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ORIGINAL ARTICLE

Association between DNA methylation of *HSPB1* and death in low Gleason score prostate cancer

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BACKGROUND: Heat shock protein 27 (Hsp-27) encoded by gene *HSPB1* is a critical regulator of the behavioral phenotype of human prostate cancer (PCa) cells, enhanced expression being associated with highly aggressive disease and poor clinical outcome. In contrast, the protein is not expressed in PCas of low malignant potential. To gain insight into the mechanism regulating its expression, we tested the hypothesis that differential methylation of CpG islands within *HSPB1* controls transcription and subsequent translation of the gene.
METHODS: We studied prostate epithelial cell lines and tissue biopsies, including 59 BPH and 415 PCas, of which 367 were a cohort of men with up to 20 years of follow-up. Methylation across the gene (DNA methylation (DNAme)) was assayed by pyrosequencing. Hsp-27 expression was assessed by western blot and immunohistochemistry.
RESULTS: In cancer tissues, methylation increased in a 3′ direction ($P < 0.0001$) whereas in benign hyperplasia methylation was constantly below 5%, a cutoff giving a specificity of 100% and sensitivity of 50%. Although methylation of the promoter region was significantly discriminating between benign and malignant prostatic epithelia, it compared poorly with methylation of the first intron. The prognostic value of *HSPB1* DNAme was confirmed by both univariate (hazard ratio 1.77 per 50% increment, $P = 0.02$) and multivariate models. Interaction between *HSPB1* methylation and Gleason score revealed high DNAme to be a reliable prognostic marker of poor outcome in men with low Gleason score ($P = 0.014$).
CONCLUSIONS: Our data indicate CpG methylation of the first *HSPB1* intron to be an important biomarker that identifies aggressive PCas otherwise regarded as low risk by current clinical criteria but that, biologically, require immediate active management.

INTRODUCTION

Heat shock protein 27 (Hsp-27), encoded by the gene *HSPB1* located on chromosome 7q11.23 has been shown in several independent studies to be a reliable biomarker of poor clinical outcome in human prostate cancer (PCa)[1–4] as well as in human breast cancer,[5] colorectal cancer[6] and malignant melanoma.[7] Biologically, Hsp-27 is an anti-apoptotic protein[8,9] that induces intracellular homeostasis and allows cellular repair and recovery after physical and chemical insults.[10] Although Hsp-27 is constitutively expressed in most human cells, induced overexpression during carcinogenesis can lead to increased survival of the malignant cells. Therefore, it is not surprising that studies link high expression of Hsp-27 to unfavorable prognosis in many cancer types.[2,11–13] The prognostic potential has been confirmed in prostate cell lines[14] as well as in prostate tissues where overexpression has been linked with hormone resistance and poor clinical outcome.[1,2,15] During early prostate carcinogenesis, expression of Hsp-27 protein becomes universally abrogated but may be re-expressed subsequently, in which case the malignancy develops an aggressive phenotype.[1,2] Although the specific factors controlling these changes are presently unknown, one plausible mechanism is DNA methylation (DNAme) of the *HSPB1* gene. The majority of CpG dyads in the human genome are methylated with the exception of

CG-rich regions called CpG islands.[16] CpG islands mainly cover gene promoters and first exons and their hypermethylation is associated with repressed transcription of many tumor-suppressor genes.[17,18] Therefore, we test the hypothesis that the DNAme status of *HSPB1,* particularly the *HSPB1* promoter, exon and intron regions, is an important determinant of PCa behavior. Thereafter, we assess any potential relationship between DNAme and Hsp-27 protein levels. Our objectives are also to investigate the diagnostic biomarker potential, by comparing the methylation status of BPH vs PCa, and the prognostic potential of DNAme, by analyzing the association between the methylation and PCa-specific death in the well-characterized Transatlantic Prostate Group (TAPG) cohort.[19]

MATERIALS AND METHODS

Human prostate tissue specimens and cell lines

Fresh frozen prostate biopsies from 77 patients, 48 PCa and 29 BPH, were used and have been previously described in detail.[20] Formalin-fixed paraffin-embedded (FFPE) PCa biopsies from a defined subset of 388 patients were randomly selected from a large cohort with TURP of well-characterized men residing in the United Kingdom—the TAPG cohort.[19,21] Sixteen patients were excluded because of poor DNA quality and five because of only normal tissue being available, leaving 367 specimens

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eligible. A total of 30 FFPE BPH specimens were collected after TURP at St Bartholomew's Hospital, London during the period 2003–2005.

Human prostate cell lines obtained from ATCC (Rockville, MD, USA) were the PNT2 immortalized epithelial cell line, the hormone-sensitive cancer cell lines LNCaP and VCaP and the hormone resistant, strong tumorigenic cancer cell lines DU145, PC3, PC3M and PC3M3 as well as PC3M variant cell line ST3 with silenced RLP19. Cell lines were cultured as previously described.[22]

For the population-based retrospective TAPG cohort UK national ethical approval was obtained from the Northern Multicentre Research Ethics Committee followed by local ethics committee approvals from each of the collaborating hospital trusts.[19] For all other tissues, written informed consent was obtained from all patients. Ethical approval was obtained from East London and the City REC Alpha and from Changhai Hospital Ethics Committee.

DNA extraction and bisulfite conversion

DNA from frozen tissues was extracted and bisulfite converted as previously described.[20] FFPE sections were deparaffinized in xylene by submersion two times for 5 min and in absolute ethanol three times for 5 min. From each case, an hematoxylin and eosin-stained section was annotated for cancerous and normal areas by an expert pathologist (DMB). Using the annotated section as a guide and depending on estimated tumor tissue size, one to six 5 μm FFPE sections were macro-dissected.[23] DNA was extracted using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation with the Proteinase K digestion done overnight and finally the DNA eluted in $2 \times 40\,\mu l$ of elution buffer. In all, 120 ng of DNA was used in the bisulfite conversion with the EpiTect Bisulfite kit (Qiagen) according to the manufacturer's instructions for DNA extracted from FFPE tissues.

PCR and pyrosequencing

HSPB1 (reference sequence NG_008995) and 500-bp upstream sequence (Figure 1a), was searched with MethPrimer[24] set at default parameters (Figure 1b). Using PyroMark Assay Design v. 2.0.1.15 (Qiagen), assays 1–5 were designed to cover the first four CpG islands, whereas it was not possible to obtain a functioning assay for the fifth island. Assay 6 was designed in the third exon. All assays were designed to include three to six CpG positions in as short amplicon as possible and a non-CpG cytosine internal control. Due care was taken to avoid any primer overlapping CpG

dyads to prevent amplification biases. To limit the costs, all assays were initially run on a selected set of specimens (Figure 1c) and assay 5 on all available tissues (Figure 3a). PCRs were performed using a converted DNA equivalent of 1000 cells (assuming 6.6 pg DNA per diploid cell) from FFPE tissues or 400 cells from frozen tissues employing the PyroMark PCR kit (Qiagen) as previously described.[20] Assay primers and annealing temperatures are presented in Supplementary Table 1. Correct amplification was confirmed by QIAxcel capillary electrophoresis instrument (Qiagen). PyroGold reagents (Qiagen) were used for the pyrosequencing reaction and the signal was analyzed using the PSQ 96MA system (Biotage, Uppsala, Sweden).[20] All runs contained standard curves as previously described.[20]

Hsp-27 expression

The Hsp-27 expression in cell lines was demonstrated with western blot and intensity was measured as previously described.[22] The Hsp-27 expression data were available for the TAPG cohort as it was previously evaluated by immunohistochemistry.[2]

Statistical analysis

All analyses were based on a pre-defined statistical analysis plan, agreed before analysis. Mean methylation of the investigated CpG positions within each assay was used for all analysis. Methylation data were adjusted for primer bias through re-scaling methylation measurements by the median standard curve. When exploring prognostic potential, FFPE data were modified by setting all DNAme values below 5% to 0, while remaining data were left continuous. Modification was done to lower the method noise on the results; 5% value was used as it clearly separated all BPH from PCa. Cuzick trend test was used to investigate the trend of methylation status across the six assays. The Wilcoxon rank sum test was used for comparisons of DNAme from different assays in BPH vs PCa. The Wilcoxon and Cuzick trend tests were used to compare association of DNAme with available clinical variables and HSP-27 expression. Univariate Cox proportional hazards model was fitted with each variable and the statistically significant variables were included in the multivariate Cox model. Six missing extent of disease values were imputed by the median value of 1 to avoid reducing of the sample size.[25] All P-values are two-sided and considered significant if < 0.05. Statistical analyses were performed in Stata version 11 (StataCorp, College Station, TX, USA) and R version 2.12.2
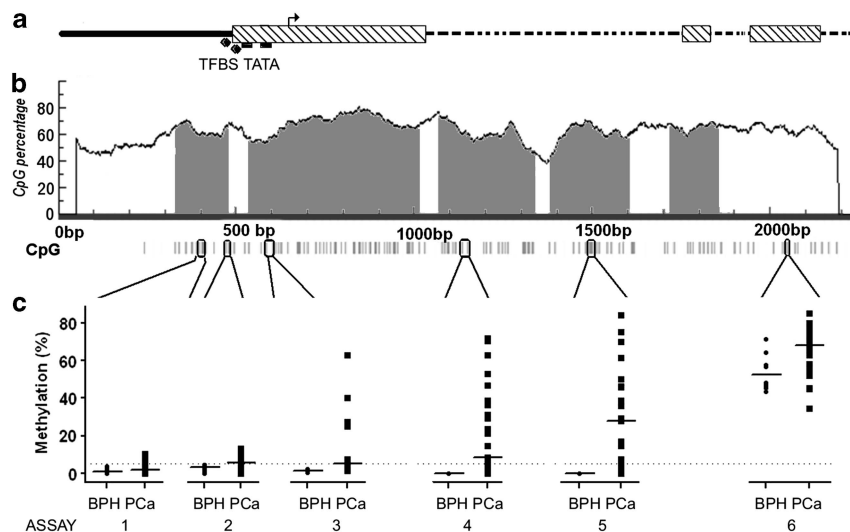
Figure 1. Schematic overview of (**a**) the HSPB1 gene, (**b**) the CpG islands and the investigated sites and (**c**) the measurement of six methylation assays. (**a**) Transcription factor-binding sites (TFBS) and TATA box are located within the 200-bp region upstream of the transcriptional start site at position 156 (arrow). The HSPB1 gene, 1740-bp, and 500-bp upstream of the gene were searched with Methprimer, (**b**) identifying 5 CpG islands (gray) located in (**a**): the first in the promoter region (black solid line), a second covering exon 1 (striped box), a third and fourth within intron 1 (dotted line) and a fifth in exon 3. (**b**) CpG positions investigated by assay 1–6 are circled in the CpG map. (**c**) Median of methylation in fresh frozen 10 BPH (circle) and 27 prostate cancer (PCa) (square) show the increasing separation between BPH and PCa going in 3′ direction, differences between BPH and PCa were all significant by the Wilcoxon test with the maximum difference ($P < 0.0001$) provided by assay 5. The increase in DNAme across the six assays was highly significant ($P < 0.0001$). The dashed line indicates 5% methylation.
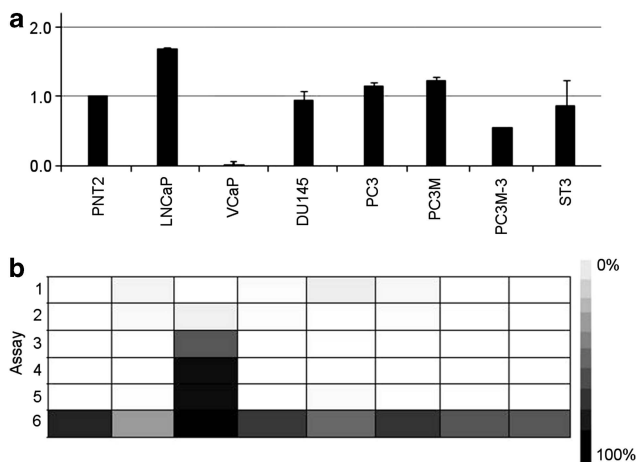
RESULTS

Methylation across the *HSPB1* gene

Methylation of CpG islands in promoter, exon 1 and intron 1 increased in PCa but remained below 5% in BPH tissue (Figure 1c). Assay 1 could not distinguish between BPH and PCa ($P = 0.05$). Assay 2, covering transcription factor-binding sites,[26] showed some separation of these categories with a low median methylation of 5% in PCa and 3% in BPH ($P = 0.009$). In assay 3, interrogating CpG positions close to the TATA box (Figure 1b),[27] the median methylation of PCa was 5% compared with 1% methylation in BPH ($P = 0.001$). In intron 1, the median methylation increased to 8% in assay 4 ($P = 0.0004$) and 28% in assay 5 ($P < 0.0001$) in PCa, whereas 0% was observed in BPH with both assays (Figure 1c). In each PCa where DNAme measured high with assay 5, an increasing trend in the 3' direction was observed with corresponding assays 1–4. Downstream of the CpG islands, in exon 3, highest methylation was observed in both BPH and PCa with median 52% and 68%, respectively, ($P = 0.03$; Figure 1c). As the largest methylation difference between BPH and PCa was observed with assay 5, it was further used to investigate both the diagnostic and prognostic potential of DNAme.

Association between DNAme and protein expression

Expression of Hsp-27 and DNAme of *HSPB1* were measured in eight cell lines (Figure 2a). Highest DNAme was observed in exon 3 in all cell lines with LnCaP measuring lowest at 40% (Figure 2b). Also, in LnCaP, highest expression of Hsp-27 was observed at 1.7-fold compared with PNT2. Protein expression was lowest (0.6-fold) in PC3M-3 and absent in VCaP. Although PC3M-3 was unmethylated in CpG islands similar to PNT2, VCaP was the only cell line where methylation was increasing and above 5% in assays 3–5. DU145, PC3, PCM3 and ST3 displayed unmethylated CpG islands and similar levels of expression to PNT2.

Wilcoxon test showed no association ($P = 0.6$) between DNAme and Hsp-27 immunohistochemistry score in the FFPE tissues (Supplementary Figure 1b).
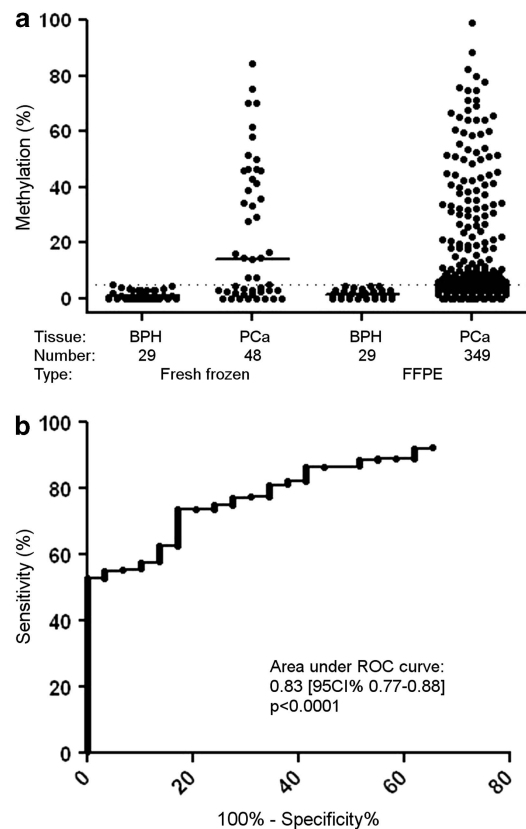
Diagnostic potential of assay 5 in frozen and FFPE tissues

Methylation was successfully measured in 349 of 367 FFPE TAPG cohort specimens, 29 of the 30 FFPE BPH and all available frozen tissues. In the fresh frozen material, the median methylation was 14% in PCa and 1% in BPH ($P < 0.0001$), whereas it was 5% in FFPE PCa and 1% in FFPE BPH ($P < 0.0001$; Figure 3a). The seemingly lower methylation in FFPE comparing with frozen PCa tissues was not significant ($P = 0.14$). Using 5% as a predefined methylation threshold to minimize false-positive results, diagnostic sensitivity in the fresh and FFPE material was 58% (95% confidence interval (CI) 43–72%) and 50% (95% CI 45–55%), respectively, with corresponding specificity 100% (95% CI 88–100%) in both material types (Figure 3b).

Prognostic potential of assay 5

The median age in the TAPG cohort was 70.5 years (interquartile range 67.3 to 73.2) and median follow-up was 9.5 years with up to a maximum of 20 years. In all, 91 out of 349 patients died of PCa.

First, the relationship between *HSPB1* DNAme and all available variables was investigated (Supplementary Figure 1) showing a significant association with Gleason score ($P = 0.003$) and extent of disease ($P < 0.0001$) but not age ($P = 0.2$), PSA ($P = 0.07$) or HSP-27 immunohistochemistry score.

Figure 3. The diagnostic potential of *HSPB1* DNA methylation (DNAme). (**a**) Methylation of *HPSB1* was significantly ($P < 0.0001$) higher in prostate cancer (PCa) tissue comparing with BPH in both fresh frozen and formalin-fixed paraffin-embedded (FFPE) samples. The dotted line shows 5% methylation. (**b**) In order to visualize the diagnostic efficacy of *HPSB1* methylation measured in the FFPE tissues in absence of an arbitrary cutoff value, the data were summarized using a receiver operating characteristic curve (ROC). Based on the sensitivity and specificity, highest possible specificity (100%) was obtained at cutoff 5%, with corresponding sensitivity 50%.

Figure 2. Heat shock protein 27 (Hsp-27) expression and gene methylation in eight investigated cell lines. (**a**) Mean Hsp-27 generic expression (fold difference) relative to the immortalized prostate epithelial cell line PNT2 $\pm$ 1 s.d. was measured by western blot. (**b**) The methylation of six investigated regions in corresponding cell lines are shown in grayscale.

Table 1. The summary statistics of clinical and pathological variables and the univariate and multivariate Cox models

Variable	N (DPCa)	Univariate analysis			Multivariate analysis		
		HR (95% CI)	χ^{2a}	P-value	HR (95% CI)	χ^2	P-value
Modified HSPB1[b]	349 (91)	1.12 (1.02, 1.23)	5.4	0.02	1.18 (0.98, 1.41)	3.2	0.075
Age		1.69 (0.94, 3.05)	3.5	0.06			
⩽65	61 (13)						
>65	288 (78)						
Gleason score		3.04 (2.35, 3.93)	79.3	<0.0001	2.36 (1.65, 3.37)	22.3	< 0.0001
<7	183 (18)						
=7	78 (24)						
>7	88 (49)						
PSA score		2.00 (1.71, 2.34)	75.1	<0.0001	1.49 (1.25, 1.78)	19.8	< 0.0001
⩽4	130 (12)						
>4–10	74 (11)						
>10–25	71 (25)						
>25–50	51 (25)						
>50–100	23 (18)						
PCa in biopsy (%)		1.85 (1.60, 2.15)	73.1	<0.0001	1.35 (1.13, 1.61)	11.1	0.001
⩽6	104 (10)						
>6–20	91 (12)						
>20–40	53 (16)						
>40–75	42 (15)						
>75–100	59 (38)						
Interaction term (HSPB1 DNAme × Gleason score)	349 (91)				0.98 (0.97, 0.99)	6.1	0.014

Abbreviations: CI, confidence interval; DNAme, DNA methylation; DPCa, died of PCa; HR, hazard ratio; PCa, prostate cancer.
[a]One-degree freedom.
[b]Continuous methylation per 10% change.

In the univariate Cox model, prostate specific death was significantly associated to Gleason score, extent of disease (proportion of TURP chips with disease), PSA level and HSPB1 DNAme level (Table 1). The hazard ratio was 1.77 (95% CI 1.13–2.79) per 50% increase in DNAme.

All significant variables in the univariate model were included in the multivariate analysis as well as all interactions between HSPB1 DNAme and all clinical variables. A significant negative interaction was only found for Gleason score and HSPB1 DNAme with hazard ratio 0.98 (95% CI 0.97–0.99) and $P = 0.014$. To illustrate this interaction, DNAme was stratified by Gleason score and endpoint status (Figure 4). Comparison of a model including HSPB1 DNAme and a model with only clinical variables showed that HSPB1 DNAme significantly improved the ability to predict prostate specific death ($\Delta\chi^2 = 6.673$, df = 2, $P = 0.036$; Supplementary Table 2).

DISCUSSION

The data from our current study support, in part, the original hypothesis by demonstrating a relationship between methylation of HSPB1 and the aggressive phenotype within a cohort of men with poor clinical outcome but low Gleason score ($P = 0.014$). DNAme increased in the 3′ direction of HSPB1, beginning with CpG positions covering the regulatory sequences in PCa but not in BPH (Figure 1). Previously, it had been reported[1,2] that progression of in situ neoplasia to frank malignancy is invariably accompanied by decline of HSP-27 protein (Supplementary Figure 2). Thereafter, subsequent development of invasive malignancy may be accompanied by re-expression of HSP-27 in the ∼30% of cases that are universally aggressive.[1,2,15] Nevertheless, this study did not reveal a simple linear relationship between DNAme and reduced HSP-27 expression (Supplementary Figure 1). In seven human prostate cell lines, DNAme of CpG islands was <10% (Figure 2), VCaP being the only cell line exhibiting a similar gradual increase in DNAme across the gene to that seen in PCa tissues. Also, VCaP was the only of eight cell lines of varying malignancy that did not express Hsp-27
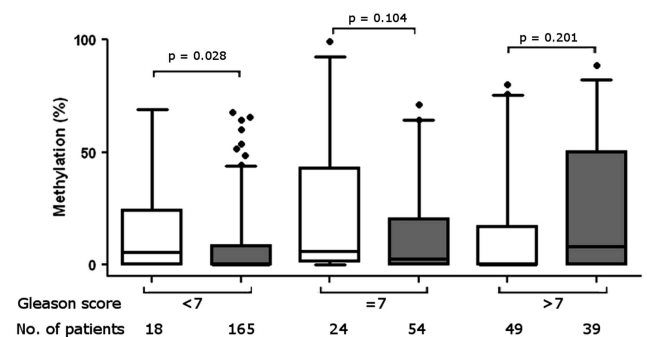
Figure 4. DNA methylation (DNAme) in patients who died (white box) of prostate cancer and patients alive or censored at the end of the study (shaded box) was stratified by Gleason score. In the low Gleason score group, higher median methylation (6%) was observed in patients who died than in patients alive or censored at the end of the study (median DNAme = 0%). In the high Gleason score group, the reverse was observed with median methylation 8% vs 0%. Whiskers of the boxplot mark the 5th and 95th percentiles, the box 25th percentile, median and 75 percentile, whereas extreme values are shown by (●).

protein, supporting silencing of the gene by hypermethylation. Hsp-27 expression was highest in LNCaP and showed decreased methylation in exon 3 comparing with the other cell lines (Figure 2). However, in PC3-M3, although the gene was not methylated, its expression was decreased by some 50%. When examined together, these data indicate a more complex regulation of Hsp-27 protein levels in vivo than simply by DNAme alone.

Although, methylation was highest outside the CpG islands as expected in both BPH and PCa, assay 6 was still able to distinguish between the tissues ($P = 0.03$). This could be a reflection of a generalized cellular defense attempt to inhibit expression of destabilizing genes through a global increase of de novo methylation in PCa.[28] In addition, the methylation difference

between BPH and PCa was most pronounced in the first intron of HSPB1 suggesting that, for diagnostic purposes, it may be valuable to assess methylation in regions outside the promoter. So far, substantial research efforts have shown that increased DNAme of the promoters of genes such as GSTP1, RARB, APC, TIG1 can be detected in PCa biopsies as well as bodily fluids and may be useful for early diagnosis, although none have been validated for routine clinical use.[29,30] Diagnostic potential of HSPB1 methylation using a 5% methylation cutoff, was comparable in frozen and FFPE tissues with 50–58% sensitivity and 100% specificity (Figure 3). Although methylation was measured in the intron alone, we believe that measuring methylation levels in the other regions would not have yielded different results because the initial data showed an increasing trend for methylation in the 3′ direction of the gene.

The prognostic value of HSPB1 DNAme was indicated by both univariate and multivariate models (Table 1). In the multivariate analysis, significant negative interaction observed between DNAme and Gleason score suggests that the increased methylation of HSPB1 is a marker of poor outcome in men with low Gleason score who would previously have been regarded as being at low risk (Figure 4). The interaction term formed a final model with PSA, extent of disease and Gleason score and was stronger than a model excluding DNAme ($P = 0.036$). Therefore, measuring HSPB1 DNAme adds valuable information when assessing an individual patient's risk of death due to PCa. Our data are in line with the previously observed absence of HSP-27 protein in patients with low Gleason score but aggressive invasive PCa.[1] In addition, the stratified hazard ratio over time showed that HSPB1 methylation is useful in identifying men at high risk of developing aggressive disease 7 years after the diagnosis (Supplementary Figure 3). This may mirror that the men that are at high risk but with low Gleason score would have a longer life expectancy when compared with those with high Gleason score. Although exciting, the interaction found between methylation and Gleason score was based only on a small number of patients (Figure 4) and validation in a larger set of samples is currently underway. A potential limitation to our study is the use of TURP specimens. Although TURP is not the standard modality for the diagnosis of PCa, analysis of these samples has allowed us to assemble a unique cohort with up to 20 years of clinical follow-up. We believe that changes in HSPB1 are intrinsic to PCa carcinogenesis and may be a driving process within this malignancy. If this is further substantiated, then assessment of HSPB1 methylation is expected to yield similar data in prostate needle core biopsies, although the terms of the risk equation may change to reflect the different specimen types.

In conclusion, HSPB1 is essentially unmethylated in BPH but with increasing neoplastic changes through to PCa, the gene becomes increasingly methylated, proceeding from the promoter in a 3′ direction. In PCas with low Gleason score, higher methylation within the HSPB1 gene independently identifies patients with poor clinical outcome and hence is an objective biomarker identifying the immediate need for active intervention in the clinical management of this cohort of patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Prostate Cancer and Prostatic Diseases website (http://www.nature.com/pcan)