The 17-Gene Genomic Prostate Score Assay Predicts Outcome After Radical Prostatectomy Independent of PTEN Status

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OBJECTIVE To compare the ability of loss of phosphatase and tensin homolog (PTEN) and Genomic prostate score assay (GPS) in predicting the biochemical-recurrence (BCR) and clinical-recurrence (CR) after radical prostatectomy (RP) for clinically localized prostate cancer (PCa).

METHODS Three hundred seventy seven patients with and without CR were retrospectively selected by stratified cohort sampling design from RP database. PTEN status (by immunohistochemistry [IHC] and fluorescence in situ hybridization [FISH]) and GPS results were determined for RP specimens. BCR was defined as Prostate Specific Antigen (PSA) $\geq$ 0.2 ng/mL or initiation of salvage therapy for a rising PSA. CR was defined as local recurrence and/or distant metastases.

RESULTS Baseline mean age, PSA, and GPS score for the cohort were 61.1 years, 8 ng/dL, and 32.8. PTEN loss was noted in 38% patients by FISH and 25% by IHC. The concordance between FISH and IHC for PTEN loss was 66% (Kappa coefficient 0.278; $P < .001$). On univariable analysis, loss of PTEN by FISH or IHC was associated with BCR and CR ($P < .05$). However, after adjusting for GPS results, PTEN loss was not a significant predictor for CR or BCR ($P > .1$). The GPS result remained strongly associated with CR and BCR after adjusting for PTEN status ($P < .001$). PTEN status and GPS results only weakly correlated. GPS was widely distributed regardless of PTEN status indicating the biological heterogeneity of PCa even in PTEN-deficient cases.

CONCLUSION GPS is a significant predictor of aggressive PCa, independent of PTEN status. After adjustment for GPS results, PTEN was not independently associated with recurrence for PCa. UROLOGY 121: 132–138, 2018. © 2018 The Author(s). Published by Elsevier Inc.

With increased understanding of molecular pathways of prostate cancer (PCa) development and progression, several biomarkers for disease aggressiveness have been identified.[1] Phosphatase and tensin homolog (PTEN), a tumor suppressor gene located on chromosome 10, has been identified as one such biomarker. PTEN expression loss has been noted in 10%-34% of PCa cases and is linked to higher Gleason grade group, advanced tumor stage, and lymph node metastases.[2–4] Another such biomarker assay, genomic prostate score (GPS), has been clinically validated as a predictor of adverse pathology and recurrence noted after radical prostatectomy (RP) for clinically localized PCa.[5,6] The GPS assay measures expression of 17 genes (12 cancer-related and 5 reference genes) representing 4 biologic pathways. In the initial gene identification study for development of GPS, expression of 732 PCa -related genes were assessed.[6] Gene selection for the assay was based on the strength of association between the expression of 732 genes studied and clinical outcomes. PTEN has been associated with advanced stage and more aggressive PCa. The predictive ability of PTEN loss has not been compared directly with GPS. Since, both GPS and PTEN occupy the similar clinical space for biomarker use in risk stratification, in this study, we compared the ability of the GPS assay and PTEN status to predict biochemical recurrence (BCR) and clinical recurrence (CR) in men with clinically localized PCa treated with RP.

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Disclosure/Duality of Interest: *Dr. Eric Klein is consultant with Genomic health. Christina Magi-Galluzzi, Sudhir Isharwal, and Sara M Falzarano have no conflict of interest. Athanasios Tsiatis, Anne Dee, Tara Maddala, Dejan Knezevic, Philip G Febbo, and Jefferey Lawrence are employee of Genomic Health.*

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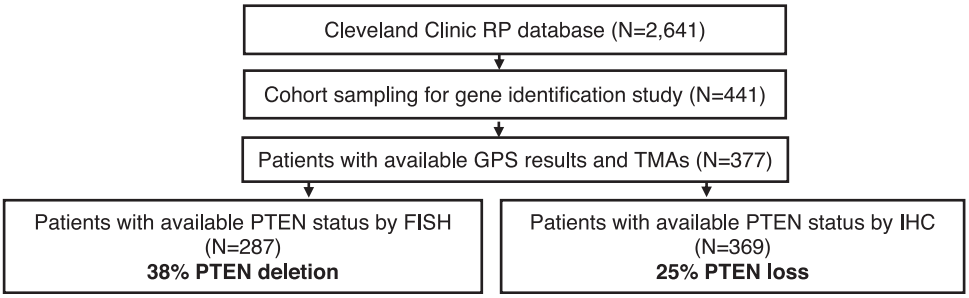


Figure 1. Sample selection for GPS and PTEN status determination.

MATERIALS AND METHODS

Study Design, Patients, and Specimens

A cohort patients (N = 2641) with clinically localized (stage T1/T2) PCa who underwent RP at the Cleveland Clinic during 1987-2004 was used to select the study population. This cohort was used for the study since it had adequate follow up to capture late local or distant recurrences. A stratified cohort sampling design was used to select all the patients with CR (local recurrence and/or metastasis) and a random sample of patients without CR with 1:3 ratio (Fig. 1).[7,8] All samples were taken from formalin fixed paraffin-embedded RP specimens and reviewed by an expert genitourinary pathologist for assignment of tumor grade and stage using the 2005 International Society of Urological Pathology Consensus guidelines.[9]

GPS and PTEN Status Determination

Each formalin fixed paraffin-embedded RP specimen was examined to identify all tumor foci, the index lesion, primary Gleason pattern (PGP) and highest (or secondary) Gleason pattern (HGP). For each patient a PGP sample, secondary (or highest) Gleason pattern sample, and an adjacent non-tumor (NT) tissue sample were evaluated.

For patients whose prostatectomy PGP was also the HGP, specimen 1 represented the PGP and specimen 2 the Secondary Gleason pattern. When possible, specimens 2 were selected from spatially distinct nodules containing the secondary pattern (separate paraffin blocks than specimens 1). For patients whose prostatectomy PGP was not the HGP, specimen 1 represented the HGP and specimen 2 the PGP.

H and E slides were marked around the periphery of each Gleason patterns (minimum size = 5 mm). NT tissue (> 3 mm distance from any tumor sample in order to avoid contamination) was also identified and marked. Six 10 μm sections of PGP, HGP, NT samples were manually-macrodissected and placed in separate tubes. Quantitative reverse transcription polymerase chain reaction was performed and gene expression was measured in primary and HGP samples and adjacent NT. The same tumor areas corresponding to PGP and HGP (or secondary) for each case were selected to obtain the 2 to 3 cores for the tissue microarrays (TMAs). This degree of sampling should reflect the PTEN status and heterogeneity within the prostate gland.

GPS results were generated using tumor RNA from micro-dissected RP specimens. For GPS score determination, RNA was extracted, purified and analyzed for the expression of 12 cancer-related genes and 5 reference genes as previously described.[6] GPS reported score of 0-100 with a higher score indicating more aggressive disease. TMA from RP specimens used to generate the GPS were also used to determine PTEN status. Two to three 1.5 mm diameter cores from selected tumor foci were taken from the donor paraffin blocks and transferred to the recipient TMA block.

PTEN status was assessed by fluorescence in situ hybridization (FISH) in 287 patients using a four-color probe set (PTEN-del-TECT, Biocare Medical, Concord CA) in TMA samples. Probe labeling, hybridization, washing, and fluorescence detection were performed according to standard procedures. Unstained slides were deparaffinized and subjected to protease digestion. Hybridization was done using the PTEN Del-Tect probe kit. This kit contained 4 fluorescent probes: 1 probe to detect the presence of PTEN, 2 probes immediately flanking the PTEN locus on both sides, and 1 centromeric probe. Up to 100 tumor cells/spot were scored and tumors were scored either as normal, hemizygous deletion for PTEN, or homozygous deletion for PTEN. Scoring was based on the presence of the 2 signals for each flanking probe and 0, 1, or 2 signals for the PTEN test probe. If greater than 20% of cells counted showed hemizygous or homozygous deletion of PTEN, the case was considered as PTEN deficient.[2]

Immunohistochemical staining for PTEN (PTEN D4.3 XP Rabbit mAB; Cell Signaling Technology, Danvers, MA) was performed in 369 patient TMA samples with appropriate external and internal positive and negative controls. Each TMA spot of tumor tissue was scored as deficient or normal for PTEN protein by comparing the staining in malignant glands with that of adjacent benign glands and/or stroma. PTEN status by immunohistochemistry (IHC) was defined as a dichotomous variable (PTEN intact and/or PTEN deficient). Cases were considered PTEN deficient if the intensity of staining was markedly decreased or entirely negative across all tumor cells compared to the surrounding benign glands and/or stroma.[2] A given TMA spot was considered noninformative if benign glands and/or stroma lacked PTEN staining.

Examples of PTEN intact and deficient cases by IHC are illustrated in the Supplemental Materials (Supplemental Figure 1).

Statistical Analysis and Outcomes

The primary outcome of the study was the progression to BCR or CR after RP. BCR was defined as a rise of PSA $\geq$ 0.2 ng/mL and confirmed in a consecutive PSA record or the initiation of salvage therapy as a result of a rising PSA. CR was defined as distant metastases (occurring in ~80%) and/or local recurrence (occurring in ~20%) confirmed by imaging or biopsy. To account for the fact that the analysis was conducted in the same cohort of subjects that was initially used to develop the GPS, regression to the mean estimates for GPS are reported throughout, which addresses over-optimism. Multivariable Cox proportional hazard regression was used to identify significant prognostic factors for recurrence. Since, GPS score is reported on a continuous scale of 0-100, we used GPS score per 20 units since it has been validated as such in the previous development

Table 1. Clinical and pathological characteristics of the cohort

Characteristic	Classifier	FISH*	IHC*	ALL†
Age in years (mean, SD)		60.7 (6.1)	61.1 (6.2)	61.1 (6.3)
Baseline PSA (ng/mL) (mean, SD)		7.8 (6.7)	8.3 (7.5)	8.0 (7.3)
GPS (mean, SD)		29.1 (13.4)	30.1 (13.8)	32.8 (34.5)
Race n (%)	White	250 (87)	316 (86)	366 (83)
	Black	23 (8)	40 (11)	53 (12)
	Other	14 (5)	13 (4)	22 (5)
Clinical Tumor Stage n (%)	T1	184 (64)	232 (63)	291 (66)
	T2	103 (36)	137 (37)	150 (34)
Pathological Tumor Stage n (%)	T2	141 (49)	170 (46)	229 (52)
	T3	146 (51)	199 (54)	212 (48)
Surgical Gleason Score n (%)	≤6 (Grade group 1)	48 (17)	62 (17)	103 (24)
	3 + 4 (Grade group 2)	143 (50)	176 (48)	192 (44)
	4 + 3 (Grade group 3)	54 (19)	77 (21)	82 (19)
	8 (Grade group 4)	22 (8)	29 (8)	34 (8)
	>8 (Grade group 5)	20 (7)	25 (7)	30 (7)

FISH, fluorescence in situ hybridization; GPS, genomic prostate score; IHC, immunohistochemistry; SD, standard deviation.
* Percentages were calculated accounting for cohort sampling weights.
† Patients in gene identification study.

and validation studies of GPS.[5,6] Recurrence-free survival curves for GPS tertiles were computed using the estimate of the standardized hazard-ratio corrected for regression to mean from a Cox proportional hazard model with cohort sampling weights. All data were analyzed using SAS version 9.4. For statistical analysis, each patient's tumor sample was scored for the presence or absence of PTEN loss by summarizing the scores of each individual sampled core from that tumor.

RESULTS

A total of 377 patients had valid GPS results and available TMAs. Baseline clinical characteristics of the study cohort are summarized in Table 1. PTEN evaluable patients were representative of the full cohort, with similar baseline demographic and pretreatment characteristics (Table 1).

From the available TMAs, 287 patients were evaluated using FISH with 120 patients (38% weighted percentage adjusted for 1:3 sampling strategy) detected to have PTEN deletions. With IHC, PTEN status was evaluated in 369 patients, with 116 (25% weighted percentage) noted to have PTEN loss. The concordance between the 2 PTEN methodologies (FISH PTEN deletion vs IHC PTEN loss) was 66% (Kappa coefficient 0.278; $P < .001$), consistent with previously published findings.[2] The proportion of cases showing loss of PTEN increased with higher Gleason Score ($P < .001$ for both FISH and IHC) (data not shown), and rates of both BCR and CR were higher in PTEN deleted than intact cases (44% vs 28% for BCR and 10% vs 6% for CR).

GPS scores had a broad range for each category of PTEN status (intact, homozygous deletion, and hemizygous deletion, Fig. 2) with only weak correlation (Spearman correlation coefficient of 0.2 for FISH and 0.3 for IHC), suggesting that GPS possesses predictive ability independent of PTEN status. This hypothesis was tested in 2 ways. First, the cohort was stratified into tertiles of risk based on GPS scores (low, intermediate, and high as described previously[10]) and rates of BCR and CR were calculated by risk group for PTEN intact and deleted cases assessed by FISH (Figs. 3 and 4). Second, univariable- and multivariable analyses including PTEN status and GPS score were performed (Table 2).

As expected, when stratified by GPS tertiles, rates of BCR and CR increased with increasing GPS risk group, and were higher in cases with PTEN loss by FISH (Figs. 3 and 4). In the group of PTEN-deficient patients, 31% were in low, 28% in intermediate, and 41% in high GPS risk categories with 10-year BCR risk of 27% for low, 38% for intermediate, and 62% for high risk category. Similarly, the patients with intact PTEN by FISH were stratified with 35% in low, 37% in intermediate, and 29% in high GPS score risk category with 10-year BCR risk of 18% for low, 26% for intermediate, and 42% for high risk category. The risk of CR also increased from low to high GPS risk category in both PTEN-intact and deficient patients (Fig. 4a and b). Of the patients with intact PTEN by FISH, 35% were in low, 37% in intermediate, and 29% in high GPS risk category with 10-year CR risk of 2% for low, 4% for intermediate, and 14% for high risk category. In PTEN deficient patients, 31% were in the low, 28% in the intermediate, and 41% were in the high GPS risk category with 10-year CR risk of 3% for low, 5% for intermediate, and 19% for high risk category. GPS risk categories were able to stratify the patients with the lowest risk of BCR and CR in the PTEN deficient group as measured by FISH and the highest risk of BCR and CR in PTEN intact cases. Similar findings were noted with the determination of PTEN status by IHC (Supplementa Figure 2).

On univariable analysis, PTEN deletion by FISH or loss of PTEN by IHC was associated with increased likelihood of BCR (hazard ratio 1.92 and 1.76; both $P < .001$) and CR (hazard ratio 1.57 and 1.80; both $P < .05$). However, on multivariable analysis, after adjusting for GPS by 20-unit increases, PTEN deletion by FISH or PTEN loss by IHC was not significantly associated with BCR or CR (Table 2), while GPS remained strongly associated with BCR and CR ($P < .001$).

DISCUSSION

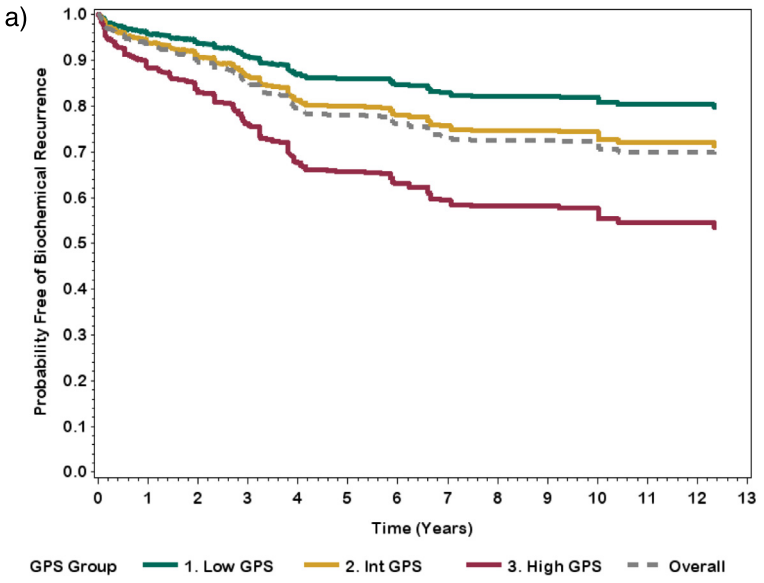
The biologic heterogeneity of localized PCa poses a clinical dilemma in selecting the optimal treatment option for individual patients. With increased molecular understanding of PCa development and progression, several tissue based assays have been developed to decrease uncertainty of outcomes based solely on clinical and pathological factors.[10] PTEN inactivation or loss results in interruption of the PI3K/AKT pathway that is responsible

Figure 2. Distribution of Genomic Prostate Score (GPS) results by PTEN status as determined by fluorescence in situ hybridization (FISH) **(a)** and immunohistochemistry (IHC) **(b)**. (Color version available online.)

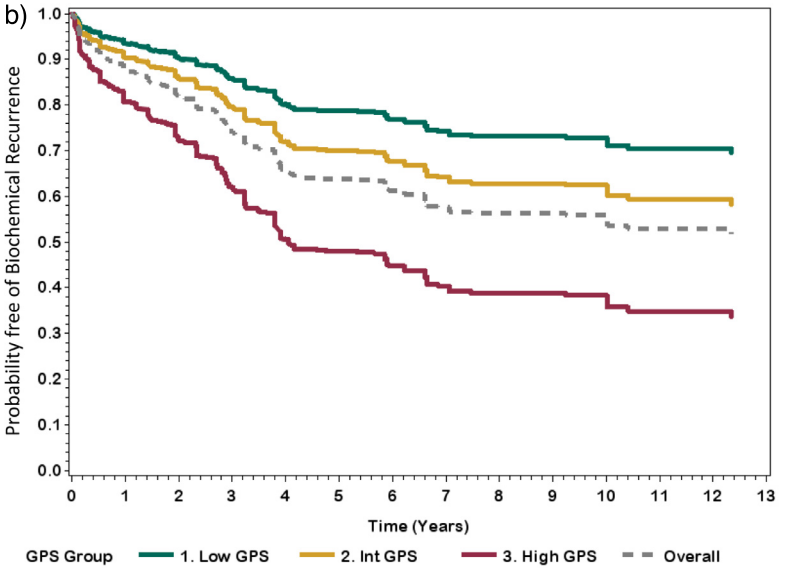
for cellular growth, survival, and polarity.[11] PTEN status can be determined at the DNA level by FISH or at the protein expression level by IHC.[12,13] Clinical-grade immunohistochemistry assay for PTEN loss has been reported to predict tumor behavior.[14] In several prior studies, PTEN deletion was associated with advanced tumor stage, higher Gleason grade, presence of lymph node metastasis, development of hormone-refractory disease, and with earlier time to BCR.[12,14,15] The GPS assay measures gene expression involved in multiple pathway of PCa development and progression.[6] It has been validated to predict clinically meaningful endpoints of adverse pathology and recurrence after surgery for PCa. In this study, our objective was to compare these 2 molecular markers available for clinical use with each other to predict the CR after RP. On comparison of the predictive ability of GPS and PTEN status in this study, GPS was a significant predictor of BCR and CR independent of PTEN status.

Loss of PTEN in our cohort ranged from 25%-38%, consistent with prior reports. PTEN expression was measured by both IHC as well as FISH.[12,16] The concordance between the 2 methods for PTEN loss was 66%. In the study by lotan et al increased concordance between PTEN IHC and FISH wild type was noted. However, out of the patients with PTEN loss by IHC only 66% had PTEN loss by FISH similar to as noted in our study.[17] The discordance between FISH and IHC noted in our study could possibly be due to intratumoral heterogeneity, alterations that are not detected by FISH assay such as truncating mutations, or epigenetic modifications influencing PTEN protein stability. In addition, the results of IHC at our centers could differ from the prior studies based on the differences in the antibody use and differences in technique for IHC.[17] As noted in previous studies, higher GPS scores and PTEN deficiency were associated with increased risks of adverse outcomes.[16] We observed a range of GPS scores in both PTEN intact and deficient cases indicating the GPS assay can further risk-stratify patients in the PTEN deficient group and thus is of additional prognostic value. The multivariable analysis including both of these variables demonstrated that the GPS score had predictive ability for both BCR and CR that was independent of PTEN status. Significantly, GPS was able to stratify both PTEN intact and deficient cases into groups with favorable, intermediate and unfavorable outcomes, suggesting that not all PTEN loss is associated with a poor prognosis.

Our study has several limitations. The main limitation was the lack of tissue availability for PTEN determination in a subset of cases, although the cases included were representative of the entire cohort as measured by



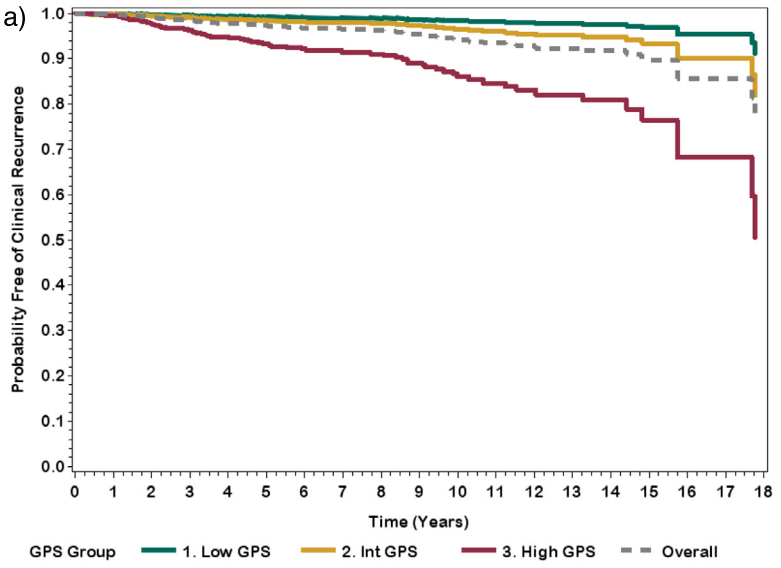
GPS Group	*Percentage of patients*	*10-year risk of biochemical recurrence (%)*
Low risk	35	18.0
Intermediate risk	37	25.6
High risk	29	42.3
Overall		27.8



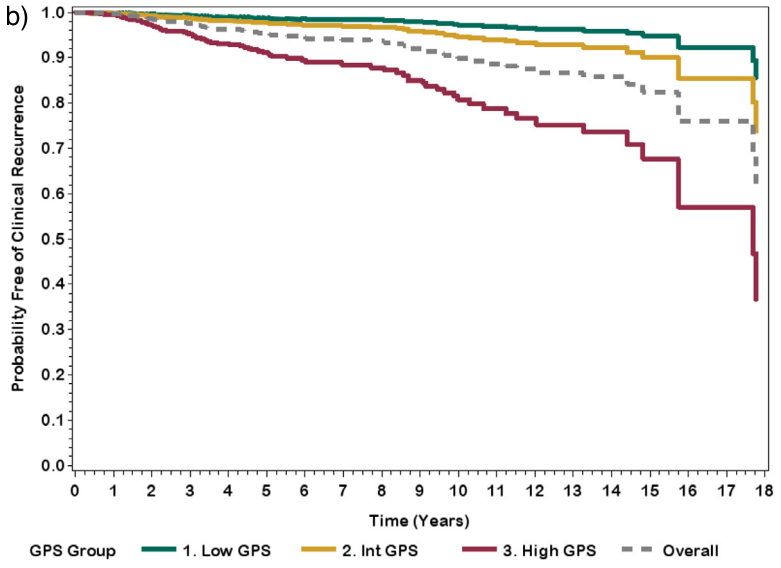
GPS Group	*Percentage of patients*	*10-year risk of biochemical recurrence (%)*
Low risk	31	27.1
Intermediate risk	28	37.6
High risk	41	61.7
Overall		44.1

Figure 3. (**a**) Biochemical recurrence free survival curve for PTEN intact assessed by FISH and GPS tertiles. (**b**) Biochemical recurrence free survival curve for PTEN deleted cases assessed by FISH and GPS tertiles. (Color version available online.)

demographics and baseline disease characteristics. Another limitation was that in this study we used the same cohort that was previously used to develop the GPS assay. We addressed this limitation by calculating RM-corrected estimates for recurrence. In addition, the outcome studied in this study was biochemical or CR that are surrogates for PCa specific mortality. CR included patients with local or distant recurrence. Although there is



GPS Group	*Percentage of patients*	*10-year risk of clinical recurrence (%)*
Low risk	35	1.7
Intermediate risk	37	3.6
High risk	29	13.9
Overall		5.9



GPS Group	*Percentage of patients*	*10-year risk of clinical recurrence (%)*
Low risk	31	2.8
Intermediate risk	28	5.4
High risk	41	19.2
Overall		10.2

Figure 4. (**a**) Clinical recurrence free survival curve for PTEN intact cases assessed by FISH and GPS tertiles. (**b**) Clinical recurrence free survival curve for PTEN deleted cases assessed by FISH and GPS tertiles. (Color version available online.)

difference in the PCa mortality based on the site of recurrence, both groups are similar in terms of need for further therapy. Out of the patients with recurrences, only a minority of patients will die from PCa. Finally, the clinical value GPS results adds over the clinicopathological characteristics in predicting recurrence was not evaluated in this study. However, even a small fraction improvement in the receiver operating characteristics as previously shown can improve the decision curve analysis significantly.[6] The major strengths of this study include

Table 2. PTEN status and GPS as predictors of recurrence of prostate cancer

PTEN Determination	Model	Variable	HR[†]	*P* Value	q Value[‡]
a: PTEN status and biochemical recurrence					
FISH	Univariable	PTEN deleted	1.92 (1.58-2.33)	<.001	
	Multivariable*	GPS per 20 units	2.09 (1.32-3.29)	<.001	<0.01%
		PTEN deleted	1.66 (0.94-2.92)	.08	
IHC	Univariable	PTEN deleted	1.76 (1.49-2.07)	<.001	
	Multivariable*	GPS per 20 units	1.62 (1.14-2.31)	<.001	<0.01%
		PTEN deleted	1.29 (0.74-2.27)	.37	
b: PTEN status and clinical recurrence					
FISH	Univariable	PTEN deleted	1.57 (1.00-2.45)	.048	
	Multivariable*	GPS per 20 units	3.97 (2.06-7.65)	<.001	<0.01%
		PTEN deleted	1.12 (0.60-2.09)	.73	
IHC	Univariable	PTEN deleted	1.80 (1.24-2.64)	.002	
	Multivariable*	GPS per 20 units	3.96 (2.33-6.73)	<.001	<0.01%
		PTEN deleted	0.90 (0.50-1.61)	.73	

HR, hazard ratio.
* Analysis accounted for cohort sampling weights.
[†] HRs for GPS are regression to mean (RM)-corrected.
[‡] q value is calculated for false positive rate.

the use of a well characterized cohort of patients undergoing RP with long term clinically relevant endpoints, a robust and validated RT-PCR based assay for measurement of GPS, measurement of PTEN status on tumor from the RP specimens rather than biopsy, and reproducible results with 2 different measures of PTEN deficiency.

The 17-gene GPS assay can significantly predict BCR and CR after RP independent of PTEN status in men with clinically localized PCa. The GPS assay can risk stratify within PTEN categories and it can help identify patients with PTEN deletion with favorable outcomes, and patients with intact PTEN with unfavorable outcomes.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.urology.2018.07.018.

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