

## Promoter Hypermethylation as an Independent Prognostic Factor for Relapse in Patients with Prostate Cancer Following Radical Prostatectomy

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**Abstract Purpose:** To analyze the prognostic significance of six epigenetic biomarkers (*APC*, *Cyclin D2*, *GSTP1*, *TIG1*, *Rassf1A*, and *RARβ2* promoter hypermethylation) in a homogeneous group of prostate cancer patients, following radical prostatectomy alone.

**Patients and Methods:** Biomarker analyses were done retrospectively on tumors from 74 prostate cancer patients all with a Gleason score of 3 + 4 = 7 and minimum follow-up period of 7 years. Using quantitative methylation-specific PCR, we analyzed six gene promoters in primary prostate tumor tissues. Time to any progression was the primary end point, and development of metastatic disease and/or death from prostate cancer was a secondary point. The association of clinicopathologic and biomolecular risk factors to recurrence was done using the log-rank test and Cox proportional hazards model for multivariate analysis. To identify independent prognostic factors, a stepwise selection method was used.

**Results:** At a median follow-up time of 9 years, 37 patients (50%) had evidence of recurrence: biochemical/prostate-specific antigen relapse, metastases, or death from prostate cancer. In the final multivariate analysis for time to progression (TTP), the significant factors were age > 60 [hazard ratio (HR), 0.4; 95% confidence interval (95% CI), 0.2-0.8; *P* = 0.01], hypermethylation of *GSTP1* (HR, 0.23; 95% CI: 0.09-0.64; *P* = 0.004), and hypermethylation of *APC* (HR, 3.0; 95% CI, 1.42-6.32; *P* = 0.004). In another multivariate analysis, a profile of hypermethylation of *APC* and *cyclin D2* hypermethylation was significant as well: if either any one was hypermethylated (HR, 1.84; 95% CI, 0.92-3.72; *P* = 0.09) or if both were hypermethylated (HR, 4.3; 95% CI, 1.52-12.33; *P* = 0.01).

**Conclusions:** Methylation status of selected genes in the prostate cancer specimen may predict for time to recurrence in Gleason 3 + 4 = 7 patients undergoing prostatectomy. These results should be validated in a larger and unselected cohort.

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Prostate cancer remains the most common cancer diagnosed in men in the United States, accounting for a third of all new cancer cases diagnosed in men this year (1). During the past 15 years, widespread clinical use of the prostate-specific antigen (PSA) test has resulted in a significant increase in the proportion of patients presenting with early stages of disease. Currently, ~80% of newly diagnosed patients present with no clinical evidence of metastatic disease (1). Data from uncontrolled studies suggest that 30% to 50% of patients treated with local modalities will show evidence of biochemical (PSA) relapse within a 10-year follow-up period (2-5).

The natural history of prostate cancer patients is heterogeneous. Many patients are good candidates for watchful waiting alone. Other patients undergoing potentially curative treatments will develop a recurrence and may benefit from immediate adjuvant treatment. Treatment decisions in these and in other stages of this disease are mainly based on older and known prognostic factors (Gleason score, stage, PSA variables, etc.). These factors are helpful but far from perfect due to significant clinical heterogeneity. Clearly, new biological markers are needed to predict more accurately the risk of relapse. Such

markers may allow a more accurate prediction of outcome for clinicians and patients and enhance the selection of patients at high risk who may benefit most from trials of adjuvant therapy.

Epigenetic changes are changes in gene expression not caused by alterations in the primary sequence of the nucleotides that compose the gene. DNA hypermethylation is the most common epigenetic change and one of the most common molecular alterations in human cancer (6, 7). A methyl group is added to cytosine only when it precedes a guanosine. CpG dinucleotides can be found in clusters called CpG islands often in promoter regions. CpG islands of many genes, including tumor suppressor genes, are unmethylated in normal tissues but are methylated to a varying degree in multiple types of cancer, causing silencing of gene transcription and inactivation of these tumor suppressor genes (8–11). Promoter regions of several genes were found to be hypermethylated in prostate cancer using conventional methylation-specific PCR (MSP; refs. 12, 13). The ratio between the number of methylated genes and the total number of genes analyzed, known as the methylation index, was found to correlate with clinicopathologic variables of poor prognosis, although it was never shown to have independent prognostic value in a multivariate analysis. However, conventional MSP (14) is also of limited usefulness for specific cancer detection because benign lesions can be weakly positive and cannot be distinguished from cancer cases. This distinction has become possible by the development of quantitative assays (quantitative MSP, QMSP; refs. 15, 16).

We evaluated the methylation status of the promoter regions of genes involved in DNA repair (*GSTP1*), cell cycle regulation (*cyclin D2* and *RASSF1A*), the main mediator of the anti-proliferative effect of retinoid (*RAR $\beta$ 2*) and tumor suppression (*APC* and *TIG1*) by QMSP. The methylation status of these specific genes was found to differentiate between prostate cancer and benign prostatic hyperplasia in previous studies (17), and decreased or absent expression of these genes is consistent with their methylation status. Several reports have shown methylation of different genes in prostate cancer showed an ability to differentiate between benign hyperplasia and carcinoma or primary and metastatic carcinoma. The purpose of this study was to evaluate the significance of the methylation status of six key genes by QMSP in predicting the outcome of prostate cancer patients following a potentially curative radical prostatectomy.

## Patients and Methods

**Study population.** We retrospectively identified 95 patients with a Gleason score of 3 + 4 = 7 for whom both tissue samples and median follow-up period of >7 years from radical prostatectomy were available. All patients underwent a radical prostatectomy for clinically localized prostate cancer at the Brady Urological Institute at Johns Hopkins University. All available tissue blocks were reviewed by a pathologist (J.E.), and 74 cases had adequate tumor present in the surgical specimen. Full clinical and pathologic data were collected and known for all patients (including age, pre-radical prostatectomy PSA, clinical and pathologic stage, Gleason grade, and surgical margin status). All patients were followed for at least 7 years, and data were recorded in a coded database (no evidence of disease, biochemical relapse, metastatic progression, and/or death). Biochemical progression was defined as PSA values of 0.2 mg/mL and increasing. The date of progression was assigned to the date of the first PSA > 0.2 mg/mL. None of the patients received any form of adjuvant treatment or

hormonal treatment at time of biochemical relapse before the development of metastatic disease. By selecting all patients with the identical Gleason grade, we could evaluate and identify epigenetic methylation profiles able to predict recurrence in a relatively homogenous group of patients. This study was granted an exemption from the Johns Hopkins institutional review board because samples were evaluated without any identifiers.

**DNA extraction.** After initial patient deidentification, all original histologic slides from the prostatectomy specimens were reviewed to reconfirm the diagnosis and the Gleason grade by a senior pathologist (J.E.). A representative block was retrieved for DNA extraction. Histologic slides from the formalin-fixed, paraffin-embedded tissue were taken. Slides were microdissected to obtain >80% neoplastic cells. DNA was extracted using standard protocols as previously described (18).

**Bisulfite treatment and quantitative methylation-specific PCR.** Sodium bisulfite conversion of unmethylated (but not methylated) cytosine residues to uracil of genomic DNA obtained from patient tissue samples was done as described previously (19). Two micrograms of DNA were used for the chemical treatment. DNA samples were then purified using the Wizard purification resin (Promega, Madison, WI), treated again with sodium hydroxide, precipitated with ethanol, and resuspended in water, and stored at  $-80^{\circ}\text{C}$ . The modified DNA was used as a template for real-time fluorogenic MSP. The primers and probes used for *GSTP1*, *RASSF1A*, *APC*, *TIG1*, *cyclin D2*, and *RAR $\beta$ 2* are described elsewhere (16, 20–24). In addition, primers and a probe were used to amplify areas without CpG nucleotides of  *$\beta$ -actin*, an internal reference gene (25). To determine the relative levels of methylated promoter DNA in each sample, the values of each gene of interest were compared with the values of the internal reference gene to obtain a ratio that was then multiplied by 1,000 for easier tabulation (target gene / reference gene  $\times$  1,000). Fluorogenic quantitative MSP assays were carried out in a reaction volume of 20  $\mu\text{L}$  in 384-well plates in an Applied Biosystems 7900 Sequence Detector (Perkin-Elmer, Foster City, CA). PCR was done in separate wells for each primer/probe set, and each sample was run in triplicate. The final reaction mixture consisted of 600 nmol/L of each primer (Invitrogen, Carlsbad, CA); 200 nmol/L probe (Applied Biosystems, Foster City, CA); 0.75 unit of platinum Taq polymerase (Invitrogen); 200  $\mu\text{mol/L}$  each of dATP, dCTP, dGTP, and dTTP; 16.6 mmol/L ammonium sulfate; 67 mmol/L Trizma; 6.7 mmol/L magnesium chloride; 10 mmol/L mercaptoethanol; 0.1% DMSO; and 3  $\mu\text{L}$  bisulfite-converted genomic DNA. PCR was done using the following conditions:  $95^{\circ}\text{C}$  for 2 minutes followed by 50 cycles at  $95^{\circ}\text{C}$  for 15 seconds and  $60^{\circ}\text{C}$  for 1 minute. Each plate included multiple water blanks, a negative control, and serial dilutions of a positive control for constructing the calibration curve on each plate. Leukocyte DNA collected from healthy individuals was used as negative control. The same leukocyte DNA was methylated *in vitro* with *SssI* bacterial methyltransferase (New England Biolabs, Inc., Beverly, MA) and used as positive control for all studied genes.

**Statistical methods.** The major statistical end point of this study was time to progression (TTP). Progression included PSA elevations of >0.2 ng/mL, metastasis, and or death. Event time distributions for this end point were estimated with the method of Kaplan and Meier and compared using the log-rank statistic or the proportional hazards regression model. The simultaneous effect of two or more factors was studied using multivariate Cox proportional hazards models.

A second major statistical end point of this study was the probability of metastasis or death. Factors associated with this outcome were based on cross-tabulations and logistic regression modeling. Cross-tabulations were analyzed using  $\chi^2$  or Fisher's exact tests where appropriate. Multivariate logistic regression models were used to determine the effects of multiple factors on the probability of metastasis or death.

All statistical computations were done using SAS or EGRET (Statistics and Epidemiologic Research Corp., Seattle, WA) PC packages. All confidence intervals (CI) are at the 95% level, and all *P*s are two sided.

**Results**

We evaluated the methylation status of several genes in radical prostatectomy specimens from 74 patients by QMSP. The median age at diagnosis was 59.5 years (range, 46-72). Demographic clinical and pathologic classic risk factors for predicting recurrence in these patients are summarized in Table 1.

Overall median follow-up time of patients without progression was 9 years. At the time of this study, 37 patients (50%) have experienced a recurrence. Fourteen patients (19%) had biochemical relapse only, whereas 16 (21.5%) developed metastatic disease and seven (9.5%) have died of prostate cancer. The median TTP for the whole group was 8 years.

The potential of methylation status in each tested gene alone or in combination for predicting time to recurrence was investigated. In a univariate Cox proportional hazard model, all clinical and pathologic risk factors and methylation status were evaluated. The significant factors for predicting TTP were age > 60, capsular extension, positive lymph node, and hypermethylation of *GSTP1*. Hypermethylation of *APC* and *cyclin D2* were of borderline significance (Table 2). In a multivariate proportional hazards model, the significant factors predicting TTP were age >60 [hazard ratio (HR), 0.4; 95% CI, 0.2-0.8; *P* = 0.01], hypermethylation of *GSTP1* (HR, 0.23; 95% CI, 0.09-0.64; *P* = 0.004), and hypermethylation of *APC* (HR, 3.0; 95% CI, 1.42-6.32; *P* = 0.004; Fig. 1). Positive lymph node nearly reached significance (Table 3A).

A profile combining the two most predictive genes (*APC* and *cyclin D2*) was evaluated as well as adjusting for age > 60 and *GSTP1*. The patients who were not in the upper quartile of methylation in either of the genes were the reference group. Patients who had methylation in the upper quartile of one of the genes had a HR for recurrence of 1.84, which was of borderline significance (HR, 1.84; 95% CI, 0.92-3.72; *P* = 0.09). Patients who harbored hypermethylation in both genes had a HR of 4.33 (95% CI, 1.52-12.33; *P* = 0.01; Fig. 2; Table 3B), higher than the HR for positive lymph nodes. Another profile including all four genes, which showed a HR > 1 when hypermethylated (*APC*, *cyclin D2*, *TIG1*, and

**Table 2.** Univariate analysis for TTP, including significant clinical/pathologic variables and all upper quadrant hypermethylation of evaluated genes

Variable	HR	% HR confidence limits		<i>P</i> > $\chi^2$
		Upper	Lower	
Age > 60	0.44	0.86	0.22	0.02
ECP	1.99	3.97	1	0.05
LN	2.29	4.87	1.08	0.03
<i>GSTP1</i>	0.34	0.88	0.13	0.03
<i>APC</i>	1.6	3.19	0.8	0.18
<i>Cyclin D2</i>	1.78	3.55	0.89	0.1
<i>RAR<math>\beta</math></i>	1.22	2.52	0.59	0.59
<i>TIG1</i>	1.27	2.58	0.63	0.5
<i>RASSF1A</i>	0.7	1.59	0.31	0.39

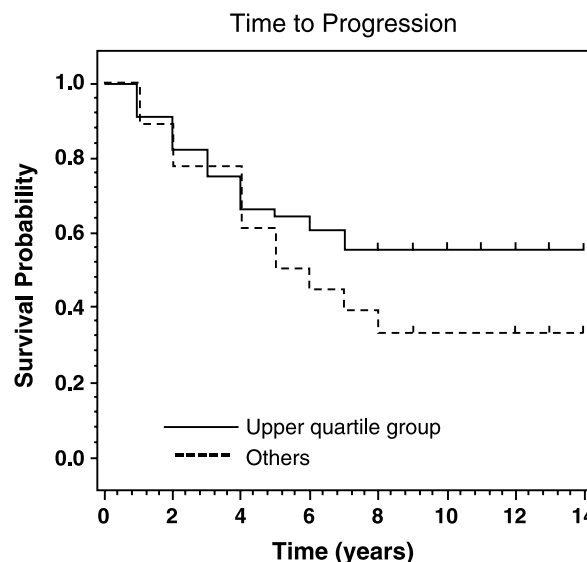
Abbreviations: ECP, extra capsular extension; LN, histologically positive lymph nodes.

*RAR $\beta$ 2*), was evaluated adjusting for age > 60 and *GSTP1*. If any one of the genes was hypermethylated, the HR was 1.32 (95% CI, 0.6-2.92), which did not reach significance (*P* = 0.49). When any two or more genes were hypermethylated, the HR reached 3.22 (95% CI, 1.42-7.26; *P* = 0.005). An additional factor was calculated combining the best-known clinical and pathologic risk factors for recurrence. Based on the Kattan nomogram for postoperative probability of freedom from recurrence at 7 years (26), each of the 74 patients was scored. Adjusting for age and the significant biomarkers *GSTP* and *APC*, higher nomogram probabilities were associated with an improved TTP outcome (HR, 0.5; 95% CI, 0.16-1.59) but did not reach significance (*P* = 0.24).

**Table 1.** Patient characteristics

No. patients	74
Age	46-72 (mean, 60)
Gleason 3 + 4 = 7	74 (100%)
Lymph nodes (+)	12 (16%)
Surgical margins (+)	21 (28%)
Extra capsular penetration	41 (55%)
Seminal vesicle (+)	15 (20%)
Organ confined	16 (22%)
Follow-up	≥8 y

NOTE: Each patient may have more than one surgical risk factor.



**Fig. 1.** TTP stratified by hypermethylation of the *APC* promoter. Patients who were in the upper quartile of methylation were compared with all other patients, and *APC* hypermethylation predicted for shorter TTP (HR, 3.0; *P* = 0.004). Adjusted estimates from multivariate model, Table 3A.

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**Table 3.** Multivariate analysis for TTP

Variable	HR	% HR confidence limits		P > $\chi^2$
		Upper	Lower	
<b>A.</b>				
Age > 60	0.4	0.8	0.2	0.01
<i>GSTP1</i>	0.23	0.64	0.09	0.004
<i>APC</i>	3.0	6.32	1.42	0.004
<b>B.</b>				
Age > 60	0.43	0.86	0.21	0.02
<i>GSTP1</i>	0.29	0.77	0.11	0.01
<i>Cyclin D2, APC</i> (1 positive)	1.84	3.72	0.92	0.09
<i>Cyclin D2, APC</i> (2 positive)	4.33	12.33	1.52	0.01

The methylation status was also evaluated in its ability to predict the probability of metastasis development or death. None of the methylation factors alone or in combination were found to significantly predict for this end point.

### Discussion

We evaluated the methylation status of the promoters of six genes, and their ability to add to known risk factors in predicting time to recurrence in prostate cancer patients following prostatectomy. These genes were chosen based on their ability to differentiate between benign hyperplasia of the prostate and prostate cancer (17). These genes were frequently found to be hypermethylated in prostate cancer and, in some studies, were related to certain clinicopathologic characteristics (12).

Patient population was unique for two reasons. First, all patients had the same Gleason grade of 3 + 4 = 7. Second, none of the patients received any additional treatment after radical prostatectomy until metastatic disease was evident. This allowed us to evaluate the significance of hypermethylation in predicting aggressiveness of prostate cancer in a relatively homogeneous group of patients without interference in the natural biological history of the disease.

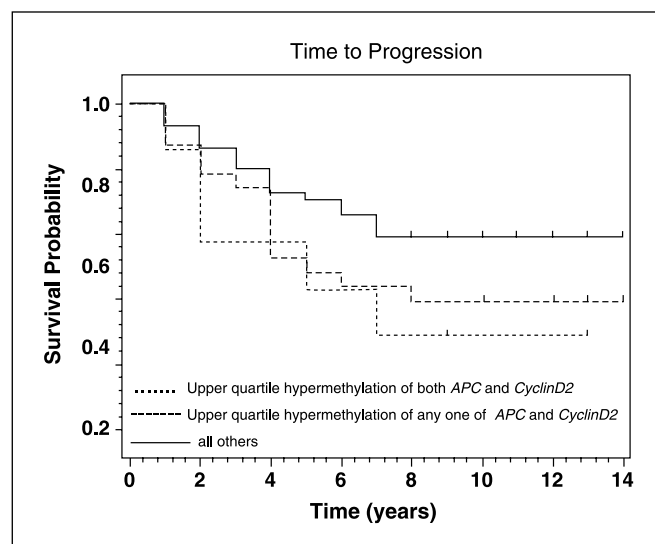
A Gleason score of 7 was chosen because it represents the most heterogeneous group of patients in terms of outcome. Most patients with a Gleason score of  $\leq 6$  will be cured with radical prostatectomy only. In patients with a Gleason score of  $\geq 8$ , the probability of recurrence is very high. In the multivariate analysis, including the most significant clinical and pathologic prognostic factors, hypermethylation of *APC* and a combined methylation profile of *APC* and *cyclin D2* were significant predictors for TTP. The HR of combined *APC* and *cyclin D2* hypermethylation was higher and more significant than the HR given by lymph node status or by the Kattan nomogram (26). The latter combines the best-known clinical and pathologic risk factors for recurrence. These findings are especially significant because we evaluated a relatively homogeneous group of patients with the same Gleason score.

Adding significant biomarkers to models predicting recurrence were shown to override commonly known risk factors by

others as well. When Kattan et al. added interleukin-6 soluble receptor and *TGF $\beta$*  to their nomogram, clinical stage lost its significance in predicting recurrence (27) in multivariate analysis. Recently, Yegnasubramanian et al. showed in a subgroup of their study population analysis that CpG island hypermethylation of *PTGS2* predicted for increased risk of recurrence following a radical prostatectomy, whereas pathologic stage lost its significance as well (28).

The upper quartile of hypermethylation of *GSTP1* was another significant predictor of longer TTP in our population. Many studies have shown *GSTP1* to be the most frequent epigenetic change in prostate cancer with a frequency of 70% to 100% (6) and an important biomarker of prostate cancer. Epigenetic silencing of *GSTP1* is considered a major step in prostate carcinogenesis. We expected that the upper quartile group might have a worse prognosis; however, our results showed the opposite. Some studies have shown a correlation between *GSTP1* methylation and the stage and grade of the cancer (17, 29), but many studies using QMSP did not show this relationship (28, 30, 31). It may be that the patients in the lower quartiles develop their prostate cancer through alternative pathways that cause more aggressive disease.

Biochemical relapse and time to this relapse is a controversial end point. On one hand, it is not as strong an end point as development of metastasis or survival. Not all patients who have a biochemical relapse develop metastasis or die from prostate cancer. On the other hand, patients undergoing a radical prostatectomy are also interested in their chance of a definite cure in addition to their chance of dying from their disease. Biochemical relapse alone causes great anxiety and a significant reduction in quality of life. Furthermore, the end point of TTP includes not only the probability of progression but also the time to its development, which by itself, is one



**Fig. 2.** TTP stratified by a hypermethylation profile of *APC* and *cyclin D2*. A profile combining the two most predictive genes (*APC* and *cyclin D2*) predicting for TTP. The patients who were not in the upper quartile of methylation in either of the genes were the reference group. Patients who had methylation in the upper quartile of one of the genes had a higher HR for recurrence, which was of borderline significance (HR, 1.84;  $P = 0.09$ ). Patients who harbored hypermethylation in both genes had the shortest TTP (HR, 4.33;  $P = 0.01$ ). Adjusting estimates from multivariate model, Table 3B.

of the predictors for further development of metastasis and death (32).

The failure of the methylation status to predict the probability of metastasis or death may be due to a relatively short follow-up. Some of the patients with a biochemical relapse at the time of the study may harbor an aggressive disease and may develop metastasis with further follow-up. Some may even have more aggressive disease than some the patients already found to have metastasis. Therefore, this end point should be optimally evaluated many years later, when full survival data of this cohort has been achieved.

In conclusion, this study suggests that hypermethylation of a limited panel of genes may help predict the time to recurrence in patients undergoing a radical prostatectomy. These results should be validated in a larger and unselected group of patients with all Gleason scores. Study of the predictive value of the

methylation status of other candidate genes (e.g., *PTGS2*) and evaluating their possible addition to these results should also be pursued. Further studies in earlier stages may provide an important tool for identifying patients who have an indolent cancer not requiring any form of treatment or in later stages in identifying those patients with biochemical relapse who may have a more aggressive course requiring early intervention. Thus, such studies may eventually help clinicians and patients in assessing the probability of cure more accurately and therefore also help in selecting better candidates for possible adjuvant treatment.

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