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The prognostic value of p53 and DNA ploidy following radical prostatectomy

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Abstract This study assesses the correlation of p53 immunoreactivity and DNA ploidy status with biochemical recurrence after radical prostatectomy. p53 protein expression and DNA ploidy were evaluated on 84 archival paraffin-embedded radical prostatectomy specimens. Patients were divided into two groups: those with low (38/84, 45%) and those with high (46/84, 55%) p53 immunoreactivity. The results were correlated with Gleason score, stage and serum PSA. Kaplan-Meier biochemical recurrence free survival and the Cox hazard-regression model were used for analysis. Multivariate analysis revealed p53, DNA ploidy, Gleason score, PSA and stage to be independent prognostic factors in this order. Kaplan-Meier analysis showed a significant difference in biochemical recurrence when p53 high expression and DNA aneuploidy were combined. The results of this study suggest that stratification for p53 expression and DNA ploidy status can provide additional prognostic information for patients with prostate carcinoma after radical prostatectomy.

Keywords p53 · DNA ploidy · Recurrence · Radical prostatectomy

The clinical course of prostate cancer (PCa) following radical prostatectomy depends on pathological staging and Gleason score [15]. Understaging is a significant problem in patients considered to have organ confined disease, and biochemical or clinical recurrence after radical prostatectomy occurs in 10–40% of these patients [15].

Although there is a clear tendency to develop molecular prognostic biomarkers for patients with clinically localized yet aggressive disease, the value of p53 nuclear accumulation and DNA ploidy as prognostic factors in clinically localized prostate cancer is controversial [3].

The first aim of the present study was to confirm the associations between p53 immunoreactivity and DNA ploidy with established prognostic markers such as Gleason score, tumour stage, serum PSA, and age. The second aim was to correlate p53 expression and DNA ploidy with progression and biochemical recurrence-free survival following radical prostatectomy.

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Materials and methods

A total of 84 patients who underwent radical prostatectomy for clinically localised prostate cancer in our department are included in this study. The mean age was 64 years (range 53–73 years) (Table 1). Pretreatment evaluation was based on rectal examination, serum PSA, TRUS guided prostatic biopsy and histological examination of the specimen, bone scan, and CT scan of the abdomen and pelvis. All patients had clinically localised disease on presentation. None of the patients received neoadjuvant or adjuvant therapy of any kind up to the time of biochemical recurrence. The mean post-treatment follow-up period was 45 months, and all patients were followed up at regular 6-month clinical intervals. Relapse was defined as biochemical disease progression with a PSA concentration greater than 0.2 ng/ml on two successive measurements.

Histopathological grading was based on the Gleason system and pathological staging on the TNM system. One pathologist, who was unaware of clinical findings, performed the pathological evaluation.

IHC analysis for p53 protein

Archival paraffin-embedded specimens of 4-μm-thick sections were stained with hematoxylin and eosin and examined for the presence of tumour cells. Only sections with high numbers of cells were used for analysis. The same paraffin blocks were cut into 4-μm-thick

Table 1 Patients and tumour characteristics

Variables	No. patients (n) (% total)		p53 high expression *n* (%)		DNA aneuploid *n* (%)	
Age (years)						
50–59	16	(19.0)	10/16	(62.5)	10/16	(62.5)
60–69	58	(69.0)	31/58	(53.5)	36/58	(62.1)
70–73	10	(12.0)	5/10	(50.0)	4/10	(40.0)
Stage (pathological)						
T2a	20	(23.8)	5/20	(25.0)	1/20	(5.0)
T2b	17	(20.2)	4/17	(23.5)	7/17	(41.2)
T3a	47	(55.9)	37/47	(78.7)	42/47	(89.4)
Gleason score						
4	14	(16.7)	3/14	(21.4)	3/14	(21.4)
5–7	29	(34.5)	10/29	(34.5)	13/29	(44.8)
>7	41	(48.8)	33/41	(80.5)	34/41	(82.9)
PSA values						
2–4	10	(11.9)	5/10	(25.0)	2/10	(20.0)
4.1–10	36	(42.9)	4/36	(23.5)	22/36	(61.1)
>10	38	(45.2)	37/38	(92.1)	26/38	(68.4)
p53 expression						
low	38	(45.2)			14/38	(36.8)
high	46	(54.8)			36/46	(78.3)
DNA ploidy						
Diploid	34	(40.5)	10/34	(29.4)		
Aneuploid	50	(59.5)	36/50	(72.0)		
Recurrence						
Without recurrence	52	(61.9)	19/52	(36.5)	22/52	(42.3)
With recurrence	32	(38.1)	27/32	(84.4)	28/32	(87.5)
Time to recurrence (months)						
24	24/32	(75.0)	24/24	(100)	24/50	(85.7)
>25	8/32	(25.0)	3/8	(38.0)	4/50	(14.3)

sections and mounted on slides. After deparaffinization, endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide in absolute methanol for 10 min at room temperature. After washing twice in phosphate buffered saline solution (PBS), the slides were covered by protein blocking agent for 10 min at room temperature, and then by the diluted (1:50) anti-p53 antibody (p53 tumor suppression protein Ab-8, Clone DO-7 + BP53-12, Neo Markers, Fremont, USA) for 30 min at room temperature. After four additional washes with PBS, sections were developed with streptavidin peroxidase for 10 min at room temperature, and then with diaminobenzidine tetrahydrochloride (DAB) (LabVision, Fremont, USA), used as chromogen, for 5–15 min in a dark room, counterstained with Mayer hematoxylin, and covered using a permanent mounting media.

Positive controls for p53 nuclear accumulation used with each run of staining included a paraffin-embedded pellet of the PCa cell line DU145 [6], which has a documented *p53* mutation; a colon cancer specimen with a *p53* missense mutation; and a tongue cancer specimen with p53 nuclear accumulation. Negative controls included a paraffin-embedded pellet of the PCa cell line PC3 [6] that does not express p53 protein.

In all cases, tumours had some degree of nuclear p53 accumulation. p53 expression was prospectively characterized as low or high when the percentage of positively stained nuclei in areas of the specimen with the higher immunoreactivity was $<30\%$ or $>30\%$, respectively.

Flow cytometric analysis

Nuclear suspensions for flow cytometric analysis (FCM) measurements were prepared from 45-μm-thick sections cut from the same paraffin blocks used for IHC analysis. After deparaffinization through graded alcohols, specimens were additionally enzymatically disaggregated, stained with propidium iodide according to the Becton Dickinson protocol, and analysed on a FACScan flow cytometer (Becton Dickinson, San Jose, California). Lymphocytes from healthy donors were used as controls for the normal diploid peak. An average of 40,000 cells was analysed from each sample at rates varying from 30 to 100 cells per second. The DNA-ploidy status was expressed using the DNA index, which is the relation between the channel of the G0/G1 peak of the neoplastic cells and the channel of the G0/G1 of the lymphocytes. A population was characterised as diploid and having a DNA index = 1 when the neoplastic cells and the lymphocytes provided the same single G0/G1 peak. Populations were characterised as aneuploid when the DNA index was different from 1 and the peak contained at least 15% of the total number of neoplastic cells.

Statistical analysis

Statistical analyses were carried out using the SPSS 8.0 program. Data were evaluated for the time to biochemical recurrence, the percentage of recurrent patients, probability, and the 95% confidence interval using the Kaplan-Meier analysis. The Cox hazard-regression model including relative risk, probability, and the 95% confidence interval was used for univariate and multivariate analysis of the prognostic factors. All P values were two-tailed and $P \leq 0.05$ was considered significant.

Results

The patient's data and tumour characteristics are summarised in Table 1. Follow-up ranged from 8 months (earliest biochemical recurrence) to 72 months (longest time without evidence of biochemical recurrence) (mean 45 months). Of the 84 patients with analysed specimens 32 (38.1%) had a biochemical recurrence during the

follow-up period. Mean time to recurrence was 22.2 months.

p53 percentage score was significantly correlated with DNA ploidy ($P = 0.0001$), Gleason score ($P = 0.0001$), stage ($P = 0.0001$), and PSA values ($P = 0.00599$). The percentage of patients with high p53 expression was found to increase with DNA aneuploidy (diploid, 29.4%; aneuploid, 72.0%), with higher Gleason scores (4, 21.4%; 5–7, 34.5% and >7, 80.5%), and with more advanced pathological stages (T2a, 25.00%; T2b, 23.53% and T3a, 78.72%).

The recurrence analysis is given in Table 2. There was a significant relationship between the percentage of patients who experienced biochemical recurrence as well as the time to recurrence, and p53 immunoreactivity ($P = 0.0001$), DNA ploidy ($P = 0.0001$), Gleason score ($P = 0.0001$) and pathological Stage ($P = 0.0001$) (Fig. 1). There was no significant difference in the PSA and age groups using univariate analysis.

When a multivariate analysis of the six factors was performed, p53 was an independent predictor of biochemical recurrence ($P = 0.0003$) with 30% relative risk after Cox proportional-hazard step-wise elimination. In addition, DNA ploidy ($P = 0.0074$), Gleason score ($P = 0.0083$), PSA values ($P = 0.0187$), and stage ($P = 0.0399$) were independent prognostic factors in this order (Table 3).

p53 status was statistically discriminatory for recurrence in younger patients (<70 years), in locally advanced PCa in definitive histopathology and in patients with poorly differentiated tumours (Gleason score >7) (Table 4).

In this cohort, high p53 expression was observed in 54.7% (46/84) of cancer cases, of which 58.6% (27/46) had a biochemical relapse (positive predictive value), while 86.8% (33/38) of the patients with a low expression did not show disease progression (negative predictive value). Aneuploidy was observed in 59.5% (50/84) of cases with cancer. The positive predictive value of DNA aneuploidy for recurrence was 56% (28/50) and

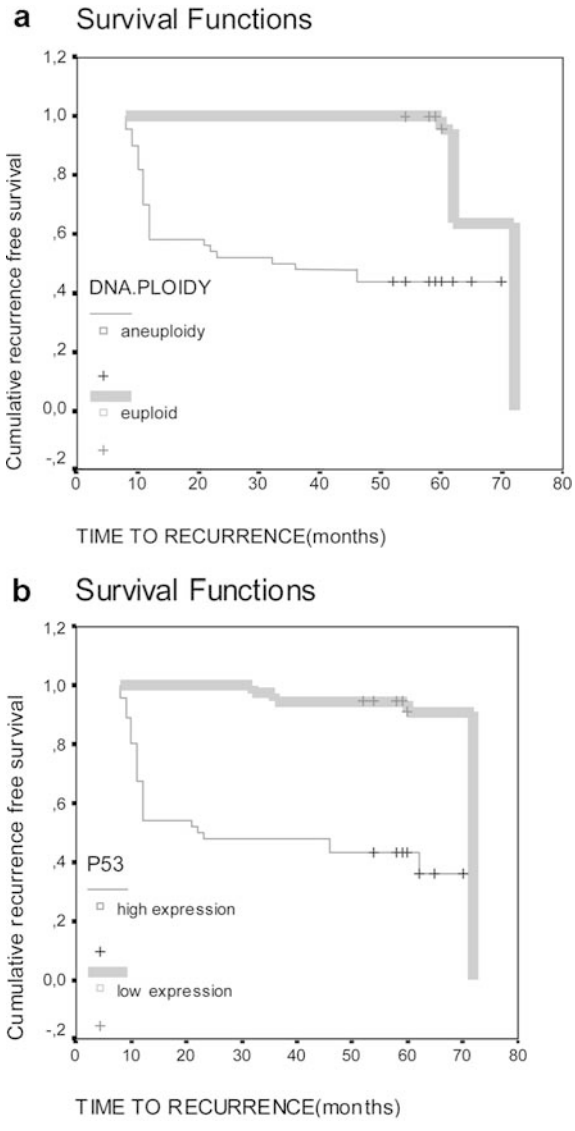

Fig.1 Kaplan-Meier plots of the estimated probability of recurrence according to **a** DNA ploidy and **b** expression of p53 ($P = 0.0001$)

Table 2 Kaplan Meier recurrence analysis

Variable	Group	*P*	Time to recurrence (months)	95% CI	%	Log rank
p53	Low expression	0.0001	69.55	66.41–72.68	86.84	23.44
	High expression		38.09	29.99–46.20	41.30	
Age (years)	50–59	0.2605	48.75	33.02–64.48	50.00	2.69
	60–69		49.74	42.58–56.50	60.34	
	70–79		56.10	48.85–63.35	90.00	
PSA	2–4	0.0599	60+		100.00	5.63
	4.1–10		53.80	44.92–62.68	58.33	
	>10		45.62	36.82–54.43	55.26	
Gleason score	4	0.0001	69.14	61.51–76.77	85.71	25.44
	5–7		68.33	64.49–72.18	82.76	
	>7		35.20	26.58–43.81	39.02	
Stage	T2a	0.0001	66.83	59.72–73.95	80.00	19.06
	T2b		58.59	54.79–62.38	88.24	
	T3a		39.34	31.19–47.49	44.68	
Ploidy	Diploid	0.0001	68.29	62.34–74.24	58.24	21.67
	Aneuploid		39.74	39.04–47.50	44.00	

Table 3 Multivariate analysis of prognostic factors analysed by Cox's hazard-regression model

Prognostic factor	Exp (B)	*P*	95% CI
p53	9.3052	0.0003	2.2750–38.0432
DNA ploidy	5.2075	0.0074	1.1779–23.0213
Gleason score	2.8206	0.0083	1.2107–6.5708
PSA values	0.4424	0.0187	0.1965–0.9961
Stage	0.1526	0.0399	0.1119–0.8874
Age	0.6717	0.2272	0.3521–1.2814

the negative predictive value was 88.2% (30/34). Combining both prognostic markers increased the positive predictive value to 72.2% (26/36) without compromising the negative predictive value (87.5%, 42/48).

Kaplan-Meier recurrence-free survival plots of the patient groups divided in terms of p53 and DNA status in combination are shown in Fig. 2.

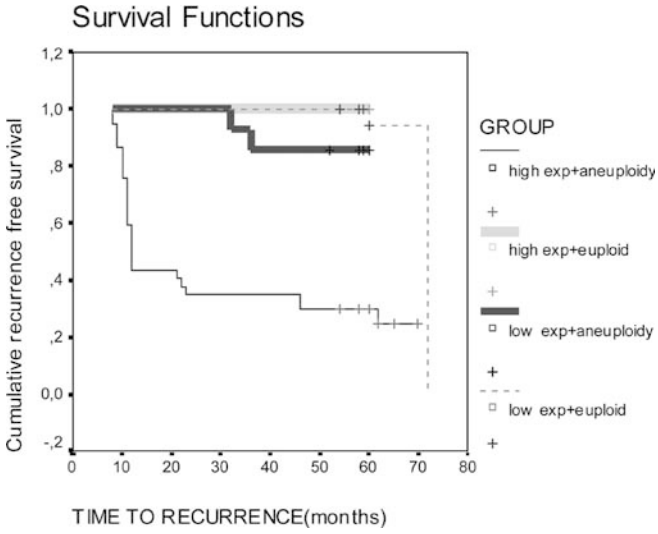

Fig. 2 Recurrence-free survival rate according to p53 expression (low/high) and DNA ploidy status (diploidy/aneuploidy)

Discussion

The accurate prediction of patients likely to have biochemical recurrence following radical prostatectomy would permit a consideration of alternative modes of therapy and/or adjuvant treatment. Currently, prognosis after radical prostatectomy is based on preoperative serum PSA and pathological findings such as Gleason score, stage, and positive margins [3]. However, the wide variability in the biological behaviour of PCa limits the prognostic value of these parameters and necessitates the identification and characterisation of other prognostic markers.

Our study illustrates a significant correlation between p53 high immunoreactivity and DNA aneuploidy in clinically localized PCa and biochemical relapse following radical prostatectomy. On multivariate analysis, both p53 expression and DNA ploidy were independent and more important prognostic factors for biochemical recurrence than PSA, stage and grade.

Our data support previously described relationships between p53 nuclear accumulation and pathological tumour stage, Gleason score and pretreatment serum PSA concentration [8]. However, the level of p53 nuclear accumulation as a prognostic factor in localized PCa remains controversial. A number of studies have shown that p53 nuclear accumulation detected by IHC is prognostic of biochemical recurrence at a variety of dichotomizing cut-off points based on the number of p53 positive nuclei [1, 9, 10, 17]. In addition to its relationship to the risk of tumour recurrence, the IHC marker p53 has been demonstrated to be a predictor of disease-specific survival following radical prostatectomy [9, 19].

However, others studies have reported no significant correlation between p53 expression in primary tumours and the time to progression and death [5, 20]. p53 was a significant postoperative marker in an initial study of Brewster et al. [4], while the same authors did not find any significance when the series was expanded [16]. Additionally, conflicting data emerge from studies evaluating the prognostic significance of p53 expression in preoperative biopsies and prostatectomy specimens [1, 16,18].

Table 4 Univariate Kaplan-Meier recurrence analysis depending on p53 status

Variable	Group	*P*	p53 low expression	p53 high expression	Overall
Age group	50–59	0.0245	72.00 (66.67%)	32.00 (40.00%)	50.00%
	60–69	0.0000	58.07 (88.89%)	36.09 (35.48%)	60.34%
	70–79	0.3173	(100.00%)	52.20 (80.00%)	90.00%
PSA	2–4	0.5029	(100.00%)	(100.00%)	100.00%
	4.1–10	0.0004	69.95 (84.00%)	17.09 (0.00)	58.33%
	>10	0.4833	50.67 (66.67%)	44.70 (54.29%)	55.26%
Stage	T2a	0.5151	69.33 (80.00%)	62.00 (80.00%)	80.00%
	T2b	0.4234	58.15 (84.62%)	(100.00%)	88.24%
	T3a	0.0005	(100.00%)	31.05 (29.73%)	44.68%
Gleason score	4	0.6015	68.36 (81.82%)	(100.00%)	85.72%
	5–7	0.4895	69.10 (84.21%)	65.20 (80.00%)	82.76%
	>7	0.0013	(100.00%)	27.24 (24.24%)	39.02%
DNA ploidy	Diploid	0.5288	71.29 (87.50%)	62.00 (90.00)	88.04%
	Aneuploid	0.0004	56.29 (85.71%)	29.97 (27.78%)	44.00%

Several studies suggest that ploidy analysis adds useful predictive information in cases of PCa [13, 14]. DNA ploidy was predictive of PSA progression after surgery in several studies [7, 12, 21]. Leibovich et al. [11], in their prospective study, suggested that the addition of p53 status to preoperative serum PSA, pathological stage, Gleason score and DNA ploidy improved the prediction of survival free of biochemical, local or systemic failure in patients with locally advanced PCa.

A number of methodological factors influence the results in studies similar to ours. Borre et al. [2] summarized the main factors that may influence immunohistochemical staining. These included the antibody used, whether paraffin slides were stored, and the tissue fixation methods. Since the monoclonal antibody that we used, DO7, recognizes an epitope shared by wild-type and mutant p53 protein, it is unclear whether all of these p53 protein positive specimens were associated with p53 mutation. In addition, the subjective nature of immunohistochemical scoring, the absence of a uniform definition of abnormality and dissimilarities in the cohort studies make a direct comparison between studies difficult [2].

We realize that our study requires cautious interpretation since it is retrospective and the number of patients is relatively small. One further potential limitation is that we did not correlated p53 status with clinical relapse. However, although the rate of development of clinical metastases varies greatly, there is a correlation between biochemical relapse and the subsequent development of clinical metastases [15].

p53 status was not predictive of outcome in older patients (>70 years), in localized PCa in definitive histopathology or in patients with better differentiated tumours (Gleason score <7). This may indicate that an effect of high p53 expression has not been apparent within our follow-up period or that these cancers are intrinsically indolent and that p53 status is not of importance in them. This may also reflect that in our cohort p53 accumulation was evaluated in patients with less aggressive features compared to other studies [15, 17].

Despite conflicting results and limitations, several immunohistochemical studies, such as ours, imply that p53 protein accumulation is an independent prognostic marker of the disease-free interval in prostate cancer. In addition, our study shows that DNA ploidy increases the positive predictive value of p53 immunoreactivity for biochemical recurrence following radical prostatectomy.

Our study indicates that patients with high p53-expression and DNA aneuploidy in radical prostatectomy specimens are at higher risk of recurrence. This suggests that these patients need a more aggressive follow-up. Adjuvant therapy following radical prostatectomy and how it impairs survival in these patients needs further consideration.

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