DNA methylation of *PITX2* predicts poor survival in men with prostate cancer

Aim: We investigated if methylation of candidate genes can be useful for predicting prostate cancer (PCa) specific death. **Patients & methods:** Methylation of *PITX2*, *WNT5a*, *SPARC*, *EPB41L3* and *TPM4* was investigated in a 1:2 case–control cohort comprising 45 men with cancer of Gleason score ≤ 7 who died (cases), and 90 men who were alive or died of other causes with survival time longer than the cases (controls). A univariate conditional logistic regression model was fitted by maximizing the likelihood of DNA methylation of each gene versus the primary end point. **Results:** A 10% increase in methylation of *PITX2* was associated with PCa related death with OR 1.56 (95% CI: 1.17–2.08; $p = 0.005$). **Conclusion:** Our study strengthens prior findings that *PITX2* methylation is useful as a biomarker of poor outcome of PCa and in addition we also suggest that it may be particularly useful in men with low Gleason score.

Keywords: DNA methylation • *PITX2* • prognostic biomarker • prostate cancer

The natural course of prostate cancer (PCa) is heterogeneous with some patients dying of metastatic disease within a few years of diagnosis while most men live for decades with indolent disease. Testing serum for PSA [1] is an increasingly used screening tool for detection of clinically localized PCa at early stages. Unfortunately, while PSA screening detects many additional cases of disease, it has poor specificity, and has produced an increasing problem in separating aggressive from indolent cancers [2,3]. Currently, the best-available prognostic tool for routine management is Gleason score [4], but drawbacks such as intra- and interobserver variability [5] as well as variability due to the difficulty of targeting needle biopsies precisely to the cancer leads to errors in sampling and visual scoring. The weakness of current tools for specifically identifying aggressive disease means that men today still face a dilemma when choosing between treatments or watchful waiting. There remains an important ambiguity in treatment benefit versus the associated harms and costs [6,7]. A large randomized study of men with clinically localized PCa showed

that radical prostatectomy did not significantly reduce PCa mortality, when compared to watchful waiting, through at least 12 years of follow-up [8]. There is an urgent need for biomarkers that identify aggressive disease in a highly accurate and cost-effective way. Various prognostic indicators such as the RNA-based cell cycle progression (CCP) score [9], epigenetic biomarkers [10] and expressed proteins [10,11] are undergoing further evaluations to determine applicability in more generalized clinical settings.

DNA methylation contributes to development and progression of PCa [12,13]. A growing body of evidence indicates that methylation has potential to improve the sensitivity for PCa diagnosis [14]. In addition, it has been suggested that DNA methylation assays could be prognostic and complementary or perhaps an alternative to other prognostic tests such as the CCP score, immunohistochemistry or other kinds of approaches [9,15,16]. Methylation assays have desirable features such as robust measurement of stable DNA and the potential of inexpensive assays [17], but have so far shown only limited success as clinical

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tools for predicting aggressive PCa [18]. A majority of the studies focusing on the prognostic value of methylation have used time to biochemical recurrence after surgical treatment as the primary surrogate end point, which does not accurately estimate the potential of the cancer in terms of risk of death if left untreated. We recently reported the prognostic value of methylation of *HSPB1* to predict men at risk of dying from PCa in a watchful waiting cohort [15]. In the current case-control study, we used the same cohort but focused on patients with localized PCa of Gleason score ≤ 7 and investigated if methylation of additional genes can predict poor survival. The aim was to find several genes than can complement *HSPB1* in a panel as predictors of poor outcome in men who would otherwise be regarded as of low risk of PCa death.

The current study was done in two phases with initial assessment of methylation of 10 genes: *WNT5a*, *CTNNB1*, *SPARC*, *EPB41L3*, *PITX2*, *TOP2a*, *CDC20*, *LINE1*, *miR-23b* and *TPM4* in fresh frozen PCa and benign prostate hyperplasia (BPH). The selection of candidates was made by searching the literature for genes that primarily showed either altered expression (*WNT5a*, *SPARC*, *EPB41L3*, *miR-23b*, *CTNNB1*) [19–22] or methylation (*PITX2*, *LINE1*, *TPM4*) [23–25] associated with poor prognosis. Of particular interest was the methylation of *PITX2* as numerous studies have suggested its ability to predict biochemical recurrence [23,26] and in addition efforts have been put into developing real time PCR assays for its detection [27]. *CDC20* and *TOP2a* were included in the first phase as they are used in the CCP score [9]. Genes that showed distinctive differences in methylation between BPH and cancers were further tested in the case-control panel comprising a selected set of 135 patients from the Transatlantic Prostate Group (TAPG) cohort [4].

Our primary hypothesis was that methylation of some of the investigated genes would differ significantly in cases compared with controls and therefore be potentially useful to identify patients with low Gleason score untreated clinically localized cancer who were at risk of dying of PCa.

Patients & methods

Patient material

The study was conducted according to REMARK criteria [28]. Fresh frozen tissues were obtained from a London specimen archive and have been fully described in an earlier paper [29]. DNA was extracted from the frozen prostate tissues from 20 patients, 10 PCa and 10 BPH and were used for the evaluation in the first phase of the study [29]. For the case-control panel in the second phase of the study, we used DNA extracted from archived formalin-fixed transurethral resections

of prostate (TURP), from the TAPG cohort as previously described [15]. In summary, the TAPG cohort is comprised of well-characterized men with symptomatic prostate cancer residing in the UK who did not receive any radical treatment (surgery or radiotherapy) at time of diagnosis or within 6 months thereafter. The primary end-point of the cohort was death from PCa and outcomes were determined through medical records and cancer registry records. Of 367 men in the TAPG cohort, 275 had a Gleason score ≤ 7 , of which 46 died of PCa while 229 were alive or died of other causes. Forty-five cases (i.e., patients who had Gleason score ≤ 7 and died of PCa) were each matched to two controls by Gleason score. Matching for follow-up was primarily based on controls alive at the end of the follow-up; however, if preferred controls were not available, controls who died of other causes were selected with the requirement that the controls always had longer follow-up than corresponding cases. Written informed consent was obtained from all patients providing frozen tissues; ethical approval was obtained from East London and the City REC Alpha. For the TURP specimens UK national ethical approvals were obtained from the Northern Multicenter Research Ethics Committee followed by local ethics committee approvals from each of the collaborating hospital trusts [4].

Methylation assays

120–200 ng of DNA was converted using EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. PCRs were performed using a converted DNA equivalent of 1000 cells (assuming 6.6 pg DNA per diploid cell) from formalin-fixed paraffin-embedded (FFPE) tissues or 400 cells from frozen tissues employing the PyroMark PCR kit (Qiagen) as previously described [29]. The primers for each assay were designed using PyroMark Assay Design v. 2.0.1.15 (Qiagen) and are described in **Supplementary Table S1** (see online at <http://www.futuremedicine.com>). The assays were designed to avoid any overlapping of CpG positions and were targeted to the same 3–6 CpG positions as previous reports for *PITX2*, *LINE1*, *TPM4* and *SPARC* [24–26], while the other assays were located near the gene promoter, details of the primers and the CpG sites interrogated are provided in **Supplementary Table S1**. To provide the internal control for total bisulfite conversion, a non-CpG cytosine was included. Each PCR reaction contained 12.5 μ l of PCR master mix, 2.5 μ l Coral red, 2 μ l of DNA, 5 pmol of each primer and water adjusting final volume to 25 μ l. An addition of $MgCl_2$ to reach 2.5 mM per reaction was added to assay *LINE1*. The thermocycling was set to 10 min at 95°C, then 45 cycles of 94°C for 30 s, optimized annealing tem-

perature (Supplementary Table S1) for 30 s and 72°C for 30 s, followed by 10 min at 72°C. Presence of the correct amplicon was confirmed on QIAxcel (Qiagen) and pyrosequencing was done as previously described [29]. All runs included standard curves as positive controls, of 0, 50 and 100% methylated human DNA and a nontemplate control. A total of 200 ng of unmethylated (Qiagen) and hypermethylated DNA (Millipore, Billerica, MA, USA) were mixed to obtain the different ratios of DNA methylation and then bisulfite converted as described above.

Statistical analysis

Supplementary Table S2 shows details of clinical variables for the specimens used in the study. Mean methylation of 3–6 investigated CpG positions within a gene was used for all the analyses. For any given gene if the methylation assay failed in some cases (due to PCR inhibitors, inadequate or damaged DNA or unknown reasons) the failed cases for that gene were excluded from the analysis. If one control failed the other control was used alone along with the case in the analysis. Finally, if both controls failed then the corresponding case was also excluded. Due to missing values there were different numbers of cases and controls for the genes in the tables (for additional information and a line listing of failed cases and controls please contact the corresponding author). Median methylation of genes in BPH and PCa tissues were compared using the Mann–Whitney U test. A univariate conditional logistic regression model, maximizing conditional likelihood, was fitted with DNA methylation of each gene versus the primary end point. The statistical analysis was performed using R v. 3.0.2 software. All statistical analyses were performed for each gene separately. All tests were two sided and $p < 0.05$ was accepted as statistically significant. To account for the high number of tested genes on the same material, the Benjamini and Hochberg step-up procedure for controlling false discovery rate (FDR) was applied with FDR of 5% [30].

Results

DNA methylation in fresh frozen tissues

The methylation of 10 investigated genes in 10 BPH and 10 PCa tissues is illustrated in Figure 1. Genes *EPB41L3*, *SPARC*, *PITX2*, *WNT5a*, *TPM4*, *miR-23b*, *TOP2a* and *CDC20* showed significantly elevated methylation in PCa tissues. The best separation of BPH from PCa was observed with methylation of *SPARC* ($p = 0.0002$; AUC 1.0). However, the methylation of *CDC20*, *TOP2a* and *CTNNB1* was low, in other words, $< 5\%$ and as such low methylation levels are prone to greater variability in measurement due the lower accuracy of the pyrosequencing method

in this range, these genes were not further investigated. At the other end of the spectrum, *miR-23b* and *LINE1* showed high methylation in all specimens with 10–20% maximum variation within the investigated cancers and controls, these genes were therefore dropped from further investigation.

DNA methylation in the case-control cohort

Methylation assays for five genes: *EPB41L3*, *SPARC*, *PITX2*, *WNT5a* and *TPM4* were completed using DNA from 135 patients, but the assays were successful for different numbers of patients for each gene (Table 1). The number of cases and the total number of patients included for the analysis of each gene is presented in Table 1. The distribution of methylation of each gene stratified in cases and controls is presented in Figure 2. The median follow-up time in the 45 cases was 7.8 years (interquartile range = 6.1 years) and 15.3 years (interquartile range = 6.8 years) in the 90 controls. A univariate conditional logistic regression was fitted with continuous methylation of each gene versus death of PCa and the odds ratio (OR) values are presented in Table 1. Prior to adjustment for multiple testing, methylation of *PITX2* and *WNT5a* was associated with PCa related death with OR 1.56 (95% CI: 1.17–2.08) and 1.28 (95% CI: 1.02–1.60), respectively (Table 1). However, after FDR adjustment at 5% was applied, only methylation of *PITX2* remained significant ($p = 0.005$) for predicting PCa related death and is interpreted as the following: for each 10% increase in methylation of *PITX2*, the risk of PCa-related death increased by a factor of 1.56.

Discussion

Assessing the methylation of *PITX2* and *WNT5a* may be suitable as prognostic biomarkers to identify men with low Gleason score who are at risk of dying from PCa. Both proposed biomarkers act in the Wnt pathway, where *PITX2* acts as a transcriptional factor which interacts with β -catenin-independent and dependent pathways, while *WNT5a* is a representative ligand activating the β -catenin-independent pathways. Of note our selection criteria for candidate genes did not actively seek biomarkers in the Wnt pathway, the genes selected were to be representative of what was available as good candidates from an objective literature search done shortly before the project was initiated.

Hypermethylation of *PITX2* in PCa has previously been shown as an independent prognostic biomarker of biochemical recurrence [23,26,27] and was also found to be associated with reduced mRNA transcription [31]. Due to the growing number of publications showing an association of *PITX2* to biochemical recurrence, methylation assays based on microarray and RT-PCR

Figure 1. The methylation of each gene in benign prostatic hyperplasia (light blue boxplot) and prostate cancer (blue boxplot). Whiskers of the boxplot mark the 5th and 95th percentiles, the box 25th percentile, median and 75th percentile.

technologies are being pursued [26,27]. Here, we report for the first time a direct association between increase in *PITX2* methylation and risk of death of PCa in men with Gleason score ≤ 7 . *PITX2* promises to be an important prognostic biomarker with a large effect size, the OR for a 10% step in methylation was 1.56, which passes initial criteria for use of a standalone prognostic biomarker with an expected OR of 1.5 or greater. Furthermore, when immunochemical biomarkers are used in clinical practice they are often scored as positive or negative. If one were to translate that to *PITX2* methylation and dichotomize the data using the median methylation in controls as a cut-off, an OR of 2.7 (95% CI: 1.1–6.7); $\chi^2 = 5.3$ was obtained.

Our DNA methylation data were analyzed as a continuous predictor and no cut-off was used to define positive/negative values. It was not our goal in this paper to find cut-offs for each gene, the definition of positive or negative values and where to draw the cut-off remains an open question needing additional research. We

anticipate that the biomarkers will be used in a multivariate classifier and their correlation to other genes in the classifier will affect where the overall cut-off is drawn. A multivariate classifier cut-off is usually not based on levels of methylation of any particular gene but rather on the sum of the entire set of gene values.

An interesting finding in the current study is the clear separation of BPH from PCa by *SPARC* methylation (Figure 1). The diagnostic potential of *SPARC* methylation has been suggested before [32] and in light of current findings, a further investigation is warranted in a large set of prostate needle core biopsies.

The main limitation of the current study is the use of TURP tissues, a method that is no longer in common practice. It allowed us, however, to assemble a cohort of untreated men with PCa with up to 20 years of follow-up and therefore we found it suitable for initial credentialing work. Nevertheless, these initial findings should be validated in needle biopsy specimens. Of relevance to the discussion of suitable study specimens,

Table 1. Effect size and significance of DNA methylation changes in five genes.							
Gene	OR (95% CI)	LR test	Unadjusted p-value	Adjusted [†] p-value	Failed [‡] assays	Patients [‡] analyzed	Cases [‡]
PITX2	1.56 (1.17–2.08)	10.85	9.9e-04	0.005	11	109	37
WNT5a	1.28 (1.02–1.60)	5.02	0.025	0.063	6	120	40
SPARC	1.10 (0.95–1.28)	1.53	0.216	0.359	11	112	38
EPB41L3	1.04 (0.88–1.22)	0.18	0.674	0.755	5	124	42
TPM4	0.97 (0.80–1.18)	0.10	0.755	0.755	12	109	37
The univariate conditional logistic regression model was fitted giving the odds ratio with 95% CI associated with a decile increase in methylation, the likelihood ratio test had one degree of freedom. Pairs were included in the analysis as long as methylation assay was successful in at least one case and one control.							
[†] The Benjamini and Hochberg step-up procedure for controlling false discovery rate was applied with false discovery rate of 5%.							
[‡] The numbers of cases or controls.							
LR: Likelihood ratio; OR: Odds ratio.							

the CCP score was first validated in these same TURP specimens [9] and subsequently validated in needle biopsy specimens, showing that TURP specimens are a suitable set for discovery and credentialing of new prognostic biomarkers for PCa. Another limitation of our study is that it is relatively small, with only 45 cases and 90 controls; however, we designed the study to be sufficiently large to meet our main goal of identifying at least one biomarker gene with a significant odds ratio. The biomarker value of the two genes identified in our study need to be validated in larger studies.

Ideally, a biomarker detected by molecular testing of bodily fluids is desired to avoid intrusive examinations and potentially harmful biopsies. Aberrant methylation of other genes has been successfully detected in urine and blood [33] indicating that methylation levels of *PITX2* may also be measurable in bodily fluids.

Conclusion

Finding biomarkers to predict aggressive disease in men with low Gleason grade who would otherwise be regarded as of low risk and are candidates for watchful waiting is of high importance. Here, we demonstrate that the methylation of *PITX2* is higher in patients diagnosed with Gleason score ≤ 7 who died of PCa (Table 1 & Figure 2). Therefore, methylation of *PITX2a* shows potential to serve as a biomarker to predict aggressive disease in men with low Gleason score and larger validation studies in prostate needle biopsies as well as bodily fluids are appropriate next steps.

Future perspective

Screening with PSA has greatly increased the detection of PCa but also identifies too many cases of indolent disease. The accuracy and quality of biop-

Figure 2. The distribution of methylation of each gene in cases (blue boxplot) and controls (light blue boxplot) in the follow-up study. Whiskers of the boxplot mark the 5th and 95th percentiles, the box 25th percentile, median and 75th percentile.

sies has been improved by better image-guided biopsy techniques, with ultrasound and MRI guided needle sampling increasingly having a beneficial effect on the diagnosis and prognosis of PCa; these improvements should decrease the invasiveness of the biopsy process by requiring fewer repeat biopsies. However, the best approach for predicting whether the patient should undergo biopsy and the nature of the subsequent treatment or of active surveillance is still under investigation. Patient management would be dramatically aided by accurate prognostic tests that can be used in urine or blood, reducing the need for excessive biopsies. Epigenetic changes in PCa are showing great promise as a source of stable biomarkers which can also be easily measured in bodily fluids. However, the identification, development and validation of optimal combinations of biomarkers, considering accuracy and costs, will take another 5–10 years. The test pan-

els may include DNA methylation biomarkers such as *PITX2* in combination with other genes and RNA expression signatures.

Financial & competing interests disclosure

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Executive summary

Results

- In men diagnosed with Gleason score ≤ 7 , methylation of *PITX2* was significantly elevated in men who died of prostate cancer (PCa) compared with men who died of other causes or were still alive at follow-up.
- *PITX2* had a large effect size with a 10% increase in methylation conferring a 1.56-times higher risk of PCa related death.
- Methylation of *EPB41L3*, *SPARC*, *PITX2*, *WNT5a* and *TPM4* were highly elevated in cancers compared with benign prostate hyperplasia tissues.
- *CDC20*, *CTNNB1* and *TOP2a* showed very low methylation levels in both cancer and BPH.
- *miR-23b* and *LINE1* were highly methylated in both BPH and cancer and were not studied as prognostic biomarkers.

Discussion

- Methylation of *PITX2* has previously been associated with biochemical recurrence in men who underwent radical prostatectomy, we show for the first time an association of elevated methylation in the gene to the risk of PCa related death.
- Our results on *PITX2* are completely separate from earlier studies by other groups and thus they represent an independent validation of *PITX2* as a PCa prognostic biomarker.
- *SPARC* showed a very large difference between BPH and PCa and may be useful as a diagnostic biomarker.

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