Published in final edited form as:

J Urol. 2014 November ; 192(5): 1542–1548. doi:10.1016/j.juro.2014.04.082.

***GSTP1* Promoter Methylation is Associated with Recurrence in Early Stage Prostate Cancer**

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Abstract

Purpose—Recurrent prostate cancer remains a major problem. Staging, grading and prostate specific antigen level at surgery are helpful but still imperfect predictors of recurrence. For this reason there is an imperative need for additional biomarkers that add to the prediction of currently used prognostic factors.

Materials and Methods—We evaluated the extent of promoter methylation of genes previously reported as aberrantly methylated in prostate cancer (*AIM1*, *APC*, *CCND2*, *GPX3*, *GSTP1*, *MCAM*, *RAR β 2*, *SSBP2* and *TIMP3*) by quantitative fluorogenic methylation-specific polymerase chain reaction. We used cancer tissue from a nested case-control study of 452 patients surgically treated for prostate cancer. Recurrence cases and controls were compared and the association between methylation extent and recurrence risk was estimated by logistic regression adjusting for patient age at prostatectomy, prostatectomy year, stage, grade, surgical margins and preprostatectomy prostate specific antigen. All statistical tests were 2-sided with $p < 0.05$ considered statistically significant.

Results—The extent of *GSTP1* methylation was higher in patients with recurrence than in controls ($p = 0.01$), especially patients with early disease, ie organ confined or limited extraprostatic extension ($p = 0.001$). After multivariate adjustment *GSTP1* promoter methylation at or above the median was associated with an increased risk of recurrence, including in men with early disease (each $p = 0.05$).

Conclusions—Greater *GSTP1* promoter methylation in cancer tissue was independently associated with the risk of recurrence in patients with early prostate cancer. This suggests that *GSTP1* promoter methylation may be a potential tissue based recurrence marker.

Keywords

prostatic neoplasms; neoplasm recurrence; local; glutathione S-transferase pi; methylation; biological markers

Currently the decision about which therapy regimen would be most beneficial for each man diagnosed with PCa is based in part on prognostic variables such as clinical stage, PSA and biopsy Gleason score. All of these tools are useful for predicting the likelihood that a patient will have organ confined or metastatic PCa at surgery as well as the probability of recurrence after surgery. However, PSA alone cannot distinguish patients with indolent disease from those with more aggressive PCa.

The Gleason score, a grading system based on tumor histological patterns, is generated by the sum of the 2 most common patterns in the gland, ranging from 2 to 10 and with the first value assigned to the most common of the 2 patterns. A higher Gleason score indicates a higher probability of nonorgan confined disease and a worse outcome after therapy.¹ Most patients who have PCa with a Gleason score of less than 6 achieve cure by undergoing radical prostatectomy while patients with a score of 8 or greater have a higher probability of PCa recurrence and death from PCa.² Men with Gleason score 7 (intermediate risk) have a variable course after treatment due to PCa heterogeneity. In several reports men with 4 + 3 tumors had a worse outcome than men with a 3 + 4 score,^{3,4} especially when this was evaluated in prostatectomy specimens rather than biopsies.⁵

Despite increased PCa detection at an early stage since the introduction of PSA testing almost a third of patients treated with radical retropubic prostatectomy experience relapse.⁶ Accurate prediction of recurrent disease at diagnosis and initial treatment and, thus, appropriate management of these cases has been difficult due to the lack of prognostic biomarkers.

The importance of epigenetic changes in the development of cancer and other diseases is increasingly appreciated. Epigenetic changes, defined as heritable changes in gene expression that occur without changes to the DNA sequence, contribute to tumor cell development and progression.⁷ Aberrant CpG island methylation, particularly of gene promoter regions, leads to transcriptional inactivation and silencing of tumor suppressor genes, representing a common phenomenon in human cancer cells and likely one of the earliest events in carcinogenesis.⁸

The best characterized epigenetic biomarker in PCa is the *GSTP1* gene, which encodes an enzyme involved in detoxification and protection of DNA from oxidants and carcinogens. *GSTP1* hypermethylation was detected in more than 90% of PCas and approximately 70% of PIN lesions,⁹ suggesting that *GSTP1* methylation may be an early event in PCa progression. Moreover, *GSTP1* methylation levels can differentiate PCa from benign

prostatic hyperplasia and high grade PIN,^{10,11} and *GSTP1* methylation is associated with different PCa stages and PCa recurrence.¹²

To continue to evaluate the potential usefulness of promoter region methylation as a tissue based biomarker of PCa recurrence risk we performed a nested case-control study of patients surgically treated for clinically localized PCa. Using QMSP we determined the extent of promoter methylation of 9 genes, including *AIM1*, *APC*, *CCND2*, *GPX3*, *GSTP1*, *MCAM*, *RARβ2*, *SSBP2* and *TIMP3*, which were previously reported to show altered methylation in PCa. To determine whether the extent of promoter methylation was independently associated with recurrence risk we considered the prognostic factors that are currently used clinically.

Materials and Methods

Study Population

We used a nested case-control study designed to test tissue based biomarkers of PCa recurrence. Several bio-markers have been evaluated to date.^{13,14} Recurrent cases and controls were selected from among the 4,860 eligible men who underwent radical retropubic prostatectomy for clinically localized PCa between 1993 and 2001 at The Johns Hopkins Medical Institutions and were followed through 2004. Men who received hormonal or radiation therapy before radical retropubic prostatectomy or adjuvant therapy before recurrence were excluded from analysis. We selected as cases all 524 men who subsequently experienced biochemical recurrence (defined as a greater than 0.2 ng/ml serum PSA increase after prostatectomy), clinical recurrence (defined as local recurrence) or systemic metastasis, or who died of PCa. For each case we used incidence density sampling to select 1 control who had recurrence by the date of case recurrence and who was matched to the case by age at surgery, race, pathological stage and prostatectomy Gleason score, as previously described.¹⁴ For this methylation study we extracted DNA from the tumor tissue of 452 men, including 259 cases and 193 controls. Approval for research on human subjects was obtained from The Johns Hopkins University institutional review boards.

DNA Extraction

Histological slides were obtained from formalin fixed, paraffin embedded tissue. Gleason scores, pathological stages and margin status were derived from the clinical surgical pathology reports. Original PCa histological slides were reviewed by members of the Brady Urological Research Institute Prostate Cancer Specimen Repository and Prostate SPORC. A biospecimen core in which a single formalin fixed, paraffin embedded block containing the highest grade/largest tumor was retrieved. The blocks were poked 6 times using a 0.6 mm tissue microarrayer puncher (Beecher Instruments, Sun Prairie, Wisconsin) and these punches were used for DNA extraction. DNA was extracted using the phenol-chloroform extraction protocol followed by ethanol precipitation, as described previously.¹⁵

Sodium Bisulfite Treatment

DNA extracted from primary tumors was subjected to sodium bisulfite treatment using the EpiTect® Bisulfite Kit according to manufacturer instructions.

Analysis

Methylation—Bisulfite treated DNA served as a template for QMSP. Amplification reactions were done in triplicate in a final volume of 20 µl containing 3 µl bisulfite modified DNA, 600 nM concentrations of forward and reverse primers, 200 nM probe, 0.6 U Platinum® Taq DNA Polymerase, 200 µM concentrations of each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate and deoxythymidine triphosphate, and 6.7 mM MgCl₂. Primers and probes were designed to specifically amplify the promoters of the 9 genes of interest and the promoter of the reference gene *ACTB* in a region without CpGs (supplementary table 1, <http://jurology.com/>) using the 7900HT sequence detector (Applied Biosystems®). Amplifications were done using the profile 95C for 3 minutes, followed by 50 cycles at 95C for 15 seconds and 60C for 1 minute.

Statistical—Differences between the recurrent cases and controls in the extent of promoter methylation were evaluated using the Wilcoxon rank sum test for the extent of methylation and the chi-square t-test for no methylation and methylation. We calculated the recurrence OR and 95% CI by the extent of promoter methylation in medians or tertiles using logistic regression, taking into account the matching factors age, race, pathological stage and Gleason score, and adjusting for preoperative serum PSA, calendar year of surgery and surgical margins status. We repeated analysis for later stage disease (ie seminal vesicle invasion or positive lymph nodes), earlier stage disease (ie organ confined disease or extraprostatic extension) and Gleason 7 disease. All analysis was done with SAS®, version 9.2. All statistical tests were 2-sided with p = 0.05 considered statistically significant.

Results

Recurrent cases and controls did not differ in the factors on which they were matched (age at diagnosis, race, pathological stage and prostatectomy Gleason score) in the parent nested case-control study (table 1). Recurrent cases had a higher mean biopsy Gleason score and higher mean PSA, and were more likely to have positive surgical margins.

The median extent of methylation of the promoter region of *GSTP1* was significantly higher in recurrent cases than in controls (p = 0.01), including those with early but not later stage disease (p = 0.001 and 0.59, respectively, table 2). Also, controls with early stage disease had a much lower *GSTP1* promoter methylation extent than controls with later stage disease (table 2). In contrast, the median *MCAM* extent was lower in recurrent cases than in controls for later stage disease (p = 0.01, supplementary table 2, <http://jurology.com/>). For the other genes the median extent did not statistically significantly differ between cases and controls overall or when stratified by early vs later stage. However, we could not rule out differences in *TIMP3* in early stage disease or in *AIM1* and *APC* for later stage disease (supplementary table 2, <http://jurology.com/>).

The extent of *GSTP1* promoter methylation, defined as at or above the control median, failed to show an association with a higher risk of recurrence after adjusting for age at surgery, preoperative PSA, positive surgical margins, surgery year, pathological stage and prostatectomy Gleason score (supplementary table 3, <http://jurology.com/>). Nonetheless, when stratified by early and later stage disease, increased *GSTP1* promoter methylation was

associated with a higher risk of recurrence in men with early stage disease (OR 1.73, 95% CI 1.00–3.02, $p = 0.05$, table 3). Men with greater methylation of *MCAM*, *SSBP2* and *TIMP3* promoters were possibly at lower risk for recurrence but none of these associations were statistically significant (supplementary table 3, <http://jurology.com/>). *MCAM* and *TIMP3* promoter methylation at or above the median was associated with a lower risk of recurrence in early (*TIMP3*) and late (*MCAM*) stage disease (supplementary table 4, <http://jurology.com/>). The extent of promoter methylation of the other genes was not associated with a risk of recurrence in men with early or later stage disease (supplementary table 4, <http://jurology.com/>). We could not control for tumor volume since it was not available as part of the clinicopathological information.

Because men with Gleason 7 disease have a variable course after treatment, we evaluated whether the extent of promoter methylation of *GSTP1* or the other genes was associated with a risk of recurrence specifically in these men. Recurrent cases with Gleason 7 disease (3 + 4 or 4 + 3) had slightly greater *GSTP1* promoter methylation than all recurrent cases combined. The same was observed for Gleason 7 controls compared to all controls. When restricted to 166 recurrence cases and 120 controls with Gleason 7 disease, we observed a borderline significant increase in *GSTP1* promoter methylation in recurrent cases compared to controls ($p = 0.06$). This attained statistical significance when it was further restricted to early stage disease ($p = 0.02$) but no difference was present in late stage Gleason 7 disease (table 4). After adjusting for age at surgery, preoperative PSA, positive surgical margins and surgery year greater *GSTP1* promoter methylation appeared to be associated with a higher risk of recurrence in men with Gleason 7 disease, including those with early stage Gleason 7 disease. However, neither result was statistically significant (table 5). We further stratified Gleason 7 cases into 3 + 4 and 4 + 3. Although we had a small number of samples, univariate and multivariate analyses revealed that increased median methylation of *GSTP1* in Gleason 7 (3 + 4) cases was associated with recurrent PCa, especially for early stage disease (tables 4 and 5).

When restricting cases to Gleason 7 disease, the extent of *MCAM* promoter methylation was again lower in recurrent cases than controls, especially for late stage Gleason 7 disease (supplementary tables 5 and 6, <http://jurology.com/>). After adjusting for age at surgery, preoperative PSA, positive surgical margins and surgery year a greater extent of methylation was possibly inversely associated with recurrent risk (supplementary table 7, <http://jurology.com/>). The extent of methylation of the promoters of the other genes was not associated with recurrence in men with Gleason 7 disease.

Discussion

In our study greater *GSTP1* promoter methylation in PCa tissue was associated with a statistically significant higher risk of recurrence after considering currently used prognostic factors. This association was present in men with early stage disease and possibly in men with a pathological Gleason sum of 7. *GSTP1* represents a promising biomarker for further study of its predictive capacity for recurrence, including in men with early stage PCa and/or Gleason 7 disease.

Several groups have evaluated *GSTP1* promoter methylation as a prognostic biomarker for PCa.^{16–21} The majority of these studies, which were mainly of prostatectomy tissue, did not successfully show an association between *GSTP1* promoter methylation and PCa recurrence.^{16,19,22} Two groups found an association between *GSTP1* methylation and PCa recurrence in bodily fluids.^{20,21} Using restriction endonuclease quantitative polymerase chain reaction Bastian et al noted that *GSTP1* methylation was an independent prognostic marker in the serum of 55 pairs of patients with recurrent or nonrecurrent PCa and a median followup of 3 years.²⁰ Using QMSP Rouprêt et al found an association between *GSTP1* promoter methylation and PCa recurrence in whole blood of 20 patients with recurrent and 22 with nonrecurrent PCa, and in 22 with benign prostatic hyperplasia.²¹ Nonetheless, these studies had several limitations, such as smaller cohorts and limited median followup. In the study by Rouprêt et al the type of treatment (radical prostatectomy, radiotherapy or hormonal therapy) was not considered, potentially altering the methylation status of the genes analyzed.

We previously reported an association between *GSTP1* methylation and a decreased risk of PCa recurrence.¹⁷ In that study the methylation extent in 74 patients with Gleason 7 PCa (3 + 4 only) and a median followup of 9 years was analyzed by QMSP. In our current cohort of Gleason 7 cases we included 3 + 4 and 4 + 3 scores. Compared to a 3 + 4 score Gleason 4 + 3 PCa is associated with a worse outcome and an increased risk of recurrence, potentially explaining the differences between these 2 studies. Population size and heterogeneity, and in the current study the fact that the significant correlation with a higher risk of recurrence was found only in patients with early stage Gleason 7 PCa may also have contributed to the observed differences.

Vanaja et al evaluated CpG methylation of several genes in 32 recurrent and 32 nonrecurrent PCa tissues, and found a significant association between increased *GSTP1* methylation and PCa recurrence.¹⁸ Nonetheless, the study cohort was significantly smaller and the method used to study methylation was different from ours.

Among the other genes analyzed in our study the *GPX3* gene, which is involved in DNA damage repair, has tumor suppressor activity in PCa cell lines and is methylated and silenced in PCa tissues.²³ *CCND2*, a gene involved in cell cycle control, has a higher methylation level in PCa tissue than in normal tissue. *CCND2* combined with the *APC* gene was a significant predictor of time to progression.^{17,24} Moreover, the *APC* and *RARβ2* genes were methylated at all PCa stages, detected in bodily fluids and also commonly tested with *GSTP1*.^{16,17,21,25,26} Our group recently reported that decreased promoter methylation of *AIM1* in PCa was associated with shorter time to progression in a cohort of 95 patients with only Gleason sum 3 + 4 = 7.²⁷ None of these genes were significantly associated with PCa recurrence in the current study in part due to tumor heterogeneity and other associated factors, such as the methylation analysis technique, environmental and endogenous factors, and, as mentioned, population size and heterogeneity.

The *SSBP2* gene regulates hematopoietic growth and differentiation. *TIMP3* is believed to have a significant role in controlling extracellular matrix remodeling and tumor invasion. In this study decreased *SSBP2* and *TIMP3* methylation levels were independent factors on

multivariate analysis and associated with an increased risk of biochemical recurrence in late and early stage PCa, respectively. Our group first reported *MCAM* methylation in PCa.^{15,28,29} In this study *MCAM* was an independent prognostic factor for predicting biochemical recurrence with decreased methylation levels in late stage PCa cases. Moreover, decreased *MCAM* methylation correlated with recurrence in late stage Gleason 7 PCa cases on univariate analysis but it failed to be an independent prognostic factor on multivariate analysis ($p = 0.06$). *MCAM*, *SSBP2*, *TIMP3* and the other genes in this study must be further tested in larger multicenter studies to determine the true value of their predictive capacity of any clinical outcome.

Our study has several limitations. Our cohort consisted of only 452 patients with PCa. Although this is one of the largest cohorts in which *GSTP1* has been evaluated in PCa, a larger and more homogeneous cohort would provide more power to detect a statistically significant association. This study identified 1 candidate marker. Thus, more studies, ideally prospective ones, are needed to identify more genes that could have higher predictive capability as a panel. Methylation analysis in tissues and/or bodily fluids before and after PCa recurrence would be ideal in future studies to fully evaluate methylation status as a predictor. Lastly, the modest OR of approximately 1.7 of the independent association between *GSTP1* methylation and recurrence risk in men with limited extension or intermediate grade PCa is statistically unlikely to substantially add to the predictive ability of the commonly used pathological prognostic factors.

Conclusions

Our nested case-control study provides evidence that *GSTP1* promoter methylation is associated with the risk of recurrence, particularly in patients with early stage PCa. *GSTP1* methylation already shows great promise in correctly triaging disease absence or presence in patients screened with PSA who undergo subsequent blinded biopsies. This study now supports a further role for *GSTP1* promoter methylation in identifying patients with more indolent cancer not requiring any form of treatment or identifying patients at higher risk for relapse who may benefit from a more aggressive therapeutic approach.

DNA methylation represents an important tool for biomarker development that is also capable of predicting recurrence, which could later be developed as clinical tests. Other methylated markers that have shown promise for PCa are *EDNRB*, *RASSF1 α* , *ER β* , *TIG1* and *PTGS2*.³⁰ A combination of methylated genes may be more accurate than individual genes for the prognosis of clinical outcome and the early detection of PCa. Thus, a panel of *GSTP1* and other bio-markers may aid in better predicting recurrent PCa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Study received approval from The Johns Hopkins University institutional review boards.

Supported by the National Cancer Institute (U01-CA84986 Early Detection Research Network), National Institutes of Health/National Cancer Institute funded Prostate SPORE (P50CA58236) Biospecimen Core, Department of Defense DAMD Grant 17-03-0273 and Patrick C. Walsh Prostate Cancer Research Fund.

Helen L. Fedor, Chantal Berez and Angelo M. De Marzo, Brady Urological Institute Prostate Bio-specimen Repository, assisted with the study.

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Abbreviations and Acronyms

AIM1	absent in melanoma-1
APC	adenomatous polyposis coli
CCND2	cyclin D2
GSTP1	glutathione S-transferase P1
MCAM	melanoma cell adhesion molecule
PCa	prostate cancer
PSA	prostate specific antigen
QMSP	quantitative fluorogenic methylation-specific polymerase chain reaction
SSBP2	sequence-specific ssDNA-binding protein
TIMP3	tissue inhibitor of metalloproteinases 3

Table 1

Characteristics of recurrent PCa cases and controls

	All Stages		p Value	Early Stage		Later Stage	
	Cases	Controls		Cases	Controls	Cases	Controls
No. subjects	259	193		184	143	75	50
Mean $\pm$ SD age	59.0 $\pm$ 6.2	59.4 $\pm$ 6.0	0.41	58.5 $\pm$ 6.4	59.1 $\pm$ 6	60.1 $\pm$ 5.8	60.5 $\pm$ 5.9
% White	84.6	87.6	0.36	84.2	85.3	85.3	94.0
% Biopsy Gleason sum:							
6 or Less	45.7	54.7		50.8	56.3	33.3	50.0
7	42.3	39.6	0.04	37.7	41.6	53.3	34.0
8 or Greater	12.0	5.7		11.5	2.1	13.3	16.0
Preop PSA (ng/ml)	12.0 $\pm$ 10.7	10.2 $\pm$ 7.0	0.03	11.0 $\pm$ 9.8	8.9 $\pm$ 6.3	14.3 $\pm$ 12.4	13.6 $\pm$ 7.8
% Pathological Gleason sum:							
6 or Less	14.7	16.6		17.9	20.3	6.7	6.0
7	64.1	62.2	0.85	61.4	59.4	70.7	70.0
8 or Greater	21.2	21.2		20.7	20.3	22.7	24.0
% Pathological stage:							
Organ confined	16.2	19.2		22.8	25.9	–	–
Extracapsular extension	54.8	54.9	0.63	77.2	74.1	–	–
Seminal vesicle invasion/lymph node	29.0	25.9		–	–	100	100
% Pos surgical margins	33.6	22.8	0.012	27.7	12.6	48	52.0

Table 2
GSTP1 methylation by pathological stage

Stages	Cases	Controls	p Value (nonparametric Wilcoxon rank sum test)
All:			
No. subjects	259	193	–
Median methylation (IQR)	1,121 (310.2–2,909)	701.1 (201.6–1,925)	0.01
Early:			
No. subjects	184	143	–
Median methylation (IQR)	1,143 (356–3,104)	653 (140–1,827)	0.001
Late:			
No. subjects	75	50	–
Median methylation (IQR)	1,099 (225–2,290)	1,059 (404–2,555)	0.59

Table 3
GSTP1 methylation extent and recurrent PCa by pathological stage

Stage	Median or Less	Greater Than Median	p Value
Early:			–
No. cases	65	116	
No. controls	77	63	
OR (95% CI)*	1.00 (referent)	1.73 (1.00, 3.02)	0.05
Late:			–
No. cases	31	42	
No. controls	20	30	
OR (95% CI)*	1.00 (referent)	1.01 (0.47, 2.15)	0.99

* Adjusted for age at surgery, preoperative PSA, positive surgical margins, surgery year, pathological stage and pathological Gleason sum.

Table 4
GSTP1 methylation in patients with Gleason sum 7 by pathological stage

Gleason Sum 7	Cases	Controls	p Value (nonparametric Wilcoxon rank sum test)
All stages			
3 + 4 and 4 + 3:			
No. subjects	166	120	–
Median methylation (IQR)	1,158 (277–2,969)	714 (258–1,925)	0.06
3 + 4 Only:			
No. subjects	49	72	–
Median methylation (IQR)	1,354 (587–3,518)	580 (131–1,916)	0.001
4 + 3 Only:			
No. subjects	37	32	–
Median methylation (IQR)	843 (179–2,818)	765 (453–1,925)	0.94
Early stage			
3 + 4 and 4 + 3:			
No. subjects	113	85	–
Median methylation (IQR)	1,224 (363–3,131)	701 (118–1,876)	0.02
3 + 4 only:			
No. subjects	36	59	–
Median methylation (IQR)	1,241 (480–3,518)	424 (107–1,241)	0.003
4 + 3 Only:			
No. subjects	23	25	–
Median methylation (IQR)	1,207 (145–3,131)	787 (424–1,972)	0.87
Late stage			
3 + 4 and 4 + 3:			
No. subjects	53	35	–
Median methylation (IQR)	1,059 (212–2,909)	1,059 (404–2,555)	0.83
3 + 4 Only:			
No. subjects	13	13	–
Median methylation (IQR)	2,196 (1,052–5,833)	2,555 (1,158–3,351)	0.81
4 + 3 Only:			
No. subjects	14	7	–
Median methylation (IQR)	820 (286–2,002)	627 (481–1,759)	0.76

Table 5
GSP1 methylation extent and recurrent PCa by pathological stage in patients with Gleason sum 7

Gleason Sum 7	Early Stage			Late Stage		
	Median or Less	Greater Than Median	p Value	Median or Less	Greater Than Median	p Value
3 + 4 and 4 + 3:						
No. cases	39	71	–	21	30	–
No. controls	47	37	–	12	23	–
OR (95% CI)*	1.00 (referent)	1.76 (0.80, 3.88)	0.16	1.00 (referent)	0.83 (0.32, 2.13)	0.70
3 + 4 Only:						
No. cases	12	21	–	2	10	–
No. controls	38	21	–	3	10	–
OR (95% CI)*	1.00 (referent)	3.76 (1.14, 12.4)	0.03	1.00 (referent)	0.66 (0.06, 7.08)	0.73
4 + 3 Only:						
No. cases						–
No. controls	11/10	12/15		6/4	8/3	–
OR (95% CI)*	1.00 (referent)	1.26 (0.23, 7.01)	0.80	Did not converge		–

* Adjusted for age at surgery, preoperative PSA, positive surgical margins and surgery year.