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Large scale genomic instability as an additive prognostic marker in early prostate cancer

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Abstract. *Background*: The clinical outcome for the individual prostate cancer patient is often difficult to predict, due to lack of reliable independent prognostic biomarkers. We tested DNA ploidy as a prognostic factor for clinical outcome in 186 patients treated with radical prostatectomy.

Methods: DNA ploidy was measured using an automatic image cytometry system and correlated with preoperative PSA, age at surgery, Mostofi grade, surgical margins and Gleason score.

Results: The mean follow up time after operation was 73.3 months (range 2–176 months). Of the 186 prostatectomies, 96 were identified as diploid, 61 as tetraploid and 29 as aneuploid. Twenty-three per cent, 36% and 62% of the diploid, tetraploid and aneuploid cases respectively, suffered from relapse during the observation time. DNA ploidy, Gleason score, Mostofi grading, surgical margins and preoperative PSA were all significant predictors of relapse in a univariate analysis. On multivariate analysis, only Gleason score and DNA ploidy proved to be independently predictors of disease recurrence. Furthermore, among the 68 cases identified with Gleason score 7, DNA ploidy was the only significant predictor of disease recurrence.

Conclusions: Our data suggest that DNA ploidy should be included as an important additive prognostic factor for prostate cancer, especially for patients identified with Gleason score 7 tumours.

Keywords: DNA ploidy, Gleason, prognosis, prostate cancer, radical prostatectomy

1. Introduction

In the period 2000–2004, prostate cancer was the most frequent type of cancer among Norwegian men, representing 26% of all male cancer cases [7].

Prostate cancer is rare before the age of 50 and about 90% of men with prostate cancer are diagnosed at the age of 60 or older. Often, the clinical progression of an individual prostate cancer is hard to predict, despite the existence of well established prognostic factors like Gleason score and preoperative serum prostate specific antigen (PSA).

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Chromosome instability is observed in a large number of cancer types. To which extent these changes are a cause or a consequence of cancer is still debated, but there is little doubt that further tumour development follows chromosome instability. Large chromosomal changes (as assessed by for example ploidy analyses) will influence chromatin structure as well as separate genes. Quantitative genomic alterations measured by DNA ploidy analysis are commonly observed in cancer [18]. Aneuploidy is a typical sign of malignancy, and is often related to a poorer prognosis for the patient [1,5,22].

Several studies based on flow cytometry [3,4,10, 23–25,27,36,39] and image cytometry [2,6,13,16,28, 32–34,37,38] have shown that the finding of a non-diploid histogram is a marker of poor prognosis in prostate cancer. Other studies have been carried out

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with varying results [8,21,31]. There are still conflicts to whether DNA ploidy analysis should be implemented in clinical routine, and there is clearly a need for robust survival studies testing DNA ploidy as an independent prognostic factor in prostate cancer.

Radical prostatectomy is an established treatment for early prostate cancer. A particular study [20] showed that radical prostatectomy, compared to watchful waiting, significantly reduced the risk of distant metastasis in early prostate cancer. Our goal is to identify effective prognostic/predictive markers, and the aim of the present study was to test the potential prognostic value of DNA ploidy on disease-free survival in prostate cancer patients. We have performed a retrospective analysis on a relatively large group of patients who underwent radical prostatectomy and our results show that DNA ploidy is an important predictor of clinical outcome for patients diagnosed with early prostate cancer.

2. Materials and methods

2.1. Patients

The study was approved by the Norwegian Regional Committees for Medical Research Ethics (REK).

A total of 193 men with prostate cancer underwent retropubic radical prostatectomy at the Norwegian Radium Hospital between 1988 and 1996. All patients were followed up either at our department or at local hospitals. PSA measurements were performed before and after operation as well as at every subsequent clinical examination. The threshold for positive versus negative PSA was set to 4, according to the standard in 1988.

Follow-up time ranged from 2 to 176 months (mean 73.3). The median age at time of operation was 64 years, ranging from 47 to 74 years. Patients were considered to have clinically evident recurrence of disease if any of the following were present: (1) evidence of local recurrence (confirmed by histological biopsies or ultrasound), or (2) evidence of distant metastasis (detected by skeletal scintigraphy and/or MR). If a patient who suffered from relapse had postoperative serum PSA $>$ 4 ng/ml before the date of either local recurrence or metastasis, the date of elevated PSA was set as the relapse date.

2.2. Material

Formalin fixed, paraffin embedded tissue and sufficient information was available for study in 186 cases. Of the seven excluded cases, six had material inadequate for analysis and one underwent a medical castration six months before operation. Data and tumour characteristics for all 186 patients are given in Table 1.

For all patients, the complete prostate gland was sectioned and embedded in several paraffin blocks per gland. Microscopic slides from each paraffin block were re-examined by a pathologist. All detectable lesions were identified and localized. This formed the basis for further assessment of the spreading of the tumours and any possible heterogeneity in relation to the marker being examined. At the time of surgery, Gleason scoring was not part of the clinical routine and therefore this was done retrospectively for all histological slides. If a gland contained several lesions, the lesion with the highest Gleason score was used. To test for inter-observer variability, the lesions with the highest Gleason scores was rescored ($\kappa = 0.5$).

2.3. Image cytometry

Tumour areas were microdissected and one or more 50 μm thick sections were prepared. The sections were enzymatically digested (Sigma protease, type XXIV, Sigma Chemical, St. Louis) for the preparation of monolayers, using a modification of Hedley's method [19]. The monolayers were stained with the Feulgen Basic Fuschin method. DNA content in tumour nuclei was measured with a DNA ploidy System composed of an Axioplan II brightfield microscope (Zeiss, Germany) equipped with a scanning stage and controller (Prior scientific, UK), a Piezo focusing unit (PI, Germany) and a 10 bit CCD camera (Hamamatsu Photonics, Japan), a PCDIG frame card (Correco Imaging, USA) and a 546 nm green filter. 1500 nuclei were automatically captured and measured in each case. To exclude possible artefacts and non-representative nuclei like doublets, necrotic or cut cells, all images of nuclei were certified by trained personnel.

DNA histograms (frequency distribution of nuclei, scaled from internal reference cells) were classified in a blinded manner. A sample was considered to be diploid if only one G0/G1 peak was present and the number of cells in the G2 peak did not exceed 15%. If the G2 peak was larger than 15%, or a peak was present in the 8C position, the sample was classified as tetraploid. The sample was considered aneuploid when a peak appeared in an area outside 2C, 4C and 8C, or when the number of euploid nuclei exceeding 5C was over 1%.

The 1% cut-off was estimated on the basis of 50 random non-tumour specimens from the same patient

Table 1
Data and tumour characteristics for all 186 patients

Variables	n	%
History of progression		
Relapse within 60 months	50	26.9
Relapse later than 60 months	12	6.5
No relapse	124	66.7
No relapse with <60 months follow-up	18	
Age at surgery (years)		
<60	42	22.6
60–69	116	62.4
⩾70	28	15.1
Preoperative PSA (ng/ml)		
0–4	30	16.1
5–10	30	16.1
11–20	65	34.9
>20	60	32.3
Missing	1	0.5
Clinical stage		
T0	3	1.6
T1	4	2.2
T2	173	93.0
T3	4	2.2
Missing	2	1.1
Histological type		
Adenocarcinoma of peripheral ducts and acini	180	96.8
Large duct adenocarcinoma	1	0.5
Primary transitional cell carcinoma	1	0.5
Mucinous adenocarcinoma	2	1.1
Missing	2	1.1
Gleason score		
2–6	37	19.9
7	69	37.1
8–10	79	42.5
Missing	1	0.5
Surgical margins		
Negative margins	93	50.0
Positive margins	93	50.0
Mostofi sum		
1	51	27.4
2	130	69.9
3	4	2.2
Missing	1	0.5
DNA ploidy		
Diploid	96	51.6
Tetraploid	61	32.8
Aneuploid	29	15.6

group. Approximately 30% of the diploid histograms had nuclei exceeding 5C, but none of them over 1%.

Lymphocytes, plasma cells and fibroblasts from each specimen were included as reference cells.

2.4. Statistical analysis

SPSS 15.0 statistical software was used for all calculations. All 186 patients were included in survival analyses. End points measured as interval to progression were estimated by the Kaplan–Meier method and the log-rank test was used to assess the prognostic value of the different variables. To determine which variables were independently correlated with progression of disease, the various parameters (significant at log-rank test) were evaluated using a Cox proportional hazards regression model. For all calculations, statistical significance was considered at $p \leqslant 0.05$.

3. Results

3.1. All patients

The cumulative disease specific survival for all 186 patients was 98.2% after 5 years and 92.1% after 10 years. The cumulative disease-free survival was 71.7% and 62.7% after 5 and 10 years. 62 patients suffered from relapse. Of these, 28 (45.2%) were identified with a local recurrence, 34 (29.0%) with a distant recurrence and 16 (25.8%) with both.

Of the 96 diploid cases, 22 (22.9%) suffered from a relapse during the observation time while 74 (77.1%) did not. Eighteen (62.1%) of the 29 patients identified with an aneuploid tumour suffered from a relapse while 11 (37.9%) did not. Of the 61 tetraploid cases, 22 (36.1%) suffered from a relapse during the observation time while 39 (63.9%) did not.

Estimated disease-free survivals according to current variables are shown in Table 2. Disease-free survival according to variables in DNA ploidy, Gleason score, surgical margins, Mostofi and preoperative PSA were all found to be statistically significant. Kaplan–Meier plots of disease-free survival, related to the respective variables, are shown in Fig. 1. In the multivariate analysis, only Gleason score ($p = 0.007$) and DNA ploidy ($p = 0.024$) were found to be of independent prognostic significance (Table 2). Patients with an aneuploid tumour had a 2.6 times higher risk of getting recurrence of disease than cases with a diploid tumour. Patients identified with a Gleason 8–10 tumour had a 7.3 times higher risk of getting recurrence of disease than patients identified with a Gleason 2–6 tumour.

Table 2
Disease free survival related to prognostic factors

Variables	Total patients (n)	Univariate analysis for disease free survival			Cox multivariate analysis for disease free survival		
		5 year disease free survival (%)	10 year disease free survival (%)	p-value (log-rank test)	p-value	Hazard ratio	95% CI
Age at surgery (years)							
<60	42	65.4	53.3				
60–69	116	70.0	62.3	0.131			
⩾70	28	88.4	77.8				
Preoperative PSA (ng/ml)					0.290		
0–4	30	81.8	70.3			1.00	
5–10	30	86.4	77.0		0.126	0.40	(0.12–1.30)
11–20	65	75.5	64.9	0.013	0.903	0.95	(0.38–2.36)
>20	60	54.5	48.5		0.880	1.07	(0.43–2.71)
Gleason score					0.007		
2–6	37	97.3	90.1			1.00	
7	69	78.6	70.1	<0.001	0.063	3.48	(0.93–13.01)
8–10	79	53.4	43.1		0.005	7.34	(1.82–29.67)
Surgical margins					0.207		
Negative	93	83.1	73.7	0.001		1.00	
Positive	93	60.0	51.3			1.47	(0.81–2.68)
Mostofi sum					0.732		
1	51	86.2	81.2			1.00	
2	130	65.4	55.0	0.011	0.527	0.75	(0.31–1.83)
3	4	75.0	75.0		0.496	0.47	(0.05–4.15)
DNA ploidy					0.024		
Diploid	96	80.5	72.9			1.00	
Tetraploid	61	67.8	61.6	<0.001	0.206	1.47	(0.81–2.68)
Aneuploid	29	49.5	31.8		0.006	2.64	(1.32–5.29)

3.2. Gleason score 7 patients

There were 69 cases with Gleason score 7. Of these, 37 (53.6%) patients were identified with Gleason $3+4$ and 31 (44.9%) were identified with Gleason $4+3$. In addition one case was identified with Gleason $2+5$. Since this case was non-comparable with the former two groups, it was excluded from the analyses including only Gleason score 7 patients.

The cumulative disease-free survival for all the 68 patients classified with Gleason score 7 was 78.3% and 69.6% after 5 and 10 years, respectively. Of the 68 patients, 19 had recurrence of disease. Kaplan–Meier analysis and log-rank tests were performed on all 68 patients identified with Gleason score 7 according to age at surgery, preoperative PSA, Mostofi, surgical margins, Gleason $3+4$ versus $4+3$ and DNA ploidy (Table 3 and Fig. 2). Only DNA ploidy ($p < 0.001$) was found to be a significant prognostic factor of disease-free survival in Gleason score 7 patients after radical prostatectomy. The multivariate analysis for recurrence of disease according to DNA ploidy for Gleason score 7 patients is shown in Table 3. Aneuploid cases had an 11.6 times higher risk of developing recurrence of disease than cases with a diploid tumour.

4. Discussion

The College of American Pathologists Consensus Statement of 1999 ranks prognostic and predictive factors in prostate cancer in three categories, based on the strength of published evidence and the expert opinions of the Prostate Working Group members [5]. Factors included in category I comprise serum PSA level, TNM stage grouping, Gleason Score and finally surgical margin status. According to the College of American Pathologists, these are factors proven to be of

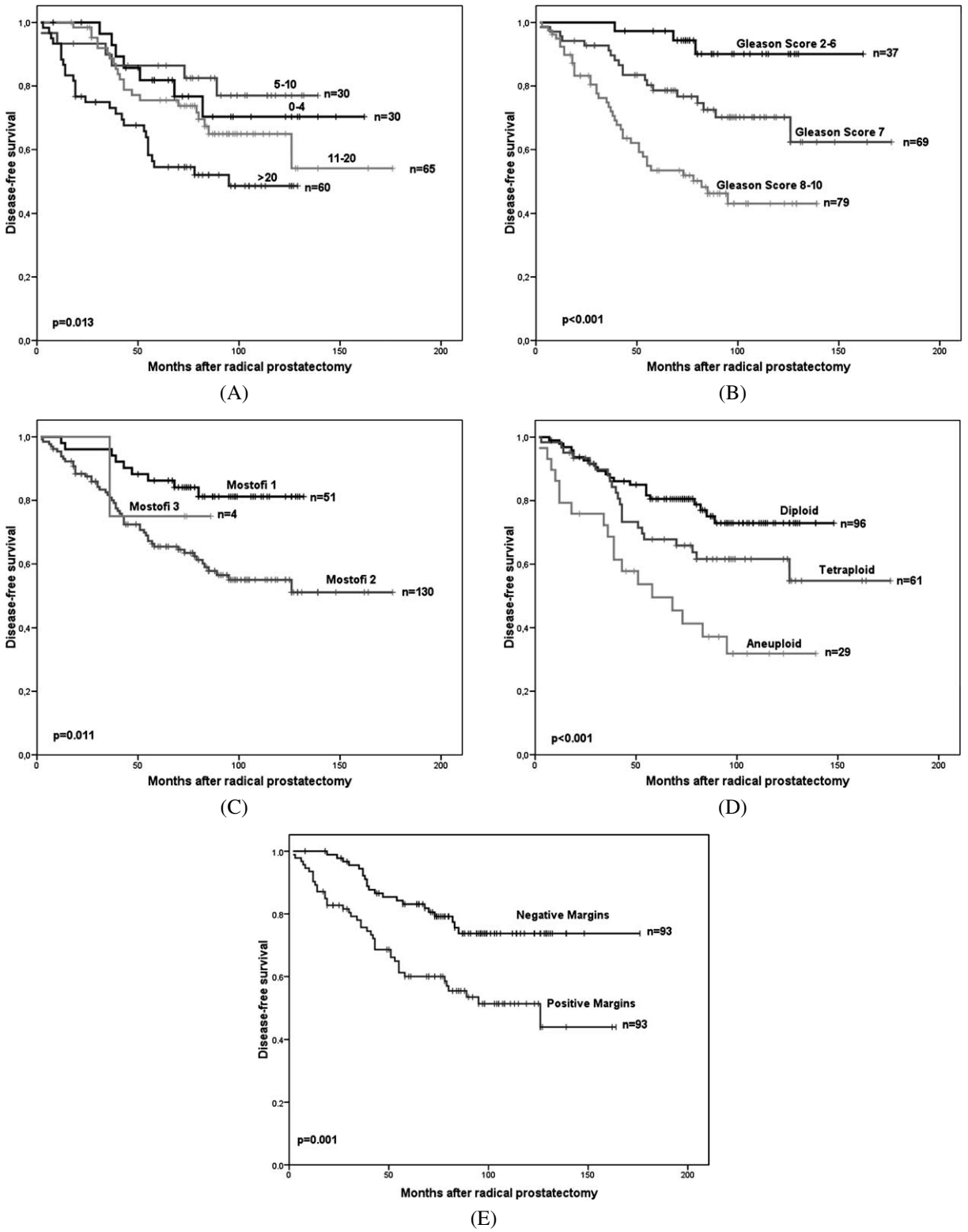
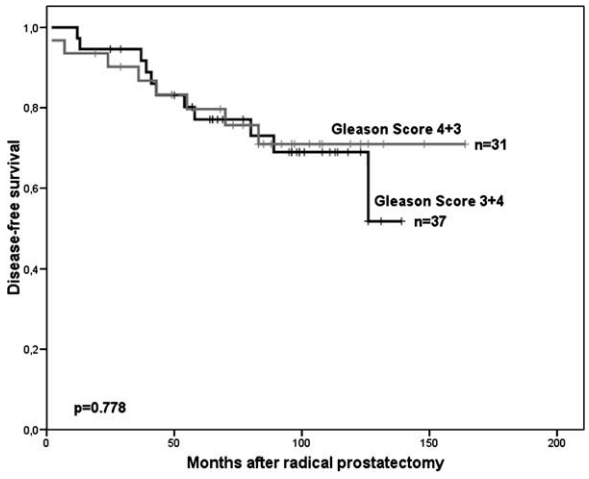


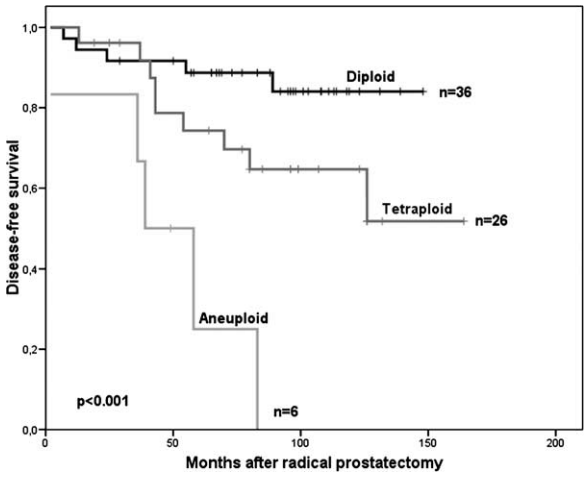
Fig. 1. Disease-free survival shown by Kaplan–Meier graphical analysis for all patients according to (A) preoperative PSA (ng/ml), (B) Gleason score, (C) Mostofi grade, (D) DNA ploidy and (E) surgical margins.

Table 3
Disease free survival related to prognostic factors for Gleason score 7 patients

Variables	Total patients (n)	Univariate analysis for disease free survival			Cox multivariate analysis for disease free survival		
		5 year disease free survival (%)	10 year disease free survival (%)	p-value (log-rank test)	p-value	Hazard ratio	95% CI
Age at surgery (years)							
<60	18	77.0	68.5				
60–69	39	72.1	64.2	0.294			
⩾70	11	90.0	90.0				
Preoperative PSA (ng/ml)							
0–4	9	100.0	100.0				
5–10	10	80.0	68.6	0.164			
11–20	28	85.2	72.3				
>20	21	56.2	56.2				
Mostofi sum							
1	20	74.7	67.9				
2	47	79.5	70.0	0.858			
3	1	100.0	100.0				
Surgical margins							
Negative	32	87.2	77.7	0.117			
Positive	36	70.0	62.1				
DNA ploidy					0.001		
Diploid	36	88.7	84.8			1.00	
Tetraploid	26	74.3	64.7	<0.001	0.085	2.63	(0.86−7.89)
Aneuploid	6	25.0	0.0		<0.001	11.59	(3.26−41.21)
Gleason score							
3 + 4	37	77.1	69.0				
4 + 3	31	70.9	70.9	0.778			



(A)



(B)

Fig. 2. Disease-free survival shown by Kaplan–Meier graphical analysis for Gleason score 7 patients according to (A) Gleason score and (B) DNA ploidy.

prognostic importance and useful in clinical patient management. DNA ploidy as a prognostic factor is included in category II together with tumour volume and histological type. According to the College of the American Pathologists, these are factors that have been extensively studied biologically and clinically, but whose importance remains to be validated in statistically robust studies. At the WHO's International Consultation meeting of 2004 [11], there were still reservations as to whether ploidy should be adopted as a part of routine clinical practice.

In our study, patients were considered to have clinically evident recurrence of disease if either local recurrence or metastasis were present. If a patient who suffered from relapse had elevated postoperative PSA before the date of either local recurrence or metastasis, the date of elevated PSA was set as the relapse date. We believe that the recurrence of disease starts at this point of time if followed by clinically evident relapse in form of histological biopsies, ultrasound, scintigraphy and/or MR. Of all the patients, 23 were identified with elevated PSA values (>4 ng/ml) without any further evidence of local recurrence or metastasis during the observation time. These were not considered to have a relapse in our study. There may be reasons why we should have included these patients in the relapse group. Maybe these patients did develop local recurrence or metastases that were not found or they left the observation for other reasons. To take this into account we also did all statistical analyses where we included these 23 patients in the relapse group. There were no significant changes from the results we present in this study for neither all 186 patients, nor the 68 patients with Gleason score 7.

Our results indicate that DNA ploidy can provide important information in predicting recurrence of prostate cancer and should always be assessed as an important additive predictor of clinical outcome. While 77.1% of all the diploid cases had no form of relapse, 62.1% of all the aneuploid cases suffered from a relapse within the observation time. DNA ploidy was an independent multivariate predictor of disease-free survival together with Gleason score. These findings support those previous studies where DNA ploidy has been shown to be an independent predictor of disease recurrence after radical prostatectomy [3,23,25, 32–34].

Gleason scores 2–4 are seldom seen in tumours other than T1. In a large study of 2404 men, only 2% were found to have Gleason 2–4 as the grade of the main tumour in radical prostatectomy [17]. Tumours with Gleason scores 5–6 are more common and generally these patients have good prognosis. Tumours of Gleason scores 8–10 often are associated with an aggressive clinical behaviour and a poor prognosis. These scores account for approximately 7% of the grades seen at radical prostatectomy [17]. Gleason score 7 tumours have a significantly worse prognosis than Gleason 6 tumours [12]. Still, Gleason score 7 tumours are heterogeneous and there tends to be a difference in clinical outcome between Gleason score $3 + 4$ and Gleason score $4 + 3$, where Gleason score $4 + 3$ has a significantly higher risk of recurrence of disease [9]. In our study, we identified 68 patients with Gleason score 7 tumours (excluding the one case with Gleason score $2 + 5$). For the Gleason score 7 patients in our study, DNA ploidy was the only significant independent prognostic factor.

With regard to all patients there was an intermediate group between DNA diploid and DNA aneuploid groups; the DNA tetraploid tumours. Tetraploidy was a predictor of better clinical outcome than aneuploidy, but still worse than diploidy. This was also the finding for the Gleason score 7 group. A tetraploid state is known to sometimes be a station on the route to DNA aneuploidy, potentially with genomic aberrations too small to be detected by ploidy analyses [14,35]. Copy number increase of chromosome arm 8q and the specific increase of the relative level of c-MYC at 8q24 have recently been shown to be associated with poor prognosis in biopsies from patients suspected of having prostate cancer, and may represent a quantitative change not detected by ploidy analyses [29,30]. Not to be able to distinguish the "good" tetraploid from the "bad" is a weak point in general for DNA ploidy analysis. This also applies for our study.

At its best, Gleason grading is the gold-standard prognostic factor for radical prostatectomy specimens. Unfortunately, Gleason grading is a subjective method and depends on the pathologist's education and experience [15,26]. In our study we found a kappa value at 0.5 when rescoring the Gleason values, considered to be a fair agreement. The results of DNA ploidy imaging are to a much smaller degree referable to subjective opinions and observer variability.

The use of radical prostatectomy as the primary treatment for early prostate cancer can be questioned. Patients with indolent prostate cancers and elderly patients often benefit from more conservative treatment or no treatment at all. There is clearly a need for good markers that can help predicting clinical outcome before commencing a treatment. In the present study we

tested factors potentially identifiable at a preoperative time (apart from surgical margins). It has been shown to be a good correlation of DNA ploidy status between needle biopsies and radical prostatectomy specimens using image analysis [32]. Future studies should try to assess the prognostic value of DNA ploidy in clinical outcome for prostate cancer patients using needle biopsies.

We have shown that DNA ploidy is an independent prognostic marker for disease-free survival in early prostate cancer. For cases identified with Gleason score 7 tumours, DNA ploidy was the only independent marker for clinical outcome. In our opinion, DNA ploidy is an important, objective method not yet fully exploited in prostate cancer, and should be used as an additive marker to Gleason score in clinical routine. The method of DNA ploidy image analysis can be established without problems in any pathology laboratory, or the service can be requisitioned in public and commercial laboratories.

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Conflict of interests

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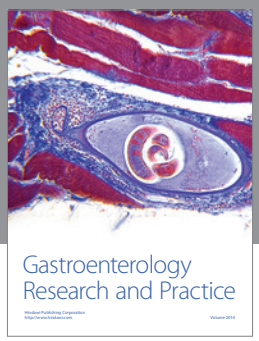

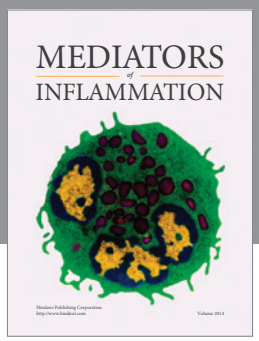

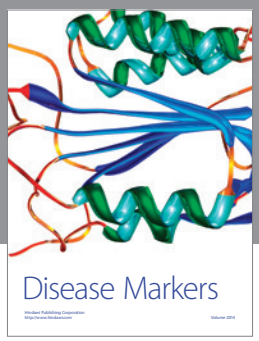

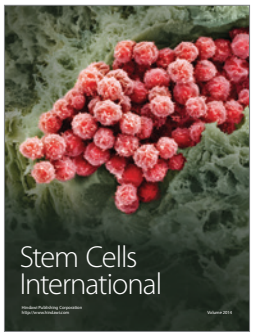

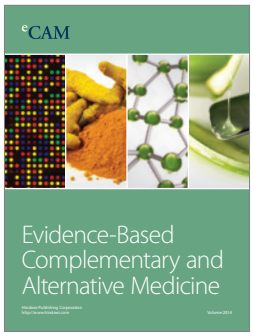

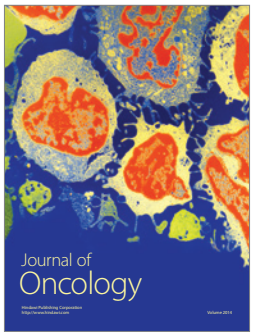

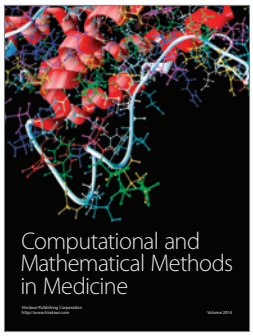

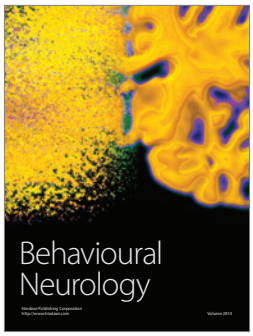

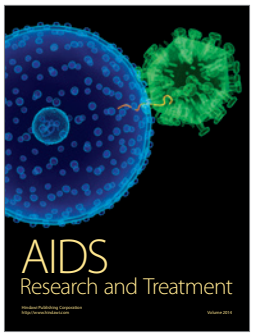

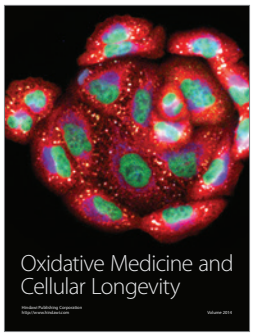

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