p53 mutations have no additional prognostic value over stage in bladder cancer

J.A.M. Vet[1], P.P. Bringuier[1], P.J. Poddighe[2], H.F.M. Karthaus[3], F.M.J. Debruyne[1] & J.A. Schalken[1]

Departments of [1]Urology and [2]Pathology, University Hospital, Nijmegen, The Netherlands; [3]Department of Urology, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands.

Summary Evidence is accumulating that the tumour-suppressor gene p53 is involved in the development of bladder cancer. Therefore we studied p53 mutations in 47 bladder cancers obtained from 45 patients using polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP) analysis. Eight out of 24 invasive tumours appeared to have a p53 mutation, while no p53 mutations were found in the superficial tumours. All the p53 mutations were found in grade 3 tumours. The tumours with altered p53 showed a higher frequency of allelic loss (FAL) than the tumours without a mutation (55.8% vs 21.1%, $P<0.05$, χ^2 test). This increase in FAL suggests a correlation between p53 mutations and genetic instability. A significant correlation between mutated p53 and poor survival in the whole group studied was found ($P<0.001$, log-rank test). However, within the group of muscle-invasive tumours the occurrence of p53 mutations had no additional prognostic value. Therefore, even though p53 mutations were found in aggressive tumours, the clinical usefulness of its detection seems limited. Nevertheless, these results imply that p53 is involved in the clinical behaviour of bladder cancer; its role in the progression of superficial cancer to invasive disease merits further attention.

Bladder cancer is the fifth most common cancer in the western male population, with an annual incidence of 20 cases per 100,000. The incidence in women is lower: about five cases per 100,000 are diagnosed annually (Raghavan et al., 1990). Transitional cell carcinoma of the bladder is divided into two groups: (a) superficial (pTis, pTa, pT1) and (b) muscle invasive (pT2, pT3, pT4) disease. Most superficial bladder cancers are associated with a good prognosis, however 10–25% clinically progress to a more aggressive state, showing an increase in grade and/or infiltration into the muscle layer. pT1 tumours invade the lamina propria and have a higher incidence of progression than pTa tumours, which are confined to the urothelium. Patients with muscle-invasive tumours usually present de novo and have a worse prognosis. Non-random chromosomal changes have been observed in bladder cancer, for example in cytogenetic studies monosomy of chromosome 9 has been reported (Gibas et al., 1984; Vanni & Scarpa, 1986; Smeets et al., 1987; Hopman et al., 1988). Using restriction fragment length polymorphism analysis (RFLP), allelic loss of chromosomes 9, 11 and 17 in bladder cancer has been demonstrated (Tsai et al., 1990). Abnormalities of chromosome 11p appeared to be more frequent in invasive than in superficial tumours, and monosomy of chromosome 9 was not correlated with grade or stage. Loss of heterozygosity (LOH) of chromosome 17p occurred only in high-grade (G3) tumours (Olumi et al., 1990). The p53 gene is considered the candidate tumour-suppressor gene on chromosome 17p, since in cancer development one of the two alleles is frequently lost and the remaining is mutated (Baker et al., 1989; Hollstein et al., 1991). Recently, it has been suggested that p53 acts as a cell cycle control protein at the level of G_1 to S phase transition (Kastan et al., 1991; Livingstone et al., 1992; Yin et al., 1992). The loss of this function may result in increased genetic instability (Yin et al., 1992). In bladder cancer it has been shown, by means of subcloning and sequencing of exons 5–9, that 17p allelic loss is strongly associated with p53 mutation (9 out of 10 cases), and out of 18 invasive tumours 61% had a p53 mutation (Sidransky et al., 1991).

This association of p53 mutations and invasive tumours was confirmed in another study by using the technique of PCR–SSCP (Fujimoto et al., 1992).

We studied p53 mutations in a group of 45 patients with bladder cancer, using PCR–SSCP analysis. Besides a correlation with grade and stage, we investigated the prognostic significance of p53 mutations. Since p53 mutations are thought to be associated with genetic instability (Kastan et al., 1991; Livingstone et al., 1992; Yin et al., 1992) we also studied the correlation of p53 mutations and genetic instability evaluated by the frequency of allelic loss.

Materials and methods

Tumour specimens

Twenty-three snap-frozen superficial carcinomas (pTa–pT1) and 24 muscle-invasive carcinomas (pT ≥ 2) obtained from 45 patients were analysed. Superficial tumours comprised two recurrences of previously analysed tumours. Among the invasive tumours, two were squamous cell carcinomas. All the other tumours were transitional cell carcinomas. Pathological and clinical data for the patients are summarised in Table I. Genomic DNA was extracted from step-sectioned tumours (>70% tumour cells) (Miller et al., 1988).

PCR–SSCP analysis

PCR–SSCP analysis was performed to investigate p53 mutations in exons 5–8 (Orita et al., 1989). The intron primers for amplification of exons 5–8 were:

exon 5 S: 5'-tca ctt gtg ccc tga ctt-3'
       AS: 5'-gag gaa tca gag gcc tgg-3'

exon 6 S: 5'-gag acg aca ggg ctg gtt-3'
       AS: 5'-gag acc cag ttg caa acc-3'

exon 7 S: 5'-cca agg cgc act ggc ctc-3'
       AS: 5'-gag gca agc aga ggc tgg-3'

exon 8 S: 5'-cct tac tgc ctc ttg cttc-3'
       AS: 5'-tga atc tga ggc ata act-3'

A 250 ng aliquot of genomic DNA was subjected to 35 cycles of PCR (95°C, 57°C, 72°C for 0.5, 2 and 1.3 min respec-

Correspondence: J. Schalken, Urological Research Laboratory, University Hospital Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands.

Table I p53 mutations, clinical and pathological data for each patient

Case no.	Age (years)	Stage/grade	p53 mutations Exon/codon	Amino acid change	Survival (months)	Treatment[a]
1	77	a/1-2			31	TURT
2	75	a/1	(p)		>41	TURT
3	72	a/1			>38	TURT
4	68	a/1			>41	TURT,C
5	66	a/3			23	TURT,Ct,R,Ch
6	66	a/2			>57	TURT,Ct
7	49	a/1-2			>45	TURT,BCG,Ct
7 rec.		a/1-2				
8	62	a/1			>64	TURT
9	53	a/1			>61	TURT,BCG
10	42	a/1			>65	TURT,BCG,Ch
11	88	a/2			52	TURT,Ch
12	44	a-1/2			>39	TURT
13	65	a-1/1			>22	TURT
14	68	1/3			>55	TURT,BCG
15	86	1/2			>58	TURT,Ch
16	76	1/2			9	TURT,Ch
17	53	1/2			>37	TURT,BCG
18	64	1/1			>55	TURT
19	85	1/2	(p)		>45	TURT,Ch
19 rec.		a/3	(p)			
20	71	1/2	(p)		>36	TURT,Ch
21	68	1/2			>36	TURT,Ch,BCG
22	81	2/2			9	TURT
23	73	2/2			8	TURT,Ch,Ct
24	49	2/3			>36	TURT,R,iridium
25	79	2/3	5/158	CGC→CTC (Arg→Leu)	4	Ct
26	81	2/2			6	TURT
27	53	2/3			7	TURT,Ch
28	70	2/3			3	TURT,Ch
29	47	2/3	8/282	del. G→frameshift	8	TURT,Ch
30	62	≥2/3			>27	TURT,Ch,R
31	65	≥2/3	5/179	CAT→TAT (His→Tyr)	>66	TURT,Ct
32	83	≥2/3			21	TURT,R
33	79	≥2/3			10	TURT,Ct
34	59	≥2/3	7/259	GAC→GTC (Asp→Val)	3	TURT,R
35	73	2-3/3	8/285	GAG→AAG (Glu→Lys)	2	TURT,R
36	73	2-3/3			9	TURT
37	76	2-3/2,SCC			32	TURT,Ct,R
38	68	2-3/3	6/215	AGT→GGT (Ser→Gly)	2	TURT
39	63	3/3			5	TURT,R
40	74	3/3	8/285	GAG→AAG (Glu→Lys)	10	Ct,Ch
41	50	3/2,SCC			5	TURT
42	70	3b/2			>28	Ct
43	75	3b/3			>40	TURT,R,Ct
44	44	4/3	5/166	TCA→TGA (Ser→Stop)	29	TURT,Ct
45	82	4/2			5	TURT

[a]Ch, chemotherapy; Ct, cystectomy; R, radiotherapy; TURT, transureteral resection of the tumour; SCC, squamous cell carcinoma; rec, recurrent; (p), polymorphism codon 213.

tively). Exons 5, 6 and 8 were amplified in 50 µl containing: 50 mM potassium chloride 10 mM Tris–HCl (pH 8.8), 1.75 mM magnesium chloride, 250 µM deoxynucleotide triphosphates, 10 pmol of each 5'-end labelled primer and 1.5 units of Taq polymerase (Perkin Elmer/Cetus). Exon 7 was amplified in the same buffer containing 1.5 mM magnesium chloride.

Five microlitres of the PCR product was diluted in 15 µl of loading buffer (96% formamide, 20 mM EDTA, 0.05% bromophenol blue and xylene cyanol), boiled for 3 min and then quenched (10 min) on ice before loading (2 µl per lane). Each sample was applied to a 5% polyacrylamide/Tris-borate EDTA (0.5 ×) gel with and without 10% (v/v) glycerol. Subsequently, electrophoresis was performed at room temperature for 16 h at 6 W or 3 W respectively.

Sequence analysis

Direct sequencing of the double-stranded PCR products that showed a shift on the SSCP gels was performed as described previously (Kusukawa et al., 1990). Amplified PCR products were purified using the magic PCR-preps DNA purification system (Promega). The PCR primers were used for sequenc-

ing in the ds-DNA cycle sequencing system (Life Technologies). Electrophoresis was performed on 6% polyacrylamide gels containing 7 M urea.

Restriction fragment length polymorphism

The DNA probes used were as follows: chromosome 9q, EFD 126 (Nakamura et al., 1987); chromosome 11p, H-ras (Pulciano et al., 1982); chromosome 16q, pV962 (Mansouri et al., 1988) and 79.2.23 (Bufton et al., 1986); chromosome 17p, 144D6 (Schwartz et al., 1988); chromosome 18q, 15.65 (Fearon & Vogelstein, 1990). RFLP analysis was performed on the 24 patients from whom normal DNA was available (Poddighe et al., submitted). The RFLP results were used to determine the frequency of allelic loss (FAL), also called fractional allelic loss (Vogelstein et al., 1989).

Statistical analysis

The Kaplan–Meier method was used to estimate survival probability as a function of time. Differences in survival were analysed by a log-rank test. The χ^2 test was used for the other correlations.

Results

p53 mutations in bladder cancer

Table I summarises the results of the PCR–SSCP and sequence analysis of the p53 gene in bladder tumours of 45 patients. Eight mutations were found in invasive tumours: three in exon 5, one in exon 6, one in exon 7 and three in exon 8. Seven out of the eight mutations found were point mutations, while one appeared to be a deletion of a G, leading to a frameshift. We found four transitions – two G to A, one A to G and one C to T – and three transversions – one C to G, one G to T and one A to T. In three patients with a superficial tumour a similar shift in the SSCP pattern of exon 6 was observed. One of these also showed loss of heterozygosity of chromosome 17p. After sequencing it appeared that the shift in the SSCP pattern was due to a silent alteration of CGA to CGG at codon 213. This polymorphism has been described previously (Mazars *et al.*, 1992). No mutations were found in the squamous cell carcinomas.

Table IIa   Relationship between p53 mutation and grade

Grade	p53 mutation		Mutations (%)
	Negative	Positive	
1	11	0	0
2	15	0	0
3	11	8	42

Table IIb   Relationship between p53 mutation and stage

Stage	p53 mutation		Mutations (%)
	Negative	Positive	
Superficial	21	0	0
Invasive	16	8	33

Table IIIa   Allelic loss of chromosomes 9q, 11p, 16q, 17p and 18q and p53 mutations in bladder tumours

Case no.	Stage grade	p53 mut	9q	11p	16q	17p	18q
1	a 1	–	●	○	●	○	○
2	a 1	–	○	○	○	○	NI
3	a 1	– (p)	○	○	○	○	○
4	a 1	–	○	○	○	○	NI
5	a 1	–	○	NI	○	NI	○
6	a 1–2	–	○	○	○	○	○
7	a 1–2	–	●	●	○	○	○
7 rec.	a 1–2	–	●	●	○	○	○
8	1 2	– (p)	●	○	○	●	ND
8 rec.	a 3	– (p)	●	○	○	●	●
9	a 2	–	○	NI	○	●	●
10	a 2–3	–	NI	○	○	○	NI
11	1 2	– (p)	●	○	○	○	NI
12	1 2	–	●	●	○	○	NI
13	1 2	–	○	○	○	○	NI
14	1 2	–	○	○	○	○	○
15	1 2	–	NI	●	○	○	●
16	1–2 1–2	–	○	NI	○	○	○
17	2 3	–	○	○	●	○	NI
18	2 3	+	NI	NI	○	●	○
19	>2 3	–	NI	NI	○	○	NI
20	>2 3	+	●	○	○	●	○
21	3 3	–	●	○	○	NI	ND
22	3 3	–	NI	○	○	○	ND
23	3 3	+	NI	NI	●	●	ND
24	4 3	+	●	NI	○	●	○

–, no p53 mutation; +, p53 mutation; ○, no LOH, ●, LOH; NI, not informative; ND, not determined, rec., recurrent tumour. (p), polymorphism at codon 213.

Table IIIb   Frequency of allelic loss according to p53 mutation

p53 mutation	Number of tumours	FAL (%)
Negative	22	21.1
Positive	4	55.8

Correlation between tumour stage/grade and p53 mutations

All the p53 mutations were found in grade 3 tumours, as is shown in Table IIa ($P < 0.001$). No mutations were found in the group of superficial tumours, while a p53 mutation was found in 8 out of 24 (33%) invasive tumours ($P < 0.001$) (Table IIb). This is in good agreement with previous observations (Sidransky *et al.*, 1991; Fujimoto *et al.*, 1992).

Relation between p53 mutations and frequency of allelic loss

As a measure of genetic instability, we used frequency of allelic loss (FAL). FAL was based on RFLP analysis using probes for chromosomes 9q, 11p, 16q, 17p and 18q (Table IIIa). LOH of 17p was found in seven tumours. Four tumours showed a p53 mutation, while in two a polymorphism at codon 213 was found. No mutations were found in the tumours without 17p LOH. The tumours with a p53 mutation show a FAL of 55.8%, while those without a mutation have a FAL of 21.1% ($P < 0.05$) (Table IIIb).

Correlation of p53 mutation and survival

The survival according to p53 mutation for the whole group of 45 patients is shown in the Kaplan–Meier curve (Figure 1a). The patients with a p53 mutation survived for a shorter period of time ($\chi^2 = 11.25$, $P < 0.001$). There was no significant association between the presence of a p53 mutation and decreased survival among patients with invasive disease ($\chi^2 = 1.46$, NS) (Figure 1b).

Discussion

In this study, we examined mutations in the p53 gene by PCR–SSCP analysis. We show that the occurrence of p53 mutations correlates with grade and stage, which is in concordance with previous studies (Sidransky *et al.*, 1991;
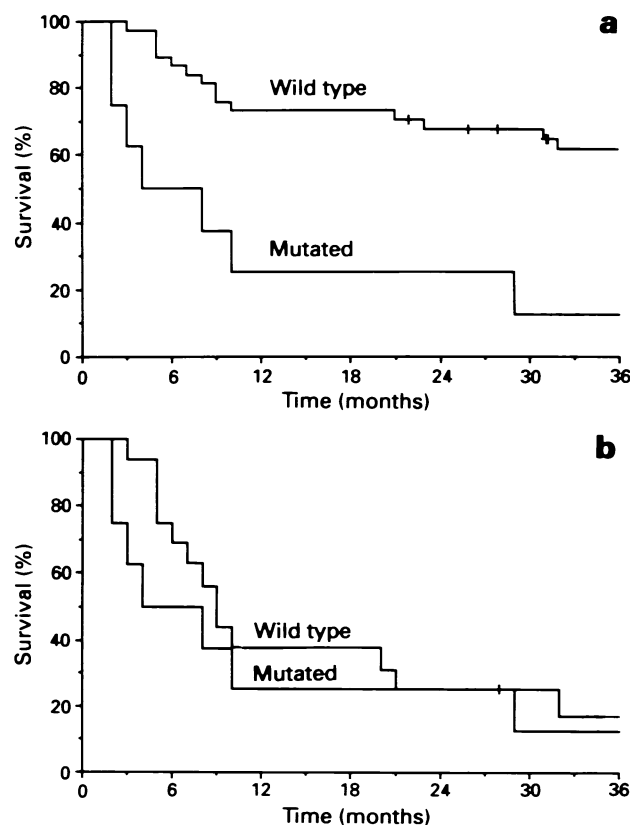

Figure 1   Three year survival (%) of bladder cancer patients according to the presence or absence of p53 mutations. **a**, All patients ($P < 0.001$, log-rank test). **b**, Patients with muscle-invasive tumours (P = not significant).

Fujimoto et al., 1992). A p53 mutation was found in 8/24 (33%) of the invasive tumours, which were all grade 3.

The p53 tumour-suppressor gene is known to be mutated in many types of cancer (Hollstein et al., 1991) and during tumour progression one of the two alleles is often lost, resulting in a reduction of growth control (Baker et al., 1989). However, some p53 mutations are known to be dominant negative: the protein produced by the mutated allele has the ability to bind and inactivate the remaining wild-type product (Vogelstein, 1990). In our study we did not find any mutation without LOH of 17p. On the contrary, one patient with 17p LOH had no p53 mutation. It could be that the mutation is outside the region of the p53 gene studied or that a second locus distinct from p53 is involved (Saxena et al., 1992).

Wild-type p53 has been suggested to be a cell cycle control protein, since progression from G_1 to S phase is often blocked in cells expressing high levels of this protein (Kastan et al., 1991; Livingstone et al., 1992; Yin et al., 1992). It has been shown that cells without wild-type p53 protein fail to show growth arrest (when subjected to conditions unfavourable for the S phase completion) and gene amplification occurs (Yin et al., 1992), which can be considered a form of genetic instability. As p53 mutations are known to occur in bladder cancer (Sidransky et al., 1991; Fujimoto et al., 1992), we used frequency of allelic loss as an indicator of genetic instability (Vogelstein et al., 1989). The high frequency of allelic loss found in tumours with a p53 mutation (55.8%, $P<0.05$) suggests a correlation with genetic instability. This increase in allelic loss in tumours with a p53 mutation could be explained by an altered G_1-S cell cycle checkpoint, which can be the result of the loss of the wild-type p53 function. The loss of certain alleles, e.g. those harbouring tumour-suppressor genes, could provide selective advantages during tumour progression by generating variants with a more aggressive phenotype (Fearon & Vogelstein, 1990). FAL showed no significant correlation with grade or stage.

In breast cancer p53 mutations are inversely correlated with survival (Allred et al., 1993). This study is the first report investigating the prognostic significance of p53 mutations in bladder cancer. The results demonstrate that p53 mutations are an unfavourable prognostic factor ($P<0.001$) for the whole group studied. However, among patients with invasive tumours there was no significant association between the presence of a p53 mutation and a decreased survival. Therefore, patients without a p53 mutation do not necessarily have a good prognosis. There are other features that can lead to an inactive p53 protein, such as complexing with other proteins (Momand et al., 1992), and other genetic events can occur that result in a poor prognosis. Epidermal growth factor receptor (EGFR) positivity has been shown to be associated with tumour progression and decreased survival (Neal et al., 1990). Neal et al. also found no significant difference between EGFR positivity and survival among

patients with invasive tumours. Furthermore, it has recently been shown that decreased E-cadherin expression correlates with the clinical aggressiveness of bladder cancer (Bringuier et al., 1993).

The variable order of appearance of genetic alterations in oncogenesis suggests that accumulation, rather than the order of occurrence, is important for tumour progression (Fearon & Vogelstein, 1990). The increased frequency of allelic loss we find in the tumours with a p53 mutation can lead to tumour progression and poorer prognosis. But, as mentioned above, there are other mechanisms which can lead to tumour progression and these may override or bypass the function of p53.

Recently immunohistochemical studies have shown p53 overexpression to be correlated with poor survival (Lipponen, 1993; Sarkis et al., 1993). In pT1 bladder tumours Sarkis et al. (1993) found a clear correlation between nuclear overexpression of p53 protein and disease progression. The study of Lipponen (1993) showed a significant correlation between p53 overexpression and decreased survival for the entire cohort and for the muscle-invasive tumours. However for the pTa and PT1 tumours only a trend was found. In muscle-invasive tumours, we found no association between p53 mutations and decreased survival. This difference stresses again the discordance of p53 immunohistochemistry and p53 mutation analysis (Thompson et al., 1992). The discrepancy in prognostic value for p53 mutations in superficial disease found in the two immunohistochemical studies might be the result of the use of different antibodies and the scoring system used. We found no p53 mutations in superficial tumours, which might be explained by the relative rarity of grade 3 tumours in this group.

The results of our study indicate that the presence of a p53 mutation is an unfavourable prognostic factor for the whole group studied, but it has no additional prognostic value for the group of muscle-invasive tumours. The step at which p53 mutations occur in the tumour progression cascade of bladder cancer is still unclear. In this respect grade 3 superficial tumours deserve more attention. In general, superficial bladder cancer has a good prognosis, but 10-25% of these tumours progress to an invasive stage. Therefore, the correlation between prognosis and p53 mutation in this subgroup is of particular interest.

Furthermore, the discordance of immunohistochemical and p53 mutation analysis indicates that comparative analysis of p53 mutations and overexpression of the p53 oncoprotein in progression of the pTa and pT1 tumours is necessary.

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