unit represents the CpG sites in the annotated gene sequence. The correlation of methylation score and clinicopathologic findings were analyzed using Spearman's rank correlation test. A p value of $<.05$ was considered as statistically significant.

RESULTS

Methylation score for diagnosis of prostate cancer

To determine the methylation score for diagnosis of prostate cancer, we evaluated methylation profiles of genes in 64 tumor samples and 32 benign samples. The individual 17 CpG unit methylation of genes that distinguish tumor from the benign tissues were depicted in the cluster diagram [Figure 1(A)]. The number followed by the CpG unit represents the CpG sites in the annotated gene sequence of the amplicon. The mean methylation levels of these CpG units were significantly increased in tumor group compared with the benign [Figure 1(B)]. The DLDA model with the local maximum of 0.9209 at top 13 CpG units using group-2 benign and tumor samples as the test sets was chosen as best fit for the Mdp score. The distribution of methylation similarity score, a cumulative score of the CpG units for diagnosis of prostate cancer, was shown in 32 benign and 64 tumor tissues [Figure 1(C)]. The Mdp score was higher in the tumor tissues when compared with the benign samples, which enabled us to easily distinguish the tumor from the benign tissues. A significant increase in the Mdp score ($p < .05$) was observed in both preoperative serum PSA of less than 10 ng/mL and greater than 10 ng/mL groups of the cancer patients [Figure 1(D)]. To assess the potential utility of hypermethylation of genes as molecular markers of prostate cancer, we determined the optimal sensitivity and specificity of the CpG units methylation by receiver-operating characteristic (ROC) curves. The sensitivity and specificity of the individual CpG units and the Mdp score for diagnosis of cancer was shown in Table 4. CpG sites 10 and 11 of RARB2 gene were the top most significantly ranked, with a sensitivity and specificity of 90.0% and 92.59%, respectively, for diagnosis of prostate cancer.

Methylation score for prediction of prostate cancer recurrence

To test the ability of CpG site methylation for prediction of recurrence, we compared 32 organ-confined prostate cancer tissues without recurrence of disease after radical prostatectomy and 32 primary prostate cancer tissues with recurrence of the disease. We found that methylation of 19 CpG units of genes and their mean methylation levels were significantly different in the recurrence group compared with the nonrecurrence group [Figures 2(A) and (B)]. The DLDA model with the local maximum of 0.600 at the top 11 CpG units using the recurrence tumor samples was chosen as best fit for Mrp score. The distribution of methylation similarity score for recurrence prediction was shown in recurrence and nonrecurrence tissues [Figure 2(C)]. A significant increase in the Mrp score was observed in local and systemic recurrence cases [Figure 2(D)]. Mrp score to predict the local and systemic recurrence showed sensitivities of 80.0% and 72.7% and specificities of 81.2% and 75.0%, respectively (Table 5).

Correlation of Mdp and Mrp scores with the clinicopathologic factors of prostate cancer

To evaluate methylation scores as an independent or a supplementary approach to the clinicopathologic parameters for diagnosis of prostate cancer and prediction of recurrence, we performed Spearman's rank correlation test to study the association of Mdp and Mrp scores with the preoperative serum PSA levels, Gleason score, and GPSM score. A GPSM score is a prognostic model using the weighted sum of the pathological Gleason score, preoperative PSA, seminal vesicle involvement, and marginal status to predict biochemical progression after radical prostatectomy. The 5-year progression-free survival was 95% for scores <5 , 60% for 10, and 32% for >12 (8). This predictive model allows identification of patients who are at high risk for cancer progression. Our analysis revealed that both Mdp and Mrp scores had a significant correlation with PSA levels ($p = .00086$ and $p = .000054$, respectively). However, significant correlation with pathological Gleason score was observed for only Mrp score ($p = .004$). The Mrp score is statistically higher in the cases having a Gleason score of 8 or 9 (unequal-variance t -test; $p = .005$) compared with those with Gleason score of 6 ($p = .28$) [Figure 3(A)]. Higher Mrp score was observed in patients with higher GPSM scores; however, this trend was

not statistically significant [Figure 3(B)], which suggests that Mrp score is an independent predictor of progression. Mrp score was highly significant with the overall recurrence of cancer ($p = .00049$). Unequal-variance t -tests were used to determine which recurrence groups were statistically different. Both Mdp and Mrp scores were significantly higher in local ($p = .017$ and $p = .0016$) and systemic recurrence cases ($p = .014$ and $p = .02$) compared with the nonrecurrent tumors; however, they were not significant in cases with only PSA recurrence. The ploidy of tumor tissues did not show any correlation with either Mdp or Mrp scores.

Correlation of recurrence with the clinicopathological factors

We further analyzed the association of prostate cancer recurrence with the Gleason score, GPSM score, and Mrp score. Gleason score based on pathology was significantly higher in local recurrent cases compared with the nonrecurrent cases (unequal-variance t -test; $p = .0032$), similarly for systemic recurrence and nonrecurrent cases ($p = .00057$). The mean GPSM score in PSA, local, and systemic recurrence groups was higher than the score in the nonrecurrent group ($p = .00174$) [Figure 3(C)]. A significant difference in the GPSM score was observed between the nonrecurrent and the systemic recurrence cases ($p < .0001$). ROC curve analysis revealed that the combination of Mrp score with GPSM score and the preoperative PSA levels has shown an augmentation of sensitivity (80.0%) and specificity (81.2%) with AUC = 0.852 for prediction of recurrence when compared with the individual parameters of risk assessment [Figure 3(D)].

DISCUSSION

Methylation in the promoter region of the genes plays an important role in regulation of gene expression. Rapid and sensitive detection of DNA methylation changes would aid in diagnosis and risk stratification of cancer (15–17). Methylation patterns in the process of tumorigenesis are often very heterogeneous. Methods to analyze the DNA methylation have been hampered by limitations such as no quantification of the methylation variable regions, limited number of analyzed CpG sites, vary labor-intensive methods, and not quantifying the individual CpG site methylation. Our results demonstrate that EpiTYPER technology is able to overcome these limitations. Methylation analysis using the MassARRAY system is an ideal tool for discovery of methylation, for discrimination between methylated and unmethylated regions, and for quantifying the methylation levels to follow the progression of cancer. We quantitatively assessed the methylation of the genes that are underexpressed in prostate cancer (21,38–41), and identified specific CpG site methylation changes of genes associated with development and progression of prostate cancer.

Methylation of genes, GSTP1, PDLIM4, and RARB2 has been reported in prostate cancer by using MSP method (42–45). We have identified methylation of 17 CpG units of genes, RARB2, GSTP1, PITX2, and FLNC that showed a significant difference in benign prostatic hyperplasia and prostate cancer. We determined the methylation scores for diagnosis of prostate cancer with a sensitivity and specificity of 87.3% and 87.1%, respectively. Enokida et al. were the first to demonstrate multi-gene methylation score (M score) by MSP method as a tool for prostate cancer detection with a sensitivity and specificity of 75.9% and 84.1%, respectively, for the genes GSTP1, APV, and MDR1 (20). The increase in sensitivity and specificity in our study might be due to the identification of specific CpG sites methylation that are of high relevance to the cancer. Our finding highlights the necessity for a comprehensive quantitative methylation analysis before developing a high-throughput assay targeting only a small number of potential methylation sites for implementation in clinical diagnostics. The functional consequences of this variation, of CpG sites methylation, in molecular terms (e.g., accessibility for transcription factors) have to be addressed in future studies. In the prostate cancer patients with PSA levels of 10 ng/mL or less, the Mdp score was significantly increased ($p \leq .05$)

compared with adjacent benign tissues. These results indicate that analysis of the CpG site methylation of genes in patients with marginal PSA elevation would serve as adjuvant molecular markers for diagnosis of prostate cancer.

Predicting the probability of recurrence will enable us to distinguish between patients who have an indolent cancer not requiring any form of treatment and patients with biochemical relapse who may have a more aggressive course requiring early intervention. Therefore, we compared the methylation patterns in 32 indolent tumors without recurrence of cancer and 32 aggressive tumors with recurrence of disease within 5 years of prostatectomy. We further evaluated the methylation status of the CpG sites to determine their ability to add to the known risk factors in predicting the progression of prostate cancer. Our results showed statistically significant differences in the frequency of methylation at individual CpG units of PITX2, PDLIM4, KCNMA1, GSTP1, FLNC, EFS, and ECRG4 in recurrence and nonrecurrence subtypes of tumors. We found that Mrp score is significantly higher in more advanced tumors in the following order: systemic recurrence → local recurrence → biochemical recurrence → nonrecurrent cases.

GSTP1 promoter hypermethylation is an early event in prostate carcinogenesis (46–49). There are some controversial reports about GSTP1 methylation with the stage and grade of the prostate cancer (43,50–53). In our study, the CpG sites for all the genes used to derive the Mdp and Mrp scores were different, except for the two CpG sites of GSTP1 (GSTP1 CpG site 19 and 21) that appeared in both Mdp and Mrp scores. The observed difference in the CpG site methylation of GSTP1 associated with recurrence of cancer needs further investigation. We found a significant hypermethylation of RARB2 in benign versus cancer tissues and not in recurrent versus nonrecurrent tumors that indicates that RARB2 methylation could be an early event in the prostate cancer development. The genes FLNC, EFS, KCNMA1, and ECRG4 were significantly down-regulated in our and other microarray expression profiling of prostate cancer tissues ($p < .0001$) (21,38,54). FLNC is an actin-cross-linking protein involved in reorganizing the actin cytoskeleton. Hypermethylation of FLNC in gastric mucosa was reported to correlate with higher risk levels of gastric cancers (55). PITX2, the homeodomain transcription factor methylation, was reported to predict the risk of distant disease recurrence in tamoxifen-treated, node-negative breast cancer patients (56). Our results revealed that 15 CpG sites of the FLNC in the 8 CpG units were highly methylated ($p < .042$) in local recurrence cases. The methylation of FLNC and PITX2 in prostate cancer might have practical implication for predicting the risk of local recurrence. EFS is a docking protein that plays a central coordinating role for tyrosine-kinase-based signaling related to cell adhesion (57). For prediction of systemic progression, we found that methylation of CpG sites were restricted to FLNC and EFS ($p \leq .03$) genes involved in cell attachment. ECRG4 is a tumor suppressor gene downregulated by promoter methylation during the development and progression of esophageal cancer (58). This is the first report to show that methylation of FLNC, PITX2, EFS, and ECRG4 is associated with recurrence of prostate cancer. These results warrant further validation of the methylation status of these genes in a large and unselected cohort of patients to follow the progression of cancer.

The risk of recurrence and metastatic progression for prostate cancer patients who undergo radical prostatectomy can be estimated in relative accuracy based on PSA doubling time and GPSM score. Multigene methylation analysis of GSTP1, APC, and MDR1 was reported to correlate with higher Gleason sum, higher preoperative PSA, and other advanced pathological features (20). In our study, Mrp score showed a strong correlation with worse clinicopathologic features such as higher Gleason sum, higher preoperative PSA values, local, and systemic recurrence. Remarkably, the combination of Mrp score with preoperative PSA and GPSM score improved the theoretical prediction of recurrence. We also observed an apparent association of the Mrp score with the GPSM score, although not statistically significant. The higher GPSM

score associated with systemic recurrence cases in our current study confirmed with our previous report that GPSM score is an independent predictor for progression of prostate cancer (12). The results of this study suggest that DNA methylation analysis could augment the ability of currently available predictors of prostate cancer progression. However, this study was not able to identify any CpG site methylation that might be associated with PSA doubling time or mortality with prostate cancer because the vast majority of samples were right censored at the time of data analysis. In this study design, patients were selected on the basis of the end point outcome of cancer; therefore, the analysis of survival curves would not be appropriate to consider. These findings warrant further analysis of the genes in a large study cohort to explore the implications of the CpG site methylation for prediction of prostate cancer risk of recurrence.

In conclusion, our study showed for the first time nonrandom and differential CpG site methylation patterns between nonrecurrence and recurrence tumor tissues. The correlation of PITX2, FLNC, and EFS CpG sites methylation with the malignant progression needs further investigation. These results suggest that analyzing the individual CpG site methylation of genes will identify tumor-type-specific changes associated with risk stratification of patients. The strength of the methylation score depends on the number of the genes analyzed simultaneously. Incorporating the methylation status of additional candidate genes into the Mdp and Mrp scores possibly further improve the sensitivity and specificity for diagnosis of cancer and assessment of progression.

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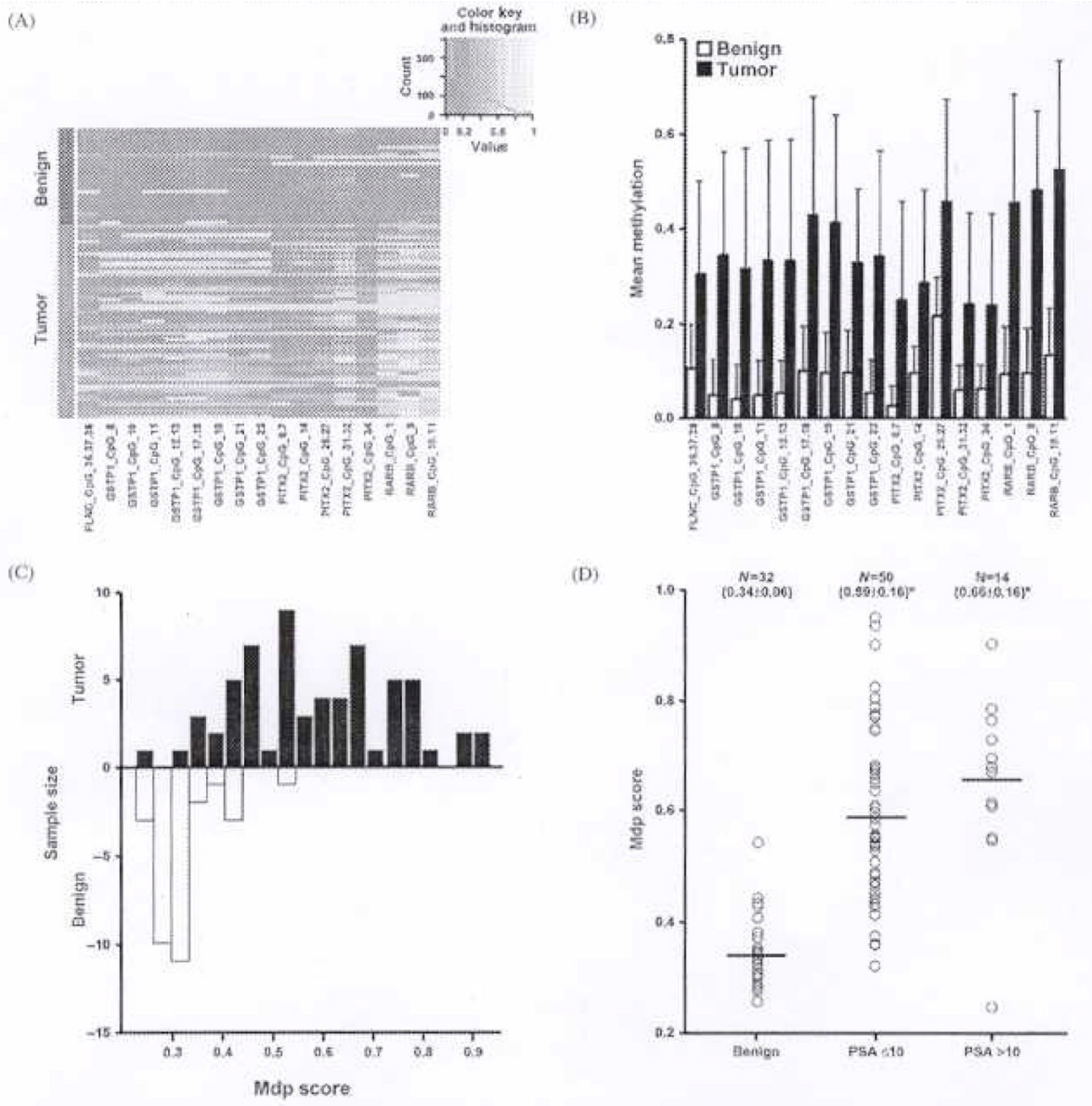


Figure 1. (A) Cluster diagram depicting CpG units methylation of genes in benign ($n = 32$) and tumor ($n = 64$) tissues. Each row represents a tissue and each column a CpG unit of the gene. Frequency of methylation levels is color scaled from red to yellow. Red denotes no methylation, while bright yellow denotes complete methylation. Gray represents technically inadequate or missing data. (B) Group mean methylation of the CpG unit of genes in the benign and tumor tissues. (C) Distribution of methylation similarity scores for diagnosis of prostate cancer (Mdp) in benign (black bars) and tumor tissues (white bars). (D) Mdp score in benign and tumor tissues with preoperative serum PSA value of <10 ng/mL and >10 ng/mL groups. $n =$ number of tissues in each group. Mean and standard deviation are shown in parentheses. $*p < .05$.

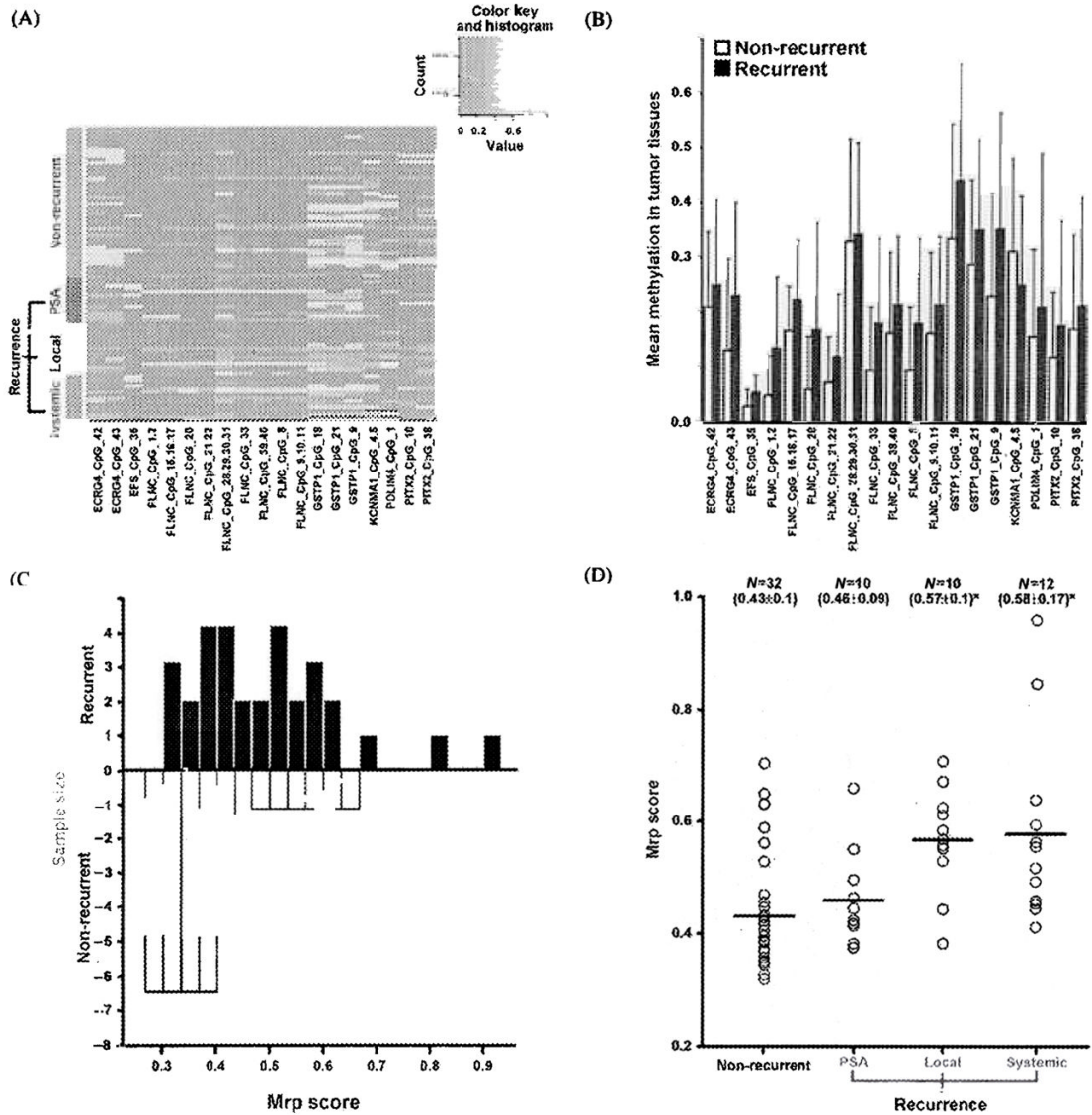


Figure 2. (A) Cluster diagram depicting CpG units methylation of genes in nonrecurrence and recurrence tumor tissues. Each row represents a tissue and each column a CpG unit of the genes. Green represents nonrecurrent ($n = 32$) tumors. Blue, yellow, and red represents recurrent tumors ($n = 32$ total) with PSA failure ($n = 10$), local recurrence ($n = 10$), and systemic progression ($n = 12$), respectively. Frequency of methylation levels is color scaled from red to yellow. Red denotes no methylation, while bright yellow denotes complete methylation. Gray represents technically inadequate or missing data. (B) Group mean methylation of the CpG units of genes in nonrecurrence and recurrence tumors (p value < 0.05). (C) Distribution of methylation similarity scores for recurrence of prostate cancer (Mrp) in nonrecurrence (black bars) and recurrence (white bars) tumor tissues. (D) Mrp score in nonrecurrent and recurrent tumors with PSA failure, local, and systemic progression. $n =$ number of tissues in each group. Mean and standard deviation are shown in parentheses. $*p < .05$.

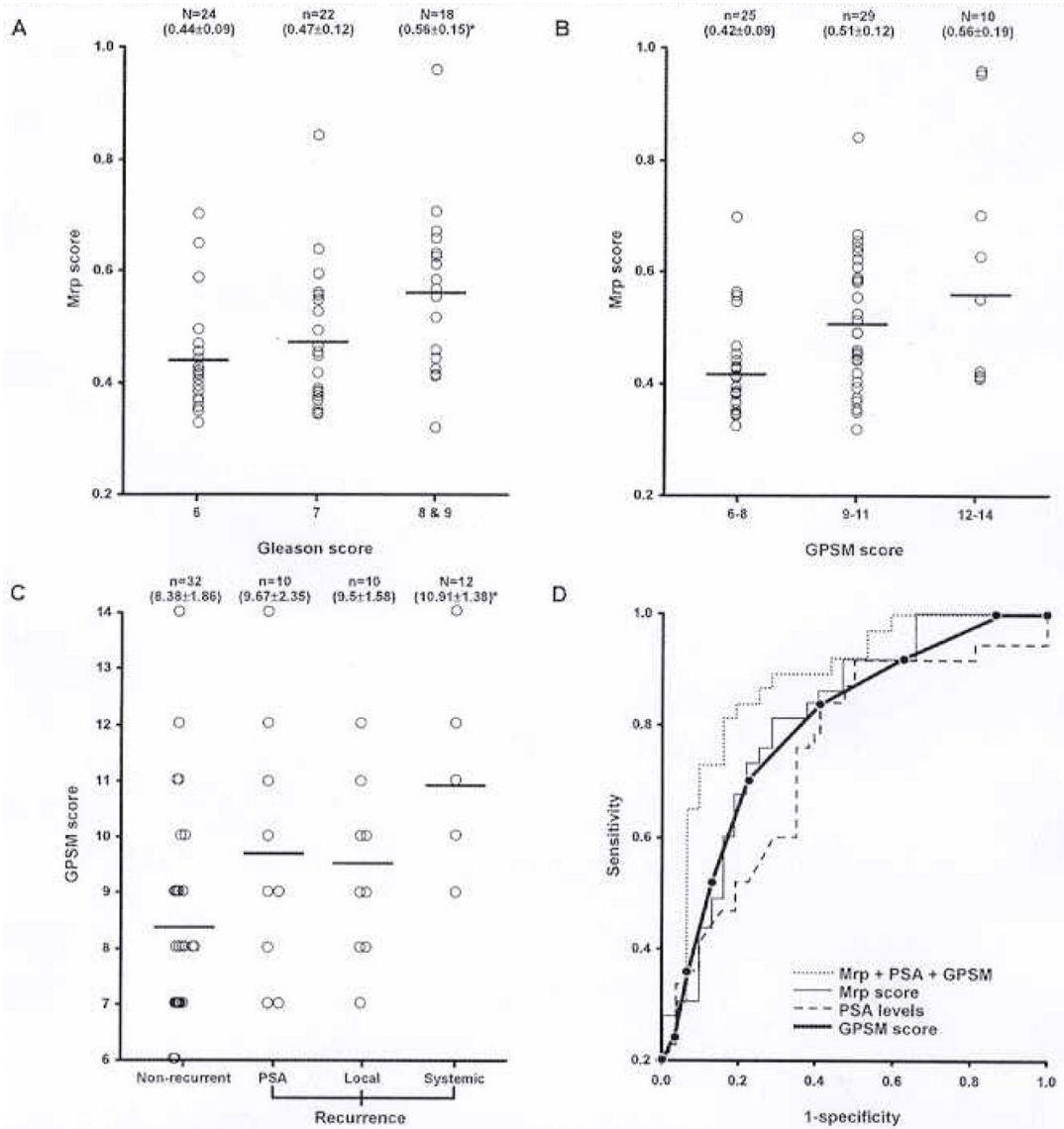


Figure 3.

(A) Correlation of Mrp score with the Gleason score of prostate cancer tissues. Each circle represent a tumor tissue in the arbitrarily grouped Gleason score. (B) GPSM score of the tumor tissues in nonrecurrence and recurrence tumors. (C) Relationship of the Mrp score with GPSM score. Horizontal bars represent the mean in each group. *n* = number of samples. Mean and standard deviation are shown in parentheses. **p* < .05. (D) ROC curves for prediction of recurrence with GPSM score, preoperative PSA, Mrp score, and the combination. GPSM score: Sensitivity = 63.3%, specificity = 78.1% (AUC = 0.755). Preoperative PSA: Sensitivity = 66.7%, specificity = 65.6% (AUC = 0.686). Mrp score: Sensitivity = 71.0%, specificity = 71.9% (AUC = 0.773). Combined: Sensitivity = 80.0%, specificity = 81.2% (AUC = 0.852).

Table 1

Prostate tissues used for methylation analysis

Tissue Group	Total Number	Mean PSA Recurrence Years	Mean Local Recurrence Years	Mean Systemic Recurrence Years	Mean Gleason Score	Mean GPSM Score
Benign prostate	32					
Prostate cancer						
Nonrecurrence (>5 years of follow-up)	32				6.53	8.37
Recurrence (within 5 years of follow-up)	32					
PSA recurrence	10	1.71 years (n = 10)			7.1	9.6
Local recurrence	10	1.87 years (n = 10)	1.75 years (n = 10)		7.6	9.5
Systemic recurrence	12	0.677 years (n = 12)		1.51 years (n = 12)	8.1	

Value in parentheses indicates the number of patients in the recurrence group who developed PSA and local and systemic recurrence, respectively.

Table 2

Target region of the genes used for methylation analysis

Gene	Length (bp)	CpG Sites	CpG Units	Chromosomal Location	Amplicon Location (Transcription Start Site)
FLNC	363	40	20	chr7: 128257721 – 128258084	Plus 2 to plus 365
EFS	371	46	19	chr14: 22904250 – 22904621	Minus 432 to minus 61
ECRG4	505	48	21	chr2: 106048379 – 106048884	Minus 166 to plus 339
RARB2	391	14	12	chr3: 25444596 – 25444987	Minus 162 to plus 229
PITX2	424	37	24	chr4: 111762893 – 111763317	Plus 14640 to plus 15064
GSTP1	347	23	19	chr11: 67107924 – 67108271	Plus 62 to plus 409
PDLIM4	238	11	7	chr5: 131621562 – 131621800	Plus 276 to plus 514
KCNMA1	261	19	14	chr10: 79066039 – 79066300	Minus 1544 to minus 1283

CpG sites present in the CpG units of the sequence analyzed are listed with the corresponding amplicon location of the sequence and the amplicon location relative to the transcription start site.

Table 3

Primers used for PCR amplification of the bisulfite-converted DNA

Gene	Forward Primer	Reverse Primer
FLNC	TTTGGAGGGAGAGAGAGTTAGAGAG	CACTTAAAATACTCATTACACCAAC
EFS	GGTAGGTTAGTTAGGTAGGAGGAGGAG	CAAACCCCAACCCCTAAAAACAC
ECRG4	GTGGTTAAGTATTTTTGGTTTTGGAGTTTA	ATCCTCTACCACCCCTTAACCCTAC
RARB2	TTGGTTGTTTGTTTTTGTAGGGTTGT	ATTCCCAAAAAATCCCAAATTCTC
PITX2	GGTGTGTATTTTTAGTTTGTGTTTGGAG	AATACCCTTCTACCCACATCCCATAT
GSTP1	GGGGTTTAGAGTTTTAGTATGGGGTTAAT	TAAACTACAACCCCAACCCCTACCT
PDLIM4	GGTTTTTAGGTAGAGGTAGGTAGTTTTAGT	TCATTTTACCCCAAATCTTCAAACAA
KCNMA1	GGGAAGGGAAGATATTTTTAGGGATA	TCTATTCTTTACCCCAACCAC

Table 4

Sensitivity and specificity of the Mdp score and the individual CpG units methylation of genes for diagnosis of prostate cancer

CpG unit	Paired <i>t</i> -Test (<i>p</i> Value)	Sensitivity (95% Confidence Interval)	Specificity (95% Confidence Interval)
MdpScore	1.90E-19	87.3 (76.50–94.30)	87.10 (70.17–96.37)
RARB2_CpG_10.11	6.14E-10	90 (73.47–97.89)	92.59 (75.71–99.09)
RARB2_CpG_1	1.36E-09	87.1 (70.17–96.37)	89.29 (71.77–97.73)
RARB2_CpG_9	7.89E-09	90 (73.47–97.89)	88.89 (70.84–97.65)
GSTP1_CpG_21	1.77E-07	88.46 (69.85–97.55)	87.10 (70.17–96.37)
GSTP1_CpG_10	1.74E-06	92.31 (74.87–99.05)	93.55 (78.58–99.21)
GSTP1_CpG_22	1.74E-06	92.31 (74.87–99.05)	93.55 (78.58–99.21)
GSTP1_CpG_17.18	1.75E-06	87.5 (67.64–97.34)	86.21 (68.34–96.11)
PITX2_CpG_31.32	1.93E-06	77.42 (58.90–90.41)	79.31 (60.28–92.01)
GSTP1_CpG_19	2.62E-06	92 (73.97–99.02)	89.66 (72.65–97.81)
GSTP1_CpG_8	5.81E-06	81.48 (61.92–93.70)	76.67 (57.72–90.07)
FLNC_CpG_36.37.38	6.47E-06	80.65 (62.53–92.55)	80.00 (61.43–92.29)
PITX2_CpG_14	7.84E-06	70.97 (51.96–85.78)	73.33 (54.11–87.72)
PITX2_CpG_6.7	1.21E-05	70.97 (51.96–85.78)	75.86 (56.46–89.70)
PITX2_CpG_34	1.21E-05	70.97 (51.96–85.78)	75.86 (56.46–89.70)
GSTP1_CpG_11	1.37E-05	84.62 (65.13–95.64)	83.33 (65.28–94.36)
GSTP1_CpG_12.13	1.61E-05	83.33 (62.62–95.26)	83.33 (65.28–94.36)
PITX2_CpG_26.27	2.83E-05	67.74 (48.63–83.32)	68.97 (49.17–84.72)

CpG units were ranked by *t*-Test, *p* value comparing tumor (*n* = 64), and benign (*n* = 32) tissues.

Table 5
Sensitivity and specificity of the individual CpG units methylation of genes associated with recurrence of cancer

CpG Unit	<i>t</i> -Test (<i>p</i> Value)	Sensitivity (95 % Confidence Interval)	Specificity (95% Confidence Interval)
All recurrence prediction			
FLNC_CpG_20	.01112	71.43 (41.92–91.61)	68.75 (41.34–88.98)
FLNC_CpG_1.2	.01179	71.43 (41.92–91.61)	68.75 (41.34–88.98)
FLNC_CpG_33	.01179	78.57 (49.22–95.34)	75.06 (47.62–92.73)
FLNC_CpG_8	.01179	78.57 (49.20–95.34)	75.12 (47.62–92.73)
FLNC_CpG_21.22	.01542	64.29 (35.14–87.24)	68.75 (41.34–88.98)
GSTP1_CpG_9	.01649	75.02 (42.81–94.51)	72.73 (39.03–93.98)
PITX2_CpG_10	.01866	66.67 (38.38–88.18)	64.29 (51.42–87.24)
GSTP1_CpG_21	.01923	73.33 (44.90–92.21)	72.73 (39.03–93.98)
GSTP1_CpG_19	.01969	76.92 (46.19–94.96)	81.82 (48.22–97.72)
FLNC_CpG_15.16.17	.02248	71.43 (41.90–91.61)	62.52 (35.43–84.84)
EFS_CpG_35	.02338	62.50 (24.49–91.48)	60.02 (32.29–83.66)
Local recurrence prediction			
FLNC_CpG_15.16.17	.00761	77.78 (39.99–97.19)	78.12 (60.03–90.72)
FLNC_CpG_20	.0114	77.78 (39.99–97.19)	81.25 (63.56–92.79)
FLNC_CpG_33	.01249	77.78 (39.99–97.19)	75.04 (56.62–88.54)
FLNC_CpG_8	.01249	77.78 (39.99–97.19)	75.04 (56.63–88.54)
FLNC_CpG_1.2	.01759	77.78 (39.99–97.19)	81.25 (63.56–92.79)
FLNC_CpG_21.22	.03588	77.78 (39.99–97.19)	75.13 (56.62–88.54)
FLNC_CpG_39.40	.04213	77.78 (39.99–97.19)	78.12 (60.03–90.72)
FLNC_CpG_9.10.11	.04213	77.78 (39.99–97.19)	78.12 (60.03–90.72)
PITX2_CpG_36	.04314	70.04 (34.75–93.33)	70.12 (50.64–85.27)
Systemic recurrence prediction			
FLNC_CpG_1.2	.01762	72.73 (39.03–93.98)	68.75 (49.99–83.88)
EFS_CpG_35	.03987	72.73 (39.03–93.98)	60.14 (40.64–77.34)
PSA recurrence prediction			
PDLIM4_02_CpG_1	.00055	20.04 (2.52–55.61)	42.86 (24.46–62.82)
EFS_001_CpG_35	.01324	75.02 (34.91–96.81)	70.22 (50.62–85.27)
KCNMA1_03_CpG_4.5	.02261	40.04 (12.16–73.76)	37.93 (20.69–57.74)
ECRG4_001_CpG_42	.03367	60.21 (26.24–87.84)	64.06 (42.52–82.03)
FLNC_002_CpG_28.29.30.31	.03877	33.33 (7.49–70.07)	31.25 (16.12–50.01)
ECRG4_001_CpG_43	.04627	70.02 (34.75–93.33)	66.67 (46.04–83.48)
Mrp score			
All recurrence	.00069	71.12 (52.02–85.5)	71.90 (53.22–86.24)
Local recurrence	.00156	80.32 (44.04–97.5)	81.20 (63.62–92.82)
Systemic recurrence	.02035	72.72 (39.03–94.02)	75.14 (56.66–88.52)
PSA recurrence	.33745	60.26 (26.02–87.80)	59.42 (40.64–76.36)

All the recurrence groups including PSA ($n = 10$), local ($n = 10$), and systemic ($n = 12$) recurrences were compared with the nonrecurrence group ($n = 32$). Sensitivity and specificity of the Mrp score is shown for overall recurrence prediction, local, systemic, and PSA recurrences.