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Short Report

Prognostic value of DNA ploidy and automated assessment of stroma fraction in prostate cancer

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The combination of DNA ploidy and automatically estimated stroma fraction has been shown to correlate with recurrence and cancer death in colorectal cancer. We aimed to extend this observation and evaluate the prognostic importance of this combined marker in prostate cancer. DNA ploidy status was determined by image cytometry and the stroma fraction was estimated automatically on hematoxylin and eosin stained sections in three tumor samples from each patient to account for tumor heterogeneity. The optimal threshold for low (≤56%) and high (›56%) stroma fraction was identified in a discovery cohort (n = 253). The combined marker was validated in an independent patient cohort (n = 259) with biochemical recurrence as endpoint. The combined marker predicted biochemical recurrence independently in the validation cohort. Multivariable analysis showed that the highest risk of recurrence was observed for patients with samples that had both non-diploid ploidy status and a high stroma fraction (hazard ratio: 2.51, 95% confidence interval: 1.18–5.34). In conclusion, we suggest the combination of DNA ploidy and automatically estimated stroma fraction as a prognostic marker for the risk stratification of prostate cancer patients. It may also be a potential generic marker as concurrent results have been described in colorectal cancer.

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Additional Supporting Information may be found in the online version of this article.

Key words: prostate cancer, DNA ploidy, stroma fraction, digital image analysis, intra-tumor heterogeneity

Abbreviations: BCR: biochemical recurrence; CI: confidence interval; H&E: hematoxylin and eosin; HR: hazard ratio; IQR: interquartile range; PSA: prostate specific antigen; REK: Norwegian Regional Committees for Medical Research Ethics; TME: tumor microenvironment

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Introduction

Prostate cancer is characterized by histological changes in the architecture of the glands, which is reflected in the Gleason grading system of such tumors. This system is the most widely used and the strongest available marker of prostate cancer prognosis. However, the degree of intra- and inter-observer variance is considerable.[1,2] Large-scale genomic instability assessed by DNA ploidy is both a generic hallmark and an important prognostic marker in several cancer types, including prostate cancer.[3,4] Patients with nondiploid tumors have an increased risk of poor prognosis compared to patients with diploid tumors,[4] which is likely caused by the evolutionary advantages of genomic instability.

Prostate carcinoma has a complex tumor microenvironment (TME), which is composed of components such as smooth muscle, carcinoma-associated fibroblasts, collagen fibers, blood vessels and inflammatory cells. Several studies have investigated the importance of the TME in prostate cancer initiation and progression, and carcinoma-associated fibroblasts have been shown to promote epithelial to mesenchymal transition of cancer cells, tumor invasion, angiogenesis and metastasis.[5–7] A

What's new?

The stroma that surrounds tumor cells can have significant effects on tumor growth and behavior. Another hallmark of cancer is aneuploidy caused by genomic instability, which offers an important prognostic marker in several cancer types. In this study, the authors found that combining these two markers—stromal fraction and DNA ploidy—provided a significant predictor of biochemical recurrence in prostate cancer. The low cost, high throughput, and accuracy of digital analysis using this combined method thus promise to provide improved prognostic biomarkers in prostate cancer.

high stroma fraction has been reported as a marker of poor prognosis for colon cancer based on both manual[8] and automatic[9] estimation. The clinical significance of stroma fraction has also been investigated in different prostate cancer cohorts, where the majority of studies have demonstrated that a high stroma fraction predicted worse prognosis.[10–13]

Better and more objective stratification of patients with prostate cancer is needed, and we have used software programs to estimate DNA ploidy and stroma fraction in an objective and reproducible manner, and report the prognostic importance of the combined marker for prostate cancer patients. A discovery cohort was used for the identification of an optimal threshold of low and high stroma fraction. The combined marker was independently validated in a cohort that included 259 men treated with radical prostatectomy.

Materials and Methods

Patients

Independent discovery and validation cohorts were included in our study, which consisted of men with prostate cancer who underwent radical prostatectomy at the Norwegian Radium Hospital, Oslo; a tertiary comprehensive cancer center in Norway. The basis of prostatectomy was preoperative absence of known metastasis, age less than 75 years and life expectancy of at least 10 years. The discovery cohort is described in Supporting Information.

The validation cohort consisted of 287 men operated between 2001 and 2006. A total of 28 patients were excluded from the analyses due to: missing consent (n = 21), missing or less than six weeks follow-up (n = 4) and no tumor material (n = 3). Three tumor blocks were selected for analyses for each patient. Block one and two represented the worst Gleason score and the largest tumor area. The third block was selected randomly from the remaining tumor blocks with a tumor area >0.5 mm^2. Two patients received neoadjuvant therapy and 16 patients received adjuvant hormonal or radiotherapy within the six first months after surgery. Therapy started more than 6 months after surgery was considered as secondary treatment. The Gleason score was evaluated as a part of the clinical routine.

Our study adhered to the reporting recommendations for tumor marker prognostic studies (REMARK) reporting criteria[14] and was approved by the Norwegian Regional Committees for Medical Research Ethics South-East region (REK numbers S-07443a and 2013/476). Informed consent was obtained from the patients included in the study. Exceptions were made for diseased patients in agreement with the REK requirements.

Image cytometric analysis

Preparation of nuclear monolayers was performed according to a modified Hedley's method[15] from 50 μm thick, dissected tumor areas (Supporting Information Fig. S1). The DNA ploidy histograms were classified automatically as diploid, tetraploid or aneuploid.[4] The threshold for tetraploid samples was set to 15% of nuclei in the 4c peak in the histogram. Tetraploid and aneuploid samples were grouped as nondiploid in our study, and a tumor was classified as nondiploid if at least one of the analyzed samples were nondiploid.

Stroma fraction analysis

The measure of stroma fraction (Supporting Information Fig. S2) was performed using hematoxylin and eosin (H&E) stained histological sections scanned at 40× with a NanoZoomer digital slide scanner (Hamamatsu Photonics, Hamamatsu, Japan). The tumor areas on the 3 μm thick H&E sections from the discovery cohort were marked on the glass and scanned, whereas the 5 μm thick H&E sections from the validation cohort were scanned and tumor areas were annotated using a custom software tool. All the tumor areas were marked by a pathologist (M.P.).

The area of stroma and epithelial cells was measured within the annotated tumor area and the stroma fraction was estimated using a novel software tool (Stroma Analyzer, Room4 Group Ltd, Crowborough, UK) as described in the Supporting Information. The H&E scan with the highest estimated stroma fraction was used to represent the tumor. The optimal threshold for dichotomizing patients into low and high stroma groups was estimated in the discovery cohort by evaluating stroma fraction thresholds [1%, 2%, 3%, …100%]; the classifier that maximized the average of sensitivity and specificity was selected as the threshold of high stroma fraction.

Statistical analyses

For survival analyses in the discovery cohort, recurrence defined in accordance with Punt et al.[16] was used as endpoint. Biochemical recurrence (BCR) was calculated from surgery to BCR or to the date of the final prostate-specific antigen (PSA) registration and used as an endpoint in the validation cohort. PSA measurements within 6 weeks after surgery were not considered when identifying BCR. We used three measures of BCR to thoroughly describe the relationship between the proposed marker and outcome; a single PSA ≥ 0.4 ng/ml, PSA rise and PSA progression. PSA rise was defined as PSA ≥ 0.4 ng/ml followed by a subsequent rise by any amount at any future time point, and the date of event was set to the first

Table 1. Patients characteristics in the validation cohort—single PSA ≥ 0.4 ng/ml

Characteristic	All *n* (%)	No event *n* (%)	Event *n* (%)	*p*-value[1]
Age, median (IQR)	62 (59–66)	62 (58–66)	63 (60–67)	0.111
Preoperative PSA (ng/ml), median (IQR)	8 (6–11)	8 (6–10)	10 (8–14)	<0.001
Missing	1 (0)	1 (1)	0 (0)	
Gleason score				<0.001
≤6	78 (30)	75 (40)	3 (4)	
3 + 4	98 (38)	69 (37)	29 (41)	
4 + 3	54 (21)	37 (20)	17 (24)	
≥8	29 (11)	8 (4)	21 (30)	
Surgical margins				<0.001
Absent	165 (64)	137 (72)	28 (40)	
Present	92 (36)	51 (27)	41 (59)	
Missing	2 (1)	1 (1)	1 (1)	
Extracapsular extension				<0.001
Absent	166 (64)	141 (75)	25 (36)	
Present	89 (35)	44 (23)	45 (64)	
Missing	4 (2)	4 (2)	0 (0)	
Seminal vesicle infiltration				<0.001
Absent	228 (88)	180 (95)	48 (69)	
Present	30 (12)	9 (5)	21 (30)	
Missing	1 (0)	0 (0)	1 (1)	
Lymph node involvement				<0.001
Absent	252 (97)	188 (99)	64 (91)	
Present	7 (3)	1 (1)	6 (9)	
DNA ploidy				0.014
Diploid	188 (73)	145 (77)	43 (61)	
Nondiploid	71 (27)	44 (23)	27 (39)	
Stroma fraction				0.013
Low	198 (76)	152 (80)	46 (66)	
High	61 (24)	37 (20)	24 (34)	
Ploidy and stroma combined				0.003
Diploid and low stroma	148 (57)	118 (62)	30 (43)	
Nondiploid or high stroma	90 (35)	61 (33)	29 (41)	
Nondiploid and high stroma	21 (8)	10 (5)	11 (16)	

Due to rounding the numbers may not sum to 100%.
[1]Pearson's $\chi 2$ test and Mann–Whitney's U-test were used to evaluate associations.
Abbreviations: IQR, interquartile range.

measure of PSA ≥ 0.4 ng/ml. PSA progression was defined as either PSA rise or receipt of secondary therapy or development of distant metastasis, whichever was recorded first. Survival distributions were compared using the Mantel–Cox log-rank test in univariable analysis of categorical variables and the Wald's chi-squared test in univariable analysis of continuous variables and in multivariable analyses. Patients with missing values for any included variable were excluded from the multivariable analyses. The markers of DNA ploidy and stroma fraction were combined as in Danielsen et al.[9] to constitute three risk groups: (*i*) diploid and low stroma, (*ii*) either nondiploid or high stroma and (*iii*) nondiploid and high stroma. Pearson's χ^2 and Mann–Whitney's U tests were used to evaluate associations. Two-sided p values <0.05 were considered statistically significant. Statistical analyses were performed using R version 3.1.3 (http://www.r-project.org).

Data availability

The data that support the findings of our study are available on request from the corresponding author. The data are not publicly available due to ethical restrictions.

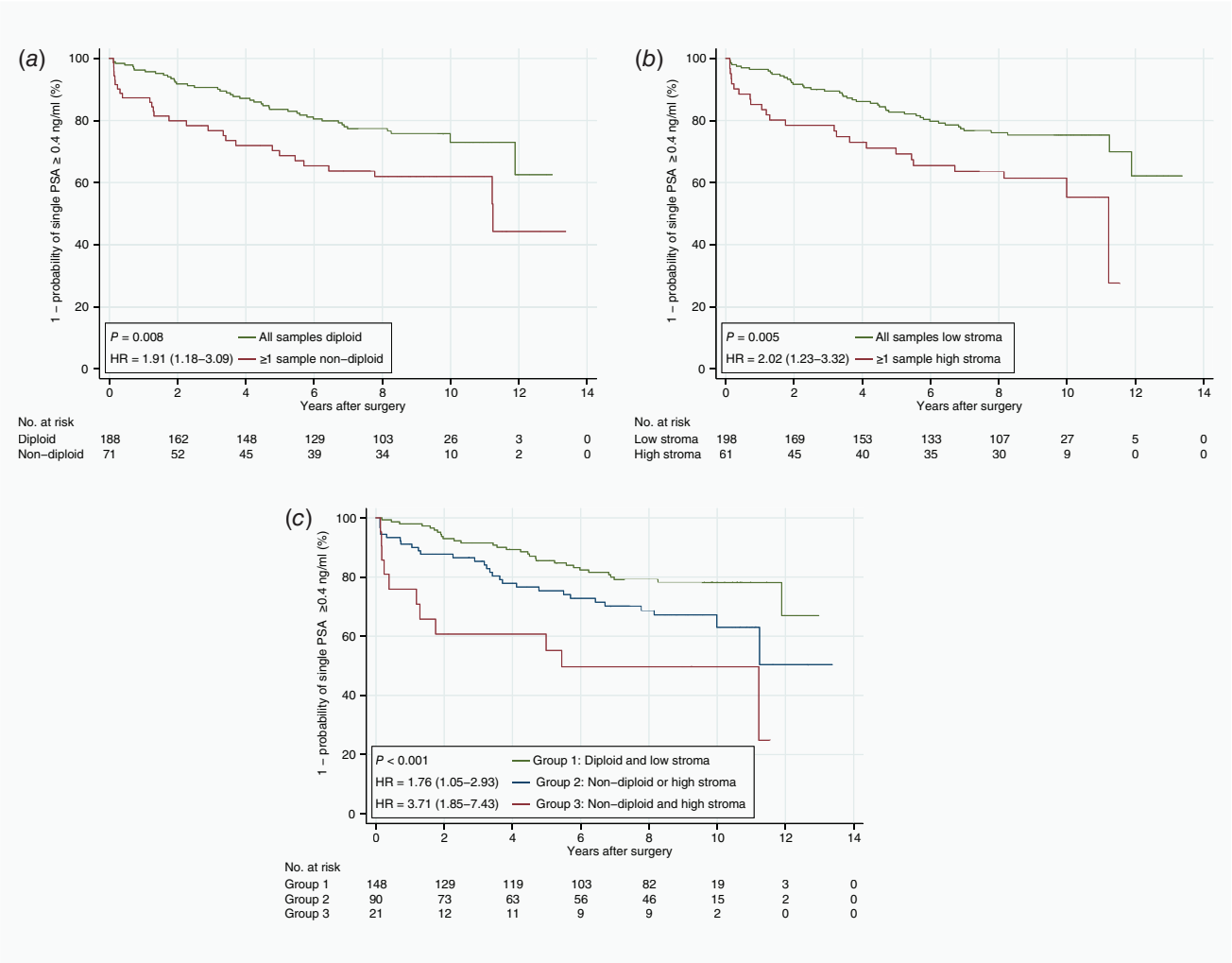

Figure 1. Kaplan–Meier analysis of recurrence in the validation cohort with single PSA ≥ 0.4 ng/ml as endpoint. (*a*) DNA ploidy, (*b*) stroma fraction and (*c*) combined DNA ploidy and stroma fraction, and 95% confidence intervals of the hazard ratios (HR) are listed in parenthesis.

Results

Discovery cohort

The clinicopathological characteristics of the patients included in the discovery cohort (n = 253) are summarized in Supporting Information Table S1. The cut-off identified as optimal in the discovery cohort was to categorize stroma fraction ≤56% as "low stroma" and >56% as "high stroma" (Supporting Information Fig. S3). Significant results were seen in the univariable analyses of DNA ploidy (hazard ratio [HR] = 2.33, 95% confidence interval [CI] 1.51–3.60), stroma fraction (HR = 1.90, 95% CI 1.23–2.94) and their combination (HR = 2.44, 95% CI 1.30–4.58 in intermediate risk and HR = 4.03, 95% CI 2.12–7.63 in high risk when compared to low risk, Supporting Information Fig. S4).

Patient characteristics

The patients in the validation cohort (n = 259) had a median age of 62 (interquartile range [IQR] 59–66) years and the follow-up time for the censored observations was 9 (IQR 8–10) years. The clinicopathological characteristics are summarized in Table 1 and Supporting Information Table S2. BCR was present in 70 patients for the single PSA event, in 60 patients for PSA rise and in 69 patients for PSA progression. A total of 60 patients had BCR for all three endpoints, 65 patients had BCR for both single PSA and PSA progression, five patients had BCR only for single PSA and four patients had only PSA progression. Median time to BCR was 3 (IQR 1–5) years for all three endpoints.

DNA ploidy and stroma fraction

Of the 777 tumor blocks scheduled for analysis, DNA ploidy and stroma fraction were measured in 775 samples, while two were excluded because they did not contain tumor. Less than 200 tumor cell nuclei were present in two samples rendering DNA ploidy status indeterminate, and valid results for both methods were present in 773 samples. Diploid DNA ploidy

Table 2. Multivariable analysis of single PSA ≥ 0.4 ng/ml in the validation cohort

	HR	95% CI	*p*-value
Age	1.00	0.95–1.04	0.851
Ploidy and stroma combined			0.034
Diploid and low stroma	Ref		
Nondiploid or high stroma	0.97	0.55–1.72	0.919
Nondiploid and high stroma	2.51	1.18–5.34	0.017
Gleason score			0.003
≤6	Ref		
3 + 4	3.83	1.11–13.18	0.033
4 + 3	3.50	0.94–13.11	0.063
≥8	9.21	2.44–34.75	0.001
Lymph node involvement	2.22	0.85–5.77	0.104
Surgical margins	1.84	1.07–3.17	0.028
Preoperative PSA (ng/ml)			0.001
≤6	Ref		
›6 and ≤10	1.01	0.40–2.54	0.991
›10 and ≤20	2.67	1.05–6.80	0.040
›20	4.07	1.10–15.10	0.036
Extracapsular extension	2.34	1.32–4.12	0.003
Seminal vesicle infiltration	1.92	1.04–3.53	0.036

n = 251, eight patients were excluded due to missing values for at least one of the included variables.
Abbreviations: CI, confidence interval; HR, hazard ratio.

classifications were seen in 655 samples, whereas 119 samples were nondiploid. The median stroma fraction was 45% (IQR 40%–51%); low stroma fraction was seen in 691 samples and high stroma fraction in 88 samples.

Nondiploid tumors were observed in 71 (27%) of 259 patients, whereas 61 (24%) of the patients had a tumor with a high stroma fraction. When the two markers were combined, diploid tumors with low stroma fraction were observed for 148 (57%) of the patients. Tumors with either nondiploid or high stroma fraction were observed for 90 (35%) patients, whereas 21 (8%) patients had nondiploid tumors with high stroma fraction.

Survival analyses

DNA ploidy (HR = 1.91, 95% CI 1.18–3.09, Fig. 1*a*), stroma fraction (HR = 2.02, 95% CI 1.23–3.32, Fig. 1*b*), and their combination (HR = 1.76, 95% CI 1.05–2.93 in intermediate risk and HR = 3.71, 95% CI 1.85–7.43 in high risk when compared to low risk, Fig. 1*c*), predicted single PSA in univariable analysis (Supporting Information Table S3). In the multivariable analysis, the combination of DNA ploidy and stroma fraction (HR = 0.97, 95% CI 0.55–1.72 in intermediate risk and HR = 2.51, 95% CI 1.18–5.34 in high risk when compared to low risk), Gleason score, extracapsular extension, seminal vesicle infiltration, surgical margins and preoperative PSA were significant predictors of BCR (Table 2). Similar results were obtained in univariable (Supporting Information Fig. S5 and Table S3) and multivariable analyses (Supporting Information Table S4) using PSA rise and PSA progression as endpoints.

In patients graded with Gleason score 7 (n = 152), neither DNA ploidy, (HR = 0.96, 95% CI 0.51–1.80), stroma fraction (HR = 1.80, 95% CI 0.97–3.34), their combination (HR = 1.09, 95% CI 0.58–2.05 in intermediate risk and HR = 1.87, 95% CI 0.76–4.60 in high risk), nor Gleason score 3 + 4 versus 4 + 3 (HR = 1.22, 95% CI 0.67–2.22) were significant predictors of BCR in univariable analyses. Significant results were obtained for extracapsular extension, seminal vesicle infiltration, surgical margins and lymph node involvement (Supporting Information Table S5).

Stroma fraction was a significant marker of BCR in univariable analysis when PSA rise and PSA progression was used as endpoints in the subgroup of patients with Gleason score 7, however, DNA ploidy and the combined marker was not significant (Supporting Information Table S5).

Discussion

We have for the first time demonstrated independent prognostic value of the combination of DNA ploidy and stroma fraction in prostate cancer. The marker stratified patients into low, intermediate and high-risk groups with 10-year PSA-free survival of 78, 63 and 50%. This novel marker may improve risk stratification of patients treated with radical prostatectomy, by offering an objective and time-efficient measure of two tumor characteristics. The marker may therefore add robustness to the set of established markers without adding additional demands on the pathologists. The importance of this combined marker has previously been described in a study of patients with colorectal cancer,[9] and the significant results in our study suggest that the combined marker has a potential as a generic marker of cancer recurrence.

PSA is a commonly used surrogate marker of clinical recurrence after radical prostatectomy, however, only a subset of the patients that develop BCR will experience disease progression and even fewer will die from the disease.[17] A standard definition of BCR does not exist and to allow for comparison with other studies, we included two commonly used BCR endpoints. A single PSA ≥0.4 ng/ml has been described as a reasonable endpoint that excludes patients with detectable PSA who are unlikely to progress,[18] whereas a PSA ≥0.4 ng/ml followed by subsequent rise has been shown to correlate well with the development of distant metastases.[19,20] Furthermore, we included PSA progression as a third endpoint, where the definition was inspired by the work of Mir et al.[20] who used PSA progression as an endpoint in their evaluation of 14 different BCR definitions. The inclusion of secondary treatments seems reasonable in order to correctly account for actual recurrence in cases where PSA registration was limited. Univariable analyses of the marker that combines DNA ploidy and stroma fraction was significant for all

endpoints. Furthermore, multivariable analysis demonstrated that the combined marker was an independent predictor of recurrence when BCR was defined as either single PSA or PSA progression. The discrepancy in results obtained by the use of different BCRs may be explained by the inherent uncertainty of estimates related to the relatively low number of patients.[21] Furthermore, as BCR is a surrogate marker of recurrence, longer follow-up or a larger cohort is necessary to decide which definition of BCR is the most accurate predictor of clinical recurrence of disease.

Several studies have investigated the importance of the tissue microenvironment (TME) and carcinoma-associated fibroblasts in prostate cancer initiation and progression.[7] Ayala et al.[10] reported that tumors with either low (<5%) or high (>50%) stroma fraction, evaluated in tissue microarray punches stained with Masson's trichrome, behaved aggressively compared to patients with a moderate (6–50%) stroma fraction. The identification of TME in H&E stained tissue sections is more difficult compared to trichrome stained tissue samples. However, TME is recognized by higher cellularity and pale eosin stain, compared to smooth muscles that are eosinophilic.[11] The stroma fraction in H&E stained tissue sections has only been reported in three independent studies that included diagnostic biopsies of prostate cancer,[11–13] where it was shown that a high stroma fraction predicted recurrence or death of prostate cancer.

We used a software-based method to obtain reproducible measures of the stroma fraction in a tumor area annotated on H&E stained tissue sections of radical prostatectomy specimens, thus avoiding inter- and intra-observer variability. We accounted for intra-tumor heterogeneity by analyzing three separate blocks from each prostate[22] and validated the method in an independent cohort. The challenges of an accurate visual assessment of stroma fraction can easily be avoided by the use of the objective measure of stroma fraction obtained by this automatic method. Furthermore, as our method quickly estimates the stroma fraction on scans of routine H&E sections, it is a convenient method that can readily be implemented in the daily clinical practice.

DNA ploidy, a marker of large-scale genomic aberration, is a prognostic marker for many cancers.[4] In line with our results, the prognostic importance of DNA ploidy in prostate cancer has earlier been demonstrated in univariable analyses, however, independent prognostic value has not always been seen in multivariable analyses,[3,4] which may be explained by the high prognostic power of Gleason grading and other clinicopathological factors.

Gleason grade is one of the best predictors of recurrence in the hands of expert pathologists. However, in a clinical setting, this subjective method depends on the pathologist's expertise and the important distinction in terms of prognosis between patients with Gleason score 3 + 4 and 4 + 3 can be hampered by the reproducibility of Gleason grading. In agreement with previous reports,[10,11] a high stroma fraction predicted a higher risk of recurrence in univariable analyses of patients with Gleason score 7 when PSA rise and PSA progression was used as endpoints, whereas the corresponding result for single PSA was borderline significant (Supporting Information Table S5).

A novel marker composed of DNA ploidy and stroma fraction might supplement the existing risk stratification tools by providing objective and more robust risk assessments for prostate cancer patients treated with radical prostatectomy. The marker combines a measure of large-scale genomic instability with a broad measure of the TME. The threshold of high stroma was determined in a cohort treated with radical prostatectomy with clinical recurrence as endpoint and validated in an independent radical prostatectomy cohort where the combined marker consistently predicted recurrence across different definitions of BCR.

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Conflict of interest

Prof. D.J.K. is a Director of Oxford Cancer Biomarkers. The other authors declare no competing interests.

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