TP53 gene mutations as an independent marker for urinary bladder cancer progression

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Abstract. This study evaluates the influence of the *TP53* genetic status on tumour recurrence and progression with a highly effective electrophoretic technique. DNA from tissue of 75 non-invasive urinary bladder cancers was PCR amplified in the *TP53* exons 5-8 and run on horizontal polyacrylamide gels under defined temperature conditions to yield specific gel shifts. Kaplan-Meier and Cox-Regression analysis were performed with tumour progression. The overall tumour recurrence in our patient population was 76.0% (57/75). Tumour recurrence frequency was 69.4% (34/49) in patients with *TP53* wild-type, and 88.5% (23/26) in patients with *TP53* mutation. There was no statistically significant difference with regard to recurrence frequency and time to recurrence. The progression-free survival was significantly shorter in patients with *TP53* mutations, and the frequency of tumour progression was significantly higher in mutated as compared to wild-type tumours. Cox-Regression analysis showed a significant and independent influence of *TP53* mutation on tumour progression in comparison with tumour grade, stage and history of prior bladder cancer. If segregated by exons, mutations in the DNA binding region of exon 8 seem to have a particular high influence on tumour progression. We conclude that genetic analysis of *TP53* can select patients at high risk of bladder tumour progression that should be followed closely and may benefit from early radical surgical procedures.

Introduction

With an estimated 61,000 newly diagnosed cases and approximately 13,180 deaths in the United States in 2007, carcinoma of the bladder is the second most common cancer of the urogenital tract (1). The incidence of urinary bladder cancer has increased in the last decades. Bladder cancer has a high rate of recurrence and a significant number of non-invasive tumours will progress to muscle-invasive disease. Due to the heterogeneity of the tumour, new markers for tumour progression are clearly needed as clinical parameters, such as tumour grade and stage are not accurate in predicting the biological behaviour and thus guiding the choice of treatment, especially in high risk cases (2-5).

TP53 mutations are the most frequent genetic alterations in human malignancies. Bladder tumours (40%) are *TP53* mutated (6). A strong association of p53 protein overexpression with a higher rate of progression and recurrence of bladder cancer has been shown (7). We have previously shown that genetic analysis of *TP53* gene can provide valuable information in regards to tumour progression and recurrence and that such analysis is possible in the urine sediment (8,9).

In this study we demonstrate with a highly effective electrophoretic technique that mutations of the *TP53* gene are a statistically significant and independent indicator for early tumour progression.

Materials and methods

Tumour tissue was obtained from 75 patients undergoing transurethral resection of urinary bladder tumour. Twenty-five of the patients had a history of prior non-invasive bladder cancer. The mean age of patients (64 males, 11 females) was 66.3 (range 44-88) years. The histological diagnosis according to the 1997 TNM classification was Ta in 46 cases, T1 in 27 cases, isolated Carcinoma *in situ* (Cis) in 2 patients and 5 patients had Cis combined with synchronous T1 carcinomas. Tumour grade was G1 in 21 cases, G2 in 47 (4 combined with Cis) cases and G3 in 5 (1 combined with Cis) cases.

Three of the 75 patients were treated with intravesical bacillus Calmette Guerin (BCG) after surgery. One patient had a mutation in exon 6, 1 in exon 7, 1 was *TP53* wild-type. One patient was treated with mitomycin C (wild-type), 1 with BCG and mitomycin C (wild-type). None of these 5 patients had progression. The other cases did not receive additional intravesical treatment after the samples were taken.

Total-DNA was isolated from frozen tumour tissues (in four cases from paraffine samples) and amplified by PCR as

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Table I. Patients with non-invasive bladder cancer and tumour progression.

No.	Age (years)	Sex	Primary histopathology	*TP53*-genotype	Affected gene map position	Codon altered	Time-to-progression (months)	Progress to
1	65	M	TaG1	Exon 6	13397 C→T	213 Arg→opal-Stop	15	T1G3M0
2	55	M	TaG1	Exon 7	Insertion 14008 C	228 Asp→Ser Frameshift	18	T3G2M0
				Exon 8				
3	66	M	T1G2	Exon 7			6	T2G2M0
4	75	M	Cis	Exon 6	13399 A→G	213 Arg Silent	8	T2G3M0
5	77	M	T1G3	Exon 7	14029 C→A		29	T3G3M0
				Exon 8	14510 G→C	281 Asp→His		
				Intron 8	14594 C→G			
6	59	M	T1G3	Exon 7	14070 G→T	248 Arg→Leu	10	T1G3M1 + Cis
7	74	M	TaG1	Exon 8	14514-15 GG→AT	282 Arg→His	15	T1G3M1 + Cis
8	58	M	T1G2	Exon 8	14501 C→G	278 Pro→Ala	5	T2G3M0 + 'Cis
9	58	F	TaG2	Exon 5	13106 G→A	143 Val→Met	33	T2G3M0
10	64	M	TaG2	Exon 7	14060-1 GG→CT	245 Gly→Leu	5	T1G3M0 + Cis
11	63	M	TaG2	Wild-type			70	T3G3M1
12	67	M	T1G2	Wild-type			6	T1G3M0
13	61	M	TaG2	Wild-type			30	T1G2M0
14	77	M	T1G2	Wild-type			27	T2G3M0

described before (10). Briefly, the critical exons 5-8 of the *TP53* gene were amplified using the following primers: exon 5, 5'-(gC)TTC CTC TTC CTA CAg TAC TC and 5'-CTg ggC AAC CAg CCC TgT CgT; exon 6, 5'-(gC)ACg ACA ggg CTg gTT gCC CA and 5'-AgT TgC AAA CCA gAC CTC Ag; exon 7, 5'-(gC)TCT CCT Agg TTg gCT CTg ACT g and 5'-gCA AgT ggC TCC TgA CCT ggA; and exon 8, 5'-CCT ATC CTg AgT AgT ggT AAT C and 5'-(gC)CCg CTT CTT gTC CTg CTT gCT T [gC refers to a 40-bp-GC-rich sequence according to Metzger *et al* (11)].

The PCR products were run on horizontal polyacrylamide gels under defined temperature conditions (TGGE, Qiagen, Hilden, Germany) to yield specific gel shifts as a screening for mutations (10). Mutations were confirmed in some cases by automated sequence analysis (Amersham Pharmacia Biotech, Uppsala, Sweden) of re-amplified TGGE bands using the nested dye-labelled primers (12).

The statistical evaluation was performed using the software SPSS for windows, version 15.0. Kaplan-Meier and Cox-Regression analysis were calculated with tumour progression defined as invasion of subepithelial connective tissue beyond the muscularis mucosae (progression from Ta to T1), muscle invasion of former non-invasive disease (progression to T2 or higher), or development of metastatic disease as the end-point. All 14 patients with tumour progression are indicated in Table I. For further analysis patients were subdivided into a 'low risk' (TaG1, TaG2) and a 'high risk' (all T1, all G3 and all Cis) subgroup of tumour progression according to their histopathological classification.

Results

In 26 of 75 patients (34.7%) one or more *TP53* mutations were detected by TGGE in tumour tissue. The mutation frequency was 23.9% (11/46) in Ta-tumours, and 55.6% (15/27) in T1-tumours. One of two tumours with isolated Cis was mutated, as were three of five T1-tumours with associated Cis.

In the 26 *TP53* mutated tumours 6 mutations were found in exon 5, 6 in exon 6, 15 in exon 7 and 4 in exon 8. Five patients had mutations in two *TP53* exons, always a combination of an exon 7 mutation with other mutated exons (one in combination with exon 5, two with exon 6, two with exon 8). In this double mutation subgroup only the 2 patients with exon 7 and exon 8 mutation suffered from tumour progression. Successful sequence analyses results of 9 from 10 patients with mutations in the TGGE and tumour progression are shown in Table I.

The overall tumour recurrence in our patient population was 76.0% (57/75). The mean time to recurrence was 32.5 months (range 3-95, median 27.0). Of the 49 *TP53* wild-type tumours 69.4% (n=34) recurred after a mean of 35.6 months (range 3-94, median 28.5). Of the 26 *TP53* mutated tumours 88.5% (n=23) recurred after a mean of 27.8 months (range 5-95, median 18.0).

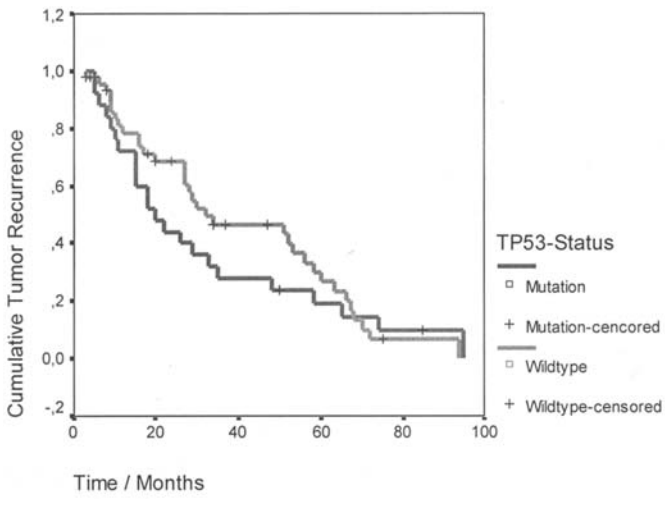

Figure 1. Kaplan-Meier analysis of tumour recurrence in non-invasive bladder cancers (*TP53* wild-type and mutation).

Kaplan-Meier analysis for tumour recurrence showed no statistically significant difference between tumours with *TP53* wild-type and mutation (statistic tests: log-rank, p=0.4487; Breslow, p=0.1289) as shown in Fig. 1. The mean time to recurrence (27.8 months in *TP53* mutation, 35.6 months in *TP53* wild-type) did not differ significantly, either.

The overall tumour progression frequency was 18.7% (14/75). The mean time to tumour progression was 19.8 months (range 5-70); 38.5% (10/26) of the *TP53* mutation patients had a tumour progression within 14.4 months (range 5-33), and 8.2% (4/49) of the wild-type tumours progressed within 33.3 months (range 6-70). The progression-free survival was significantly shorter in the mutation group (statistic tests: log-rank, p=0.0031; Breslow, p=0.0009).

Of the 29 high risk tumours (27 T1G3-tumours and 2 Cis) the tumour progression was 27.6% (8/29). The mean time to tumour progression of these 8 patients was 23.5 months (range 5-70) months. Six of the 46 low risk tumours (13.0%) progressed after a mean time of 14.8 months (range 5-30).

The number of tumours that progressed was significantly different in patients with *TP53* wild-type versus mutation if calculated for the whole study population (Fig. 2A) and if calculated separately for the low risk group (statistic tests: log-rank, p=0.0107; Breslow, p=0.0140) as it can be seen in Fig. 2B. However, in the high risk subgroup the Kaplan-Meier technique did not show a significant difference of tumour progression between patients with *TP53* wild-type and mutation (statistic tests: log-rank, p=0.1869; Breslow, p=0.0788) as shown in Fig. 2C.

The calculation of the influence of exon-specific mutations on tumour progression showed that only mutations in exon 8 reached a specific significance on tumour progression (Fig. 3). Separate evaluation of exon 8 mutations within the high risk subgroup showed a significant difference (statistic tests: log-rank, p=0.0021; Breslow, p=0.0037; figure not shown).

Examination of mutations in single exons showed different frequencies in patients with tumour progression: exon 5, 1/10; exon 6, 2/10; exon 7, 5/10; and exon 8, 4/10. This is outlined in the Kaplan-Meier computations (Fig. 3), and in the Cox-Regression analysis showing exon 8-mutations as an independent progression factor in patients of the high risk subgroup (Table II).

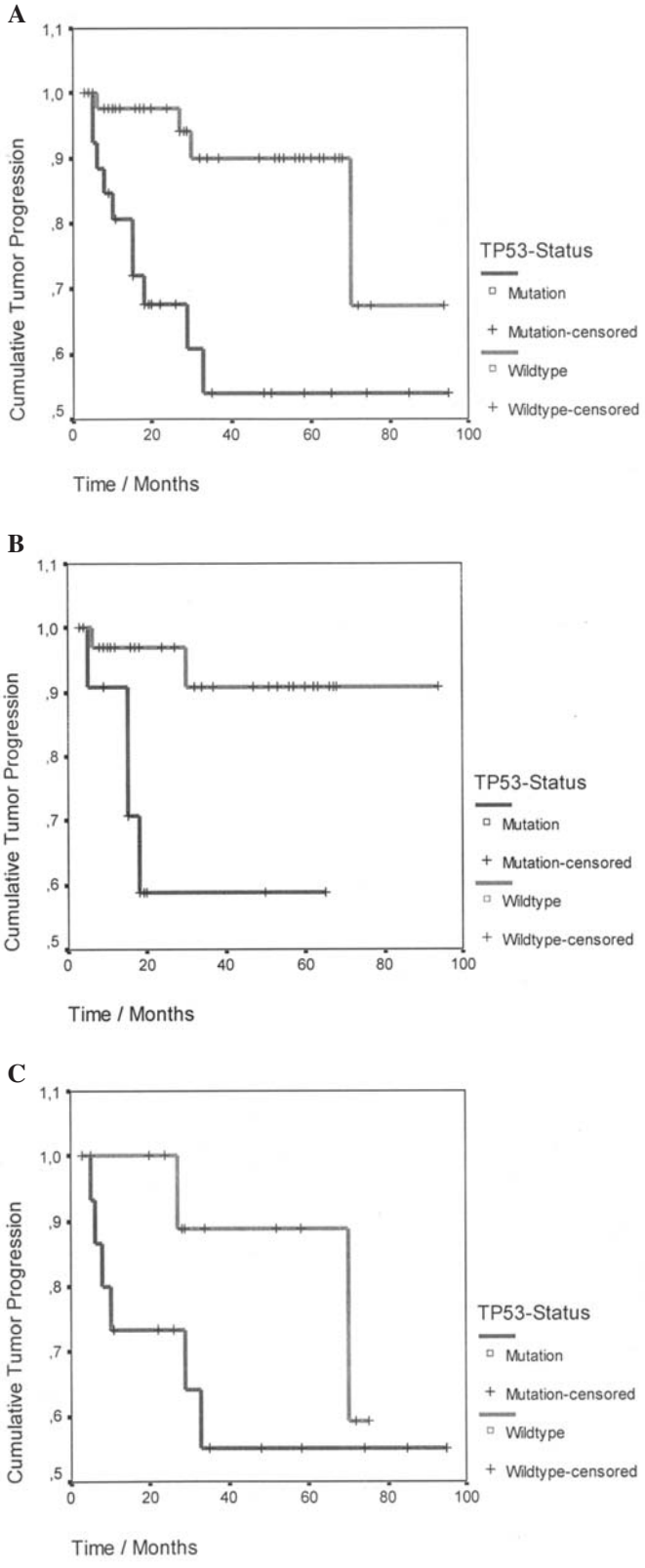

Figure 2. (A) Kaplan-Meier analysis of tumour progression for all patients (*TP53* wild-type and mutation). (B) Kaplan-Meier analysis of tumour progression for low risk tumours (*TP53* wild-type and mutation). (C) Kaplan-Meier analysis of tumour progression for high risk tumours (*TP53* wild-type and mutation).

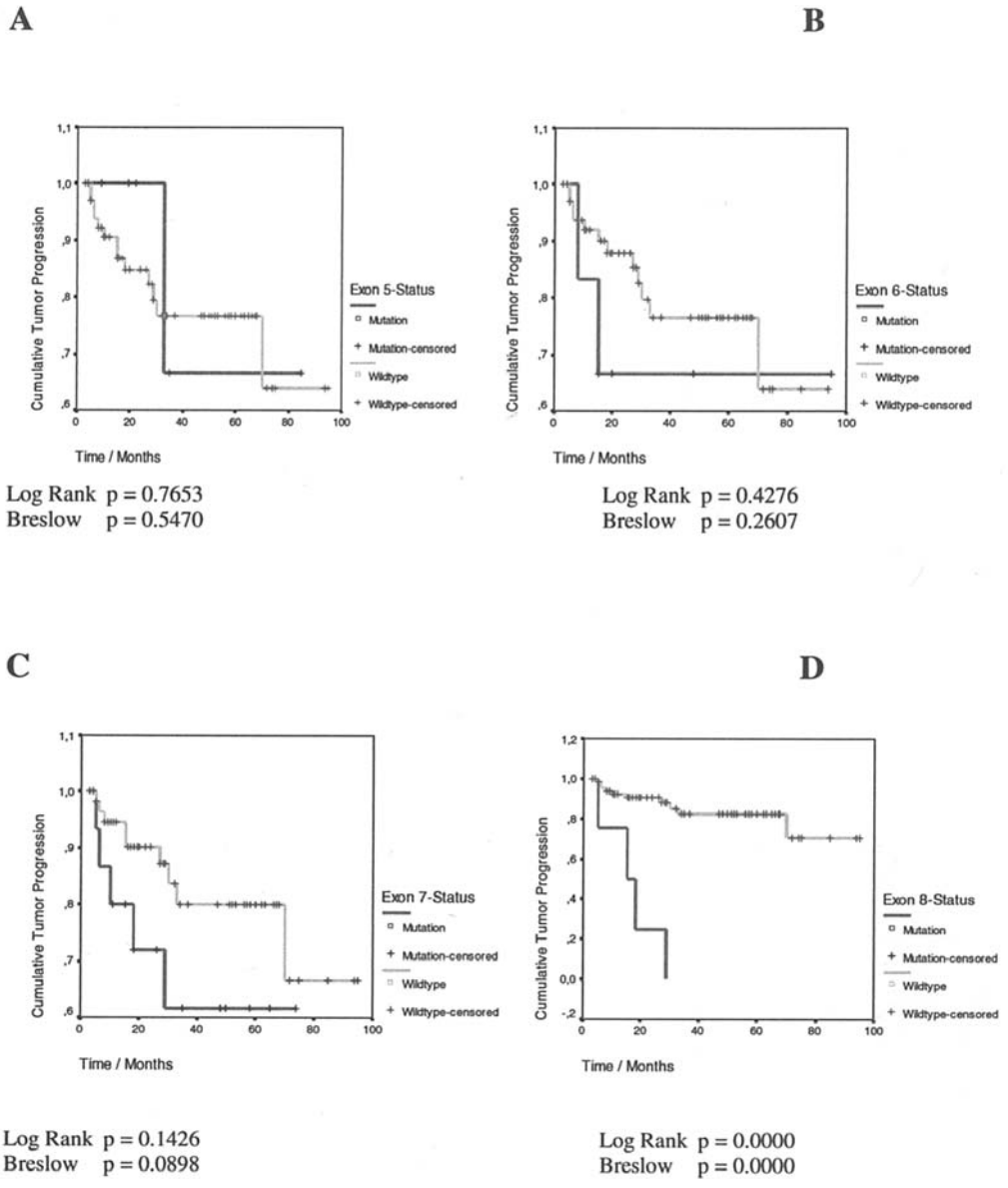

Figure 3. Kaplan-Meier analysis of tumour progression - exon-specific mutations of the *TP53* gene (A-D, exons 5-8).

Interestingly, the multivariate Cox-Regression analysis of tumour stage, grade, history of previous bladder cancer, *TP53*-status, patient age and gender as progression factors revealed, that the *TP53*-status reached statistical significance in the initial block of the Cox-Regression technique. Calculations of the exon 8-status showed stronger significance as the analysis of all 75 patients (Table II, calculatation nos. 1 and 2) or the low and high risk subgroups. The variable G2 does not reach significance in all calculations of the initial block and was excluded in the next step of the Cox-Regression analysis. The term Exp(B) in Table II refers to the increase of probability to suffer from tumour progression in case of a mutation. Exon 7 status has no influence on tumour progression with our data.

If tumour progression was defined as muscle invasion or metastases the number of cases with progression would be reduced to 10 (primary tumours: 2 Ta, 7 T1 and 1 Cis; 8 with *TP53* mutations, 2 with wild-type). In statistic tests a high statistical significance is computed (log-rank, p=0.0009; Breslow, p=0.0002). Kaplan-Meier analysis in Fig. 4 and

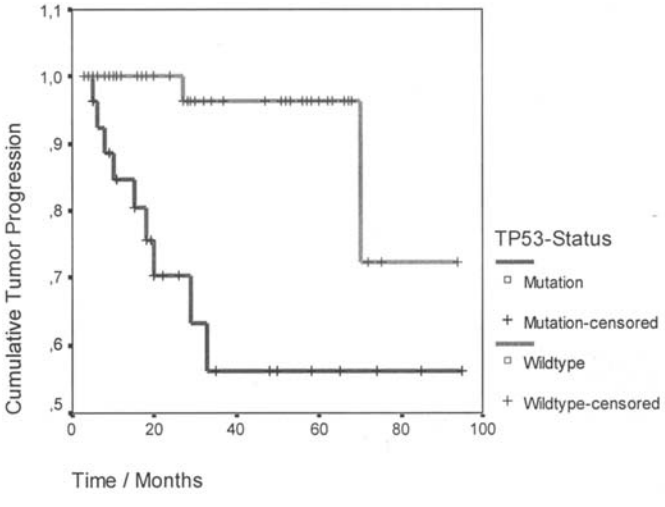

Figure 4. Kaplan-Meier analysis for tumour progression between tumours with *TP53* wild-type and mutation for progression to muscle invasion and/or metastatic disease.

Table II. Cox-Regression analysis of bladder cancer progression variables are *TP53* status, exon 8 status, patients age and sex, tumour stages, tumour grades.

Computation No.	No. of patiens	Variables	Significance in beginning block	Exp(B)	95% CI[a] of Exp(B)
1	75	*TP53*-status	0.003	4.869	1.522-15.578
		Age	0.999		
		Sex	0.514		
		Stage	0.393		
		Ta	0.257		
		T1	0.333		
		Grade	0.269		
		G1	0.825		
		G2	0.062		
		G3	0.717		
2	75	Exon 8	0.000	12.020	3.490-41.396
		Grade	0.272		
3	75	Exon 7	0.133		
		Grade	0.236		
4	46 low risk	Exon 8	0.015	22.314	1.837-271.088
		Grade	0.603		
5	29 high risk	Exon 8	0.014	8.519	1.535-47.277
		Grade	0.551		
6	Tumour progression to invasive tumours, 75	*TP53*-status	0.016	7.109	1.431-35.326
		Grade	0.337		
		G1	0.609		
		G2	0.081		
		G3	0.588		

[a]CI, confidence interval.

Cox-Regression analysis (Table II, calculation no. 6) show significance for *TP53* status and exon 8 status.

Discussion

In this study we show our experiments and calculations supporting *TP53* mutations as a tumour progression factor superior to and independent of tumour grading and staging, patient age and gender, in non-invasive bladder cancer. Only within the high risk subpopulation, if evaluated separately, this relationship was not statistically significant, which supports the results of Peyromaure *et al*, who found no prognostic value of p53 overexpression in T1G3 bladder tumours that were treated with BCG therapy (13). In our cohort 5 patients received intravesical therapy, 2 of them with *TP53* wild-type. We think that this number is too low to influence the statistical significance of our data. The low number of patients receiving intravesical therapy is certainly a point of discussion and might be explained by the fact that most patients were treated outside the study institution and that by the time this study was initiated, intravesical therapy was not yet generally accepted as a standard procedure. On the other hand, our patient population enables an evaluation independent of additional treatments and therefore a more precise definition of the role of *TP53* gene mutations.

Our data do not show a significant relationship between *TP53* mutation and rate of tumour recurrence. Although the recurrence rate in our study is at the higher end of previously published results it is still within their limits and could also be explained by the low percentage of patients who received intravesical therapy (14-16).

Llopis *et al* described, that p53 protein expression has prognostic value for survival and progression in T1 bladder tumours and can be used for early detection of T1 bladder tumours with poor prognosis (17).

Until now, no definitive molecular evidence proving or disproving the progression from non-invasive to invasive bladder tumours has been reported (18). *TP53* mutations have been shown in non-invasive stages of bladder cancer in the range of 35% with increasing frequencies of up to 70% in invasive stages (10). Interestingly, in those tumours that have not directly inactivated *TP53* it is suspected that the functionality is hampered by mutated components of signaling pathways that activate p53 (19). In an *in vitro* study it was shown that organisms with multiple *TP53* genes are tumour resistant (20).

Several groups have presented results of p53 to be a tumour progression factor in bladder cancer and several other malignancies (17,21-37). So far, the role of p53 as a prognostic indicator has been contradictory. Immunological detection of p53 overexpression has been interpreted as mutation. For example, Wu *et al* found p53 overexpression in 70% of non-invasive bladder tumours, but only the ki-67 index was a significant and independent predictor of recurrence and progression (38). They used immunohistochemical detection of p53 overexpression with a cut-off of 20% of nuclei staining positive.

Immunological detection of p53 overexpression and bladder cancer progression was presented by Kuczyk *et al* (26). A number of reports have shown, however, that despite good concordance between *TP53* mutation and p53 overexpression there is not a direct causal relationship between mutation and protein accumulation and that apparently, other events than mutation can trigger p53 stability (28,39). Dahse *et al* found that *TP53* mutations seem to occur more often in higher malignant bladder tumours with a higher tendency of recurrence and progression, although their results were not statistically significant (40). Furthermore, *TP53* mutation or p53 overexpression precedes chromosome 9 defects in Cis as a precursor for invasive cancer (41,42). Prognostic implications of *TP53* gene mutations in bladder tumours were discussed by Lorenzo-Romero *et al* (43). Many studies have analyzed p53 in bladder cancer; the prevalence of p53 alterations increases with stage and grade (6,44-46), but there is no definite evidence that p53 overexpression is an independent prognostic factor (45).

There are less publications combining molecular genetic *TP53* analysis with progression in bladder cancer. In this study we present TGGE and sequencing data of patients with a follow-up of up to 95 months. Our *TP53* exon specific mutation frequencies are in the same range as in some other urological tumours. As in most other tumours, the mutation frequency of 6.1% (4/66) of exon 8 in bladder cancer is lower than other exon mutation frequencies. The overall *TP53* mutation frequency of urothelial tissue without tumour verification is in the range of 13% (47).

The small sample number in this study probably accounts for the non-significance of exons 5-, 6- and 7-mutations, if analyzed separately, while all mutations taken together showed a high significance as a progression factor. On the other hand, it seems interesting, that exon 8 mutations reach statistical significance. This result is supported by prior work by Huang *et al* (41), who found that mutations in exon 8 were more useful indicators of prognosis for non-small cell lung cancer than mutations in other *TP53* exons. They suggested, that the worse overall survival of the patients with mutations in exon 8 was associated with mutations in codon 273 and between codons 280 and 285, which are included into the H2 alpha helix. The abnormal conformation of H2 might play an important role not only in the loss of normal function but also in the acquisition of tumourigenesis (41). Also, Skaug *et al* found exon 8 mutations were related to even still poorer lung cancer-related prognosis than mutations at other locations within *TP53* (27).

The possible function of *TP53* defects for tumour progression should be further elucidated. Some doubts exist about the loss of DNA repair capabilities in case of p53 defects. Huang *et al* (41) have outlined the 3'→5' exonuclease function of p53 wild-type protein for proofreading function of DNA-polymerase α.

Ten of eleven sequenced mutations in our study (Table I) are suspected to cause strong biological effects; three of them are non-sense mutations. The mentioned intron 8 mutation C→G in position 14594 maps 6 bases outsite the exon 8 border, therefore an influence on splicing may be possible. Codon 245 is a hotspot mutation in bladder cancer and several other tumours. All other identified mutation sequences are in regions of special functional activity of p53. The exon 8 region has DNA binding properties. Missense mutations of codons 245, 278, 281 and 282 encompass conserved regions of *TP53* (48). Codon 248 is a well known mutational hotspot in bladder cancer. Codon 143 mutations may result in p53 overexpression and increased cell proliferation (49).

In summary, our results show, that *TP53* genetic mutations are independent prognostic factors for poor progression free survival in non-invasive bladder cancer. Furthermore, mutations at certain sites of the *TP53* gene, particularly at exon 8, can cause even poorer prognosis as these sites involve the biological function of p53. Mutations in defined structural and functional domains of p53 may therefore be useful molecular biological markers for prognosis and treatment strategies of non-invasive transitional cell carcinomas. This finding is even more valuable, since *TP53* mutations can be analyzed in urine cells by non-invasive methods (8,39,50). *TP53* analyses in tumour tissue or urine cells might guide the clinician towards a more aggressive therapy, such as radical cystectomy for high risk T1G3 or Cis tumours which could otherwise undergo bladder sparing procedures and close surveillance. With newer and faster techniques for genetic analysis, this might be included into the daily routine in the future.

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