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Mutations of tumour suppressor gene P53 (*TP53*) in tumour tissue and cellular urine sediments in urinary bladder cancer

Review Article

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Abbreviations: papillary urothelial neoplasia with low malignant potetntial, (PUNLMP); papillary urothelial carcinoma, (PUC); polymerase chain reaction (PCR); single strand conformational polyphormism (SSCP) temperature gradient gel electrophoresis (TGGE); transurethral resection of bladder (TURB)

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Summary

***TP53* mutations are frequently correlated with tumour development in bladder cancer. One function of *TP53* is the suppression of apoptosis. We compared *TP53* mutation in tumour tissue and urine sediment of urinary bladder cancer patients. We examined 32 patients with urinary bladder cancer. Screening for *TP53* mutations in tumour tissue and urine sediment: Amplification of the *TP53* gene by polymerase chain reaction (PCR) for the exons 5, 6, 7 and 8; Temperature gradient gel electrophoresis (TGGE) was used to analyse the *TP53* mutations. We detected mutations of *TP53* in invasive bladder cancer in 5 of 5 cases (100%); in superficial bladder cancer we found 20 of 27 cases (74%) with mutation of *TP53*. In 14 of 25 patients (56%) we could find *TP53* mutation mobility shifts in the same exons analyzed in both tumour tissue and urine sediment. In some cases we could localize the same mutation of *TP53* in both materials. *TP53* mutations are detectable in early bladder cancer stages by PCR-TGGE and sequencing, which might influence therapeutic strategies. The identification of gene mutations in extracorporal samples, such as urine sediment, is an important area of research having implications for tumour diagnosis and monitoring. This study show that *TP53* mutation are detectable in tumour tissue and in cellular urine sediment; beside that we demonstrate that *TP53* mutation frequently occur in higher stages and grades of bladder tumours. The included review of the literature and the results of this study make clear that scientific work in the field of tumour markers in urinary bladder cancer is an important theme in urology nowadays and in future.**

I. Introduction

In western countries, urinary bladder cancer is the fourth most common cancer in men and the ninth most in women. The incidence of urinary bladder cancer has increased in the last decades. There is still a lack of tumour markers for urinary bladder cancer to improve optimal therapy and outcome (Kroft et al, 1994; Stein et al, 1998; Burchardt et al, 2000; Sánchez-Carbayo, 2004; Ecke et al, 2005). Tumour stage and grade are still the best established prognostic factors in urinary bladder cancer. Because of their limited prognostic value, the biological heterogeneity of the disease and the different clinical course, the analyses of cytogenetic and molecular changes hast vastly expanded and offers the posibility of higher risks of tumour progression (Brauers and Jakse, 1996; Dinney et al, 2004). The development of new array technologies to primary human tumours will change the scientific and clinical paradigm, providing novel predictive and therapeutic targets for the cancer patient (Sánchez-Carbayo and Cordon-Cardo, 2003).

Mutations of tumoursuppressor gene *TP53* are frequently correlated with tumour development and

progression in bladder cancer (Spruck et al, 1993; Kuczyk et al, 1995; Hartmann et al, 2002). *TP53* is localized on the short arm of chromosome 17 (McBride et al, 1986; Miller et al, 1986). *TP53* has influence on cell cycle regulation, gene transcription, DNA repair, genome stability, chromosome segregation, angiogenesis and apoptosis. Genetic alteration is the most frequent reason of a change of function of *TP53*. Loss of function may also be due to binding with viral oncoproteins or cellular gene products or maybe caused by dislocation of the protein to cytoplasma (Lowe et al, 1993; Symonds et al, 1994; Harris, 1996). A very important function of wild-type *TP53* is the induction of apoptosis (Ozbun and Butel, 1997).

The first detection of *TP53* mutations in cellular urine sediment was in 1991 by Sidransky et al. *TP53* mutation frequency in urinary bladder cancer estimated by temperature gradient gel electrophoresis (TGGE) is in the range of 40% (Schlechte et al, 1997). TGGE analyses show that about 80% of urinary bladder cancer tissue mutations can be detected in cellular urine sediments (Brenner et al, 1996; Schlechte et al, 1996).

We analyzed the effect of the *TP53* state in urinary bladder cancer by genetic analysis of tumour tissue and urine sediment with a highly effective electrophoretic technique. TGGE is a powerful and comfortable screening method to detect *TP53* mutations (Schlechte et al, 1997). It is superior in its technical performance to the widely used single strand conformational polyphormism (SSCP) due to the visualization of gel mobility shifts and extra bands (Scholz et al, 1993). Low copy numbers of DNA and RNA sequences can be analyzed easily by combining PCR and TGGE (Riesner et al, 1992).

In this paper we show and discuss the results of detecting *TP53* mutation in tumour tissue and in urine sediment. Questions about progression of patients with *TP53* mutations have already been published in former papers (Ecke et al, 2005).

II. Materials and methods

A. Tumour samples and urine sediments

We examined 32 patients with urinary bladder cancer. We collected urine sediment and tumour tissue. 22 male patients and 10 female patients (average age 66.1 years) were included. We did not take any samples from healthy individuals in this study because such results did not help for the question of detecting *TP53* mutations in tissue and cellular urine sediment of urinary bladder cancer patients. All samples were analyzed according to histopathological standard methods. Tissue specimens of approximately 10-50 mg were frozen and stored at -20°C. Cellular urine sediments were collected prior to the first transurethral resection of bladder (TURB). 10-50 ml of fresh urine were spinned (10 minutes, 3000 rpm), washed with 0.15 M sodiumchloride/0.015 M sodiumcitrate solution (pH 7.0) and stored at -20°C before analysis.

Genomic DNA was isolated by standard method using pronase and phenol extraction (Sambrook et al, 1989).

B. Screening for TP53 Mutations

DNA fragments containing one of the exons 5, 6, 7 and 8 of the *TP53* gene were amplified by polymerase chain reaction (PCR) in four separate reactions (Metzger et al, 1991; Scholz et al, 1993). For better detection of single base changes in the following TGGE, one of the primers had an attached 40 bp GC-clamp (Scholz et al, 1993; Kappes et al, 1995; Schlechte et al, 1997). TGGE was run in 8% polyacrylamide gels located on a horizontal plate. The temperature was controlled by two thermostats in order to generate a linear temperature gradient. An example for a TGGE run in exon 7 is shown in **Figure 1**. Each TGGE experiment was carried out with a DNA-negative PCR-control sample and with a positive mutation control to rule out potential contaminations. A 4-band pattern of silver staining in TGGE was estimated as indicative of mutation.

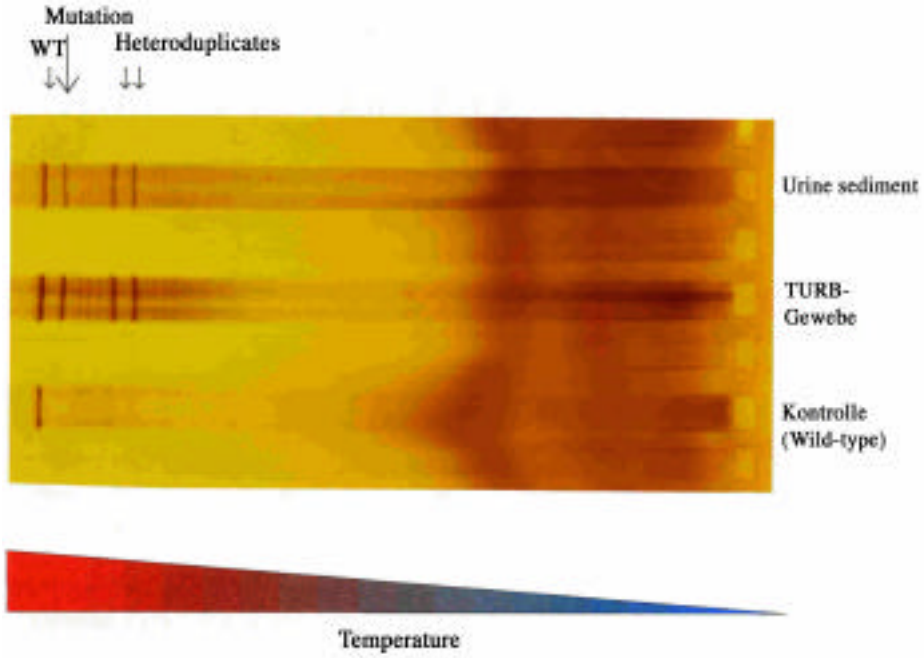

Figure 1. TGGE of *TP53* exon 7. Lane 1: Mutant DNA in urine sediment; Lane 2: Mutant DNA in tumour tissue; Lane 3: Reference wild-type DNA

C. Sequencing

Sequence analyses was performed by solid-phase DNA sequencing of amplified DNA and a laser-fluorescent electrolphoresis unit. Starting materials for sequencing were mutation-specific bands visible in silver stained TGGE gels. Mutant bands and wild-type bands were excised with sterile scalpel blades from the same gel lane. The excised fractions were reamplified. Each sequencing analysis was split in a reaction with the isolated DNA-plus-strand and a reaction with the DNA-minus-strand. An example of processed data of automated sequence analysis is presented in **Figure 2**. The quality of sequencing reaction of exons 5 and 7 were sometimes unsatisfactory with isolated TGGE-products. The reaction was then repeated by direct sequencing of primary PCR products. We used the wild-type sequence of human p53 gene as reference (HSP53G; 10.01.1992, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany). Mutation map positions are indicated according to this data library.

D. Statistical analysis

Statistical analysis included the Chi-square-Test after Pearson's correlation computed and performed by the computer program SPSS 12.0.1.

III. Results

A. *TP53* mutation for superficial and invasive bladder cancer

Thirty-two urinary bladder cancer specimens of different tumour grades and stages were analyzed by PCR-TGGE (**Table 1**). Twenty-seven were superficial urinary bladder cancers, twelve Ta and fifteen T1 stages. Five were invasive ones, including four T2 and one T3 stages.

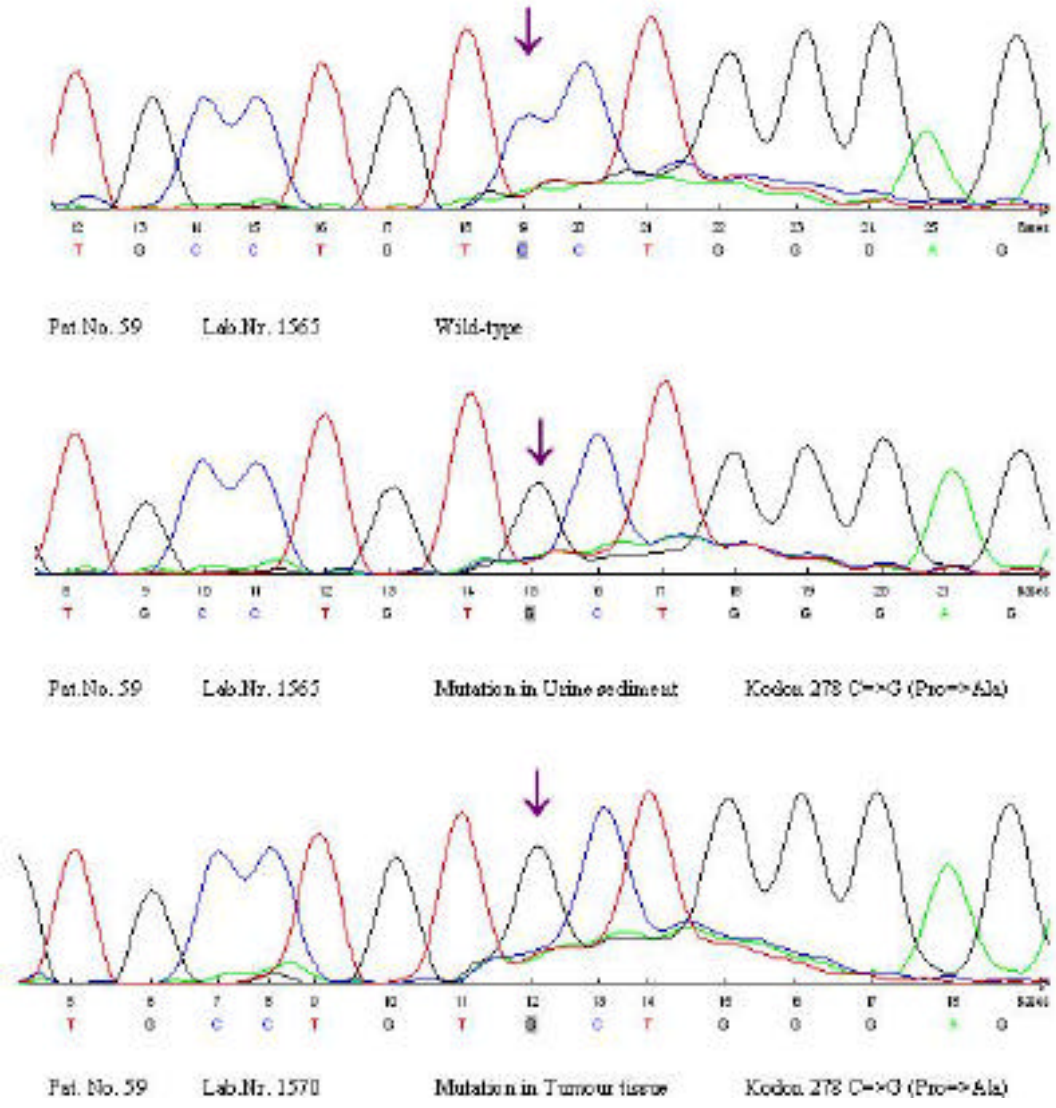

Figure 2. Sequence analysis in urine sediment / Tumour tissue in urinary bladder cancer (T3b high-grade PUC) patient no. 59 / exon 8, Positions 14494-14508 (codons 276-280)

- Upper graph: Sequence from TGGE wild-type (urine sediment)
- Middle graph: Sequence from TGGE mutant (urine sediment)
  Transversion: C=>G in Position 14501 / Kodon 278 Pro=>Ala
- Lower graph: Sequence from TGGE mutant (tumour tissue)
  Transversion: C=>G in Position 14501 / Kodon 278 Pro=>Ala

Table 1. Histological staging and grading of urinary bladder cancer patients. *TP53* mutation frequency analyzed by TGGE

Staging / Grading	PUNLMP	Low-grade-PUC	High-grade-PUC	Total
Ta	4/5	4/6	1/1	9/12
	80%	67%	100%	75%
T1	1/2	7/10	3/3	11/15
	50%	70%	100%	73%
T2	0	0	4/4	4/4
			100%	100%
T3-4	0	0	1/1	1/1
			100%	100%
Total	5/7	11/16	9/9	25/32
	71%	69%	100%	78%

Twenty of 27 superficial urinary bladder cancers (74.1%) and five of five invasive tumours (100%) showed a mobility shift indicative of a mutation in the TGGE. *TP53* mutation frequency was 78% of all 32 urinary bladder cancer specimens, but already 75% of Ta-tumours (9/12) were *TP53* mutated. For grading we used the new 2004 WHO classification (Sauter et al, 2004; Montironi and Lopez-Beltran, 2005; Seitz et al, 2005). The former G1 tumour is classified as papillary urothelial neoplasia with low malignant potetntial (PUNLMP). Differentiation between G2 and G3 are low-grade and high-grade papillary urothelial carcinoma (PUC). *TP53* mutation frequency is also increasing in tumour grading from 71% (5/7) in all PUNLMP to 100% (9/9) in high-grade PUC.

The high number of mutations is caused in our counting system. We count a patient as mutated in *TP53* if there is a mutation in tissue *or* in urine sediment, or in both. Because of the low number of patients we have not made statistical analyses between the different grades.

TP53 mutation in patients with urinary bladder cancer is significantly correlated with tumour staging. We detected mutations of *TP53* in invasive urinary bladder cancer in 5 out of 5 cases (100%); in superficial urinary bladder cancer, we found 20 out of 27 cases (74.1%) with mutation of *TP53*. Pearson's significance with the Chi-square-Test between mutation of *TP53* in superficial and invasive bladder cancer was calculated with p=0.001.

B. *TP53* mutation analyses in urine sediments and tumour tissue

Thirty-two patients with urinary bladder cancer were analyzed for *TP53* mutations by TGGE in tumour tissue and cellular urine sediments before TURB as well. Seven patients did not show any mutation in four analyzed exons in both materials. All of them are patients with PUNLMP or low-grade PUC. All high-grade PUC patients have mutation in *TP53* in tumour tissue in cellular urine sediment or in both materials. Twenty-five patients show partly corresponding results. **Table 2** shows mobility shifts in the same exons analyzed in both tumour tissue and urine sediment in 14 of 25 patients (56%) with *TP53* mutation. Only five out of 25 *TP53* mutations have the same result in all five exons. We have not found any statistically significant correlation between tumour tissue and urine sediment in patients with mutation of *TP53*.

C. Sequence analysis of tumour tissue

All sequenced *TP53* mutations of nine patients with urinary bladder cancer map in 11 positions (**Table 3**). One patient show double mutations (No. 132/T1 high-grade PUC in map positions 14070 + 14494) and one patient show three different mutations (No. 41/T2 high-grade PUC in map positions 14029 + 14594 + 14510).

Six patients have high-grade PUC. Five of six mutations in high-grade PUC are clustered in exons 5 and 8.

None of the patients show two-nucleotide mutations. Two mutations are nonsense (codons 213, 234).

Referring to gene-function at mutated location we found two L2-Loop, one L3-Loop and seven DNA-Bindings (Heinaut et al, 1998).

D. Sequence analysis of urine sediments

Sequencing of urinary samples of 12 urinary bladder cancer patients identified mutations in 13 different codons (**Table 4**). Two positions were silent alteration of codons 213 and 177 and one was a stop-mutation. One patient shows double mutations (No. 89/T1 low-grade PUC in map positions 13215 + 13210). Two of the patients show two-nucleotide mutations (No. 94, 121). Two mutations are nonsense (codons 213, 234).

Bold printed cases in **Table 4** refer to patients with identical sequencing results in their urinary bladder cancer tissue.

Referring to gene-function at mutated location we found one L2-Loop, two L3-Loop and five DNA-Bindings (Heinaut et al, 1998).

Sequence analyses of urine sample of patient number 59 is shown in **Figure 1**. The one-nucleotide mutation C=>G (transversion) specifies alanine in codon 278 instead of wild-type codon proline. This patient (male, age 59) had a primary urinary bladder cancer T1 high-grade PUC. After radical cystectomy the classification was T3bG3.

IV. Discussion

The mutation frequency in the so-called high-risk exons 5-8 of the *TP53* gene is approximately 40% in bladder cancer tissue (Schlechte et al, 1997). Mutation of *TP53* might be accelerating carcinogenesis especially by

Table 2. *TP53* mutations in tumour tissue corresponding with results of urine sediment – TGGE analysis
Bold and underlined a non-corresponding mutation in this exon

Pat. No. / Sex	Patient	Age	Pathology	*TP53* in tumor tissue	*TP53* in urine sediment
				5 6 7 8	**5 6 7 8**
26 / m	G.F.	53	Ta PUNLMP	- - - -	- - - -
46 / w	N.H.	55	Ta PUNLMP	- - - -	- - **7** -
81 / m	H.M.	67	Ta PUNLMP	5 - **7** -	5 - - -
102 / m	P.S.	65	Ta PUNLMP	- - - -	- - **7** -
111 / w	D.S.	65	Ta PUNLMP	5 - - -	5 - **7** -
9 / m	H.B.	74	Ta low-grade PUC	- - - -	- - - -
12 / w	H.B.	66	Ta low-grade PUC	- 6 - -	- 6 **7** -
114 / w	W.S.	56	Ta low-grade PUC	- - - -	- **6** **7** -
118 / w	E.T.	69	Ta low-grade PUC	- - - -	- - - -
119 / m	M.T.	65	Ta low-grade PUC	- **6** 7 -	- - 7 -
135 / m	K.Z.	59	Ta low-grade PUC	- - **7** -	- - - -
94 / m	F.P.	65	Ta high-grade PUC	- - 7 -	- - 7 -
21 / m	G.D.	59	T1 PUNLMP	**5** - 7 -	- **6** 7 -
115 / m	F.S.	68	T1 PUNLMP	- - - -	- - - -
2 / m	H.B.	69	T1 low-grade PUC	5 - - -	5 - - **8**
15 / m	J.B.	63	T1 low-grade PUC	- - - -	- - - -
16 / m	W.D.	57	T1 low-grade PUC	5 - - -	5 **6** - -
20 / w	L.D.	84	T1 low-grade PUC	- - - -	- - - -
36 / w	E.G.	64	T1 low-grade PUC	- - - -	- - - -
44 / m	G.H.	67	T1 low-grade PUC	- **6** - -	**5** - - -
50 / w	H.H.	68	T1 low-grade PUC	- - **7** -	- - - -
87 / m	K.N.	75	T1 low-grade PUC	- **6** 7 -	- - 7 -
89 / m	H.N.	82	T1 low-grade PUC	5 - - -	5 - - -
121 / m	B.T.	75	T1 low-grade PUC	- - - -	- - **7** **8**
132 / m	H.Z.	59	T1high-grade PUC	- - **7** **8**	- - - -
32 / m	W.G.	59	T1 high-grade PUC	- - **7** -	- - - -
99 / w	J.R.	59	T1 high-grade PUC	5 - - -	5 - - -
41 / m	G.H.	76	T2 high-grade PUC	- - **7** 8	- - - 8
76 / m	T.L.	72	T2 high-grade PUC	**5** - - -	- - - -
79 / w	U.M.	67	T2 high-grade PUC	5 - - -	5 - - -
122 / m	J.V.	75	T2 high-grade PUC	- - - -	**5** - - -
59 / m	G.K.	59	T3b high-grade PUC	- - - 8	- - - 8

- = Tp53 wild type in this exon

Table 3. *TP53* mutation in human bladder cancer tissue – all mapped mutation positions

PN	*	Pathology	Exon	Tp53 –Mutation Position	Nucleotide Change	Amino Acid Change	Analyzed Sequence
44	(3)	T1 low-grade PUC	6	13397	C=>T	213 Arg=>opal-stop	13296-13451
87	(3)	T1low-grade PUC	7	14028	A=>G	234 Tyr=>Cys	14000-14116
89	(1)	T1 low-grade PUC	5	13215	A=>G	179 His=>Arg	13044-13222
132	(2)	T1 high-grade PUC	7	14070	G=>T	248 Arg=>Leu	13999-14096
132	-	T1 high-grade PUC	8	14494	T=>C	275 Cys silent	14460-14565
99	(3)	T1 high-grade PUC	5	13106	G=>A	143 Val=>Met	13044-13256
41	(3)	T2 high-grade PUC	7	14029	C=>A	234 Tyr=> ochre-stop	13995–14119
41	(3)	T2 high-grade PUC	8	14594	C=>G	Intron 8	14467–14606
				14510	G=>C	281 Asp=>His	
76	(1)	T2 high-grade PUC	5	13203	G=>A	175 Arg=>His	13046-13221
79	(3)	T2 high-grade PUC	5	13134	C=>T	152 Pro=>Leu	13044-13259
59	(3)	T3b high-grade PUC	8	14501	C=>G	278 Pro=>Ala	14467-14605

PN = Patient number
* = Gene-function at mutated location (Heinaut et al, 1998)
Meaning of numbers at star-column (*)
(1) = L2-Loop
(2) = L3-Loop
(3) = DNA-Binding

Table 4. *TP53* mutation in cellular urine sediments of patients with urinary bladder cancer – all mapped mutation positions

PN	*	Pathology	Exon	Mutation position	Nucleotide Change	Amino acid Change	Analyzed Sequence
81	(2)	Ta PUNLMP	7	14035	C=>A	236 Tyr=>ochre-stop	13996-14121
119	-	Ta low-grade PUC	6	14058	G=>A	244 Gly=>Asp	13997-14097
94	(2)	Ta high-grade PUC	7	14060-1	GG=>CT	245 Gly=>Leu	13997-14118
21	-	T1 low-grade PUC	6	13399	A=>G	213 Arg silent	13295-13442
2	-	T1low-grade PUC	5	13146	G=>A	156 Arg=>His	13044-13219
16	(3)	T1 low-grade PUC	5	13106	G=>A	143 Val=>Met	13043-13256
87	(3)	T1 low-grade PUC	7	14028	A=>G	234 Tyr=>Cys	13995-14116
89	(1)	T1 low-grade PUC	5	13215	A=>G	179 His=>Arg	13044-13220
				13210	C=>G	177 Pro silent	
121	-	T1 low-grade PUC	8	14514-15	GG=>AT	282 Arg=>His	14483-14606
99	(3)	T1 high-grade PUC	5	13106	G=>A	143 Val=>Met	13043-13256
79	(3)	T2 high-grade PUC	5	13134	C=>T	152 Pro=>Leu	13044-13260
59	(3)	T3b high-grade PUC	8	14501	C=>G	278 Pro=>Ala	14488-14606

PN = Patient number
* = Gene-function at mutated location (Heinaut et al, 1998)
Meaning of numbers at star-column (*)
(1) = L2-Loop
(2) = L3-Loop
(3) = DNA-Binding

enhancement of cell proliferation, loss of apoptosis and by insufficient DNA repair (Sidransky et al 1996). Nowadays it is for sure that the *TP53* state plays a role in the progression of bladder tumours (Esrig et al, 1994; Lu et al, 2002; Moch et al, 1994; Serth et al, 1995). In our study we found a mutation frequency of 100% for invasive bladder cancer and of 74% for superficial bladder cancer confirming with published results in 1997 by Schlechte and colleagues. Mutations in *TP53* gene are correlating with infiltrating bladder cancer and are qualified as a marker.

The main aim of this study is to clarify the relationship between *TP53* mutation in urine sediments and tumour tissues of varying stages and grades of urinary bladder cancer.

The results show that *TP53* wild-type is frequently associated with non-invasive and low-grade malignancy (**Table 1**), but the mutation frequency increases with tumour stage and grade. *TP53* mutations are not necessarily a late stage event, but are most likely one of the important genetic alterations in multi-step bladder carcinogenesis. They might help differentiate those superficial tumours that need more aggressive therapy from the ones that can be treated more conservatively. Unfortunately at the moment *TP53* mutation frequency could not be used as marker for prognosis in urinary bladder cancer (Hartmann et al, 2002; Sauter et al, 2004; Ecke et al, 2005). Nevertheless it should be known that "p53 alterations do not sufficiently well discriminate good and poor prognosis groups in properly staged bladder cancers to have clinical utility" (Sauter et al, 2004). It is known that PUNLMP and low-grade PUC are genetic stabile and have less *TP53* mutation than the genetic instabile high-grade PUC (Bryan et al, 2005; Catto et al, 2004; Montironi and Lopez-Beltran, 2005). Although we could not make statistical analyses because of the low number of patients, we have 71% *TP53* mutation frequency in PUNLMP, 69% in low-grade PUC, and 100% in high-grade PUC.

Urine sediment was available from 32 patients prior to TURB. In 14 of 25 cases (56%) TGGE-mobility shifts were identified in the same exons in tumour tissue and urine sediments. Seven patients have shown *TP53* wild-type in tumour tissue and urine sediments as well. This correlates with sequencing results in tumour tissue and the urine of same patients where 5 of 12 patients (41.7%) showed identical results. These results are corresponding with the results of Schlichtholz et al, 2004 where mutation in *TP53* could be detected in tumour tissue and re-detected later in urine sediments. Though alterations in *TP53* have also been detected in the study of Dahse et al. in patients serum, plasma and urine samples, they concluded that the results of re-detection in different materials of the same patients are not sufficient to use *TP53* as a marker in practice (Dahse et al, 2002).

The sequencing results showed a spreading of the mutations over the analyzed exons, including some known mutational hotspots (codons 175, 248, 281, 282) (Xu et al, 1997). Sequence analyses was not successful in all samples. A possible reason may be the higher sensitivity of denaturing gel shifts in comparison with sequence analyses (García-Delgado et al, 1998). It is also possible that the mutant allel, was masked by its wild-type counterpart in the sequencing results of these cases (Schlechte et al, 1997).

The mutation spectrum is not likely to be sufficiently interpretable or distinctive for the identification of specific causative factors giving rise to the alterations detected in this study. But, together with a larger pool of sequence data, clues to the nature of substances or cellular events important in shaping the *TP53* mutation pattern may be

provided (Hollstein et al, 1994; Béroud and Soussi, 1998; Heinaut et al, 1998).

The new classification of urinary bladder cancer shows more than the older versions the need of biological and genetic markers (Sauter et al, 2004; Seitz et al, 2005). This study and the presented results make clear that more work in future is necessary to find out novel markers. A new research field is the use of microarray technologies. Although the large amounts of gene expression data generated have already had a tremendous impact on bladder cancer research, major challenges remain (Sánchez-Carbayo and Cordon-Cardo, 2003).

We conclude that *TP53* mutations are detectable in early bladder cancer stages by PCR-TGGE and sequencing, which might influence therapeutic strategies. The identification of gene mutations in extracorporal samples, such as urine sediment, is an important area of research having implications for tumour diagnosis and monitoring (Sidransky et al, 1991; Lu et al, 2002; Sánchez-Carbayo, 2004; Montironi and Lopez-Beltran, 2005). *TP53* mutation could be used as a marker for tumour recurrence. At the moment this method as a non-invasive diagnostic tool in outpatient samples is of limited value for clinical practice.

To answer more questions about the clinical use of genetic markers and its quality and clinical use, carefully controlled studies on large numbers of clinically well-annotated cases are needed.

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