

Clinical implications of *p53* mutation analysis in bladder cancer tissue and urine sediment by functional assay in yeast

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Short title: Functional analysis of *p53* mutations in bladder cancer

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Key words: bladder cancer, *p53* mutations, urine sediment, survival, recurrence

Abstract

In the present study we correlate the *p53* gene mutations in tumour tissue with urine sediment using functional assay in yeast, and relate the *p53* status to outcome in a group of patients with transitional cell carcinoma of the bladder. The *p53* mutations were found in 3 of 30 (10%) Ta/T1 tumour tissue samples and in 2 of 20 (10%) corresponding urine sediments. In the stage T2-T4 tumour *p53* mutations were found in tumour tissues and urine sediments in 13 of 31 (42%) and in 7 of 18 (39%) samples, respectively. In 80% (8/10) of cases, the *p53* mutations found in tumour tissue were re-detected in urine sediment. Median follow-up was 20 months. Disease recurred in 18 of the 61 patients (30%) with a median time of 5 months. In Ta/T1 tumours the frequency of recurrence was 37% (11/30) comparing to 23% (7/31) of T2-T4 tumours. The 3-year overall survival was 82% (50/61). The *p53* status was significantly associated with stage ($P = 0,0077$, two-sided Fisher's exact test), grade ($P < 0,001$), and lymph node involvement ($P = 0,027$). There was an association between the *p53* mutations and shorter overall survival ($P = 0,033$; log-rank test), however in a multivariate analysis adjusted for stage, grade, lymph node status, and age the *p53* mutation was not an independent predictor of survival. There was no correlation of the *p53* status with decreased disease-free survival ($P = 0,8$; log-rank test). The data presented indicate that yeast functional assay is a useful method for *p53* gene mutation analysis in tumour tissue and *p53* mutation can be re-detected in urine sediment, but further validation of the assay in non-invasive screening for *p53* mutations is needed.

Introduction

Bladder cancer is the second most common genitourinary malignancy after prostate cancer. In 1999, there were approximately 7100 new cases of bladder cancer in Poland, accounting for 8.6% of new cases in men (the fourth cancer in men) and 2% in women, and was responsible for about 3% of all cancer-related deaths (1). Epidemiologically transitional cell carcinoma of the bladder represents 90-95% of cases in Europe, while 5% represents mostly squamous cell carcinoma. Most superficial bladder carcinomas grow noninvasively and have a high frequency of recurrence of about 50% (2) whereas up to 15% progress to a more advanced stage. Cystoscopy and voided urine cytology are the most accepted techniques of urinary system neoplasm detection. The limitations of urinary cytology and invasiveness of cystoscopy have led to studies searching for the new bladder tumour markers that allow for non-invasive detection of bladder carcinomas. The *p53* tumour suppressor gene plays a key role in suppression of neoplastic transformation by cell cycle arrest or/and by apoptosis. Alterations of the gene *p53* are common in bladder cancer, occurring in approximately 50% of transitional cell carcinoma and are seen more frequently in high grade invasive tumours (3,4). Moreover, loss of *p53* function has been associated with progression to invasive disease and decreased survival (5).

In the present study we determined the *p53* status in tumour tissue and paired voided urine by functional assay in yeast followed by sequencing. A large number of tumours have been examined using this method, including breast (6), upper aerodigestive tract (7), hepatocellular carcinoma (8), and acute myeloid leukaemia (9), but very few bladder carcinomas have been studied (10). Additionally the presence of exfoliated cancer cells in voided urine provides a good opportunity to tests whether yeast-based functional assay can be used for non-invasive detection of the *p53* gene mutations in bladder carcinoma.

The aim of our study was; a) to investigate patients with low- and high-stage transitional cell carcinoma of the bladder (TCC) for the presence of *p53* mutations using functional assay in yeast; b) to compare the *p53* mutations in tumour tissue with these from urine sediments; c) to further evaluate feasibility and sensitivity of the *p53* gene functional analysis to detect bladder cancer cells in urine; d) to determine association between the *p53* status and clinicopathological parameters including age, sex, pathologic stage, tumour grade and lymph node involvement. Multivariate survival analysis was performed considering *p53* as an independent prognostic factor.

Materials and methods

Tumour samples and exfoliated cells. The Department of Urology at the Medical University of Gdansk provided a total of 61 bladder tumours and 50 matched voided urine samples, 30 biopsies of bladder tumours were obtained by transurethral resection of the bladder while 31 at cystectomy. The patients' median age was 62 years (ranging from: 32-85). Tissues collected included obvious tumour tissue that was confirmed by subsequent histopathological diagnosis at the Pathology Department, Medical University of Gdansk. Histological staging and grading was performed according to the fifth edition of the TNM classification (11). The clinicopathologic characteristics of patients are summarized in Table I. Samples were immediately frozen in liquid nitrogen, and stored at -80°C. Exfoliated cell were collected from 50 ml of naturally voided urine from 50 patients with bladder carcinoma. The urine was stored at 4°C until centrifugation, followed by a washing and lysis procedure. Survival was determined from the date of primary therapy. Follow-up was available for 61 patients.

cDNA synthesis. Isolation of total RNA from frozen bladder tumour tissue, adjacent non-neoplastic tissue, served as normal bladder tissue, and urine sediment was performed using RNA extraction kit (RNeasy Mini Kit; QIAGEN, USA) and stored at -20°C. In case of urine sediments sufficient amounts of RNA were obtained from 38 patients. Reverse transcription was performed on 1 µg RNA using Omniscript Reverse Transcriptase (QIAGEN, USA) and Oligo d(T)₁₅ Primer (Promega, USA) in a total reaction volume of 20 µl, following the manufacturer's instruction.

PCR. *p53* cDNA was amplified using the following two sets of primers P3 and P17 or P4 and P16 as previously described by Waridel et al.[7]. cDNA (2µl) was subjected to PCR in a 25µl reaction, using ProofStart DNA Polymerase (QIAGEN, USA) with the following conditions: 1x PCR buffer, 1x buffer Q, 1,5 mmol/l MgCl₂, 200µmol/l of dNTPs, 10 pmol of each primer. Following an initial incubation of 5 min at 94°C, 35 cycles of 30s at 94°C, 30s at 63°C and 1 min at 72°C were performed. The amplification was performed in a Model PTC-200TM Thermal Cycler (MJ Research, Watertown, MA, USA) PCR products were analysed on a 1,5% agarose gel.

Homologous recombination in yeast

The PCR product was used in a yeast-based assay (12). For gap repair pFW35 and pFW34 yeast expression vectors were used (7). The *S. cerevisiae* strain yIG397 that allows for the evaluation of *p53* transactivation function was originally constructed by Richard Iggo (12). The strain yIG397 with an ADE2 gene open reading frame (ORF) downstream from three copies of a *p53*-responsive DNA sequence found at the ribosomal gene cluster (RGC) was co-transformed using PEG-based method with linearized expression vector and various *p53* cDNA as previously described (12). Transformants were selected on leucine-deficient media containing limiting amounts of adenine (5mg/l). Colony colour was assessed after 3 days growth at 30 °C. The mutants were scored as previously described (13). Red colonies - indicative of the *p53* mutations represented in average 69% of colonies for tumour and 50% for urine sediment-isolated RNA. Normal bladder tissue gave always less than 10% of red colonies.

Recovery of *p53* plasmids from yeast and DNA sequencing

Plasmid DNA was extracted by rapid DNA isolation method described by Hoffman et al. (14). Mutations were identified on both strands of plasmids from individual colonies by direct sequencing of *p53*-PCR products. *p53* plasmids recovered from yeast were amplified with the same set of primers like for *p53* cDNA amplification prior to homologues recombination. PCR products were purified by QIAquick Gel Extraction Kit (QIAGEN, USA) and sequenced directly by BigDye terminator chemistry followed by capillary electrophoresis with an ABI 310 Genetic Analyzer according to the supplier's instruction (Applied Biosystems).

Statistical Analysis. Based on available clinical and molecular data, the following associations were examined for patients, the presence of *p53* mutation in tumour specimen *versus* sex, age, grade and stage. Univariate analysis using two-sided Fisher's exact test and multivariate analysis using logistic regression were performed. In addition two prognostic outcomes were examined: DFS, defined as the time from primary therapy to first recurrence including censored times and OS, defined as the time from primary therapy to death (from any cause) or last follow-up. The last follow-up evaluation was performed in October 2003. Total length of the study at the time of analysis was 44 months, and median follow-up was 20 months (range 2-44 months). In bivariate association clinical variables were dichotomised as

follows: age at diagnosis, ≤ 65 versus age > 65 years; tumour grade low versus moderate or high grade, stage Ta/T1 versus T2-T4.

Univariate survival curves were constructed by the method of Kaplan-Meier, and the significance of differences estimated by the log-rank test. Cox proportional hazard regression analysis was used to examine the prognostic value of each variable. Patient's age, grade, stage, lymph node and *p53* status were entered into Cox regression. For all analyses, a P value of less than 0,05 was accepted as significant and the analyses were performed with the software STATISTICA version 6 (StatSoft, Inc. Tulsa, USA)

Results

Functional analysis of the *p53* mutations in paired tumour and urine in bladder cancer.

We tested mRNA of tumour specimens from 61 bladder cancer patients for the presence of *p53* mutations. Additionally the same analysis was done in corresponding voided urine specimens from 38 patients. To determine the transcriptional activity of p53, a reporter assay was performed using a reporter construct containing the p53-responsive element upstream ADE2 reporter, as described by Flaman et al. (12). The PCR products corresponding the 5' and 3' parts of *p53* cDNA were co-transformed into yIG397 along with either linear form of pFW-34 or pFW-35 expression vectors.

In general the *p53* mutations were found in 16 of 61 patients (26%) in tumour specimens and in 9 of 38 (24%) corresponding urine sediments. In 80% (8/10) cases, the *p53* mutations found in tumour tissue were re-detected in urine sediment. In non-muscle invasive Ta/T1 stage tumours we found *p53* mutations only in 3 of 30 (10%) tumour tissues and in 2 of 20 (10%) urine sediments. In contrast, in muscle invasive tumours we found *p53* mutations in 13 of 31 (42%) tumour tissue and 7 of 18 (39%) urine sediments. In normal bladder tissue the *p53* mutations were not found (0/10). Additionally all positive cases were analysed by sequencing of the *p53* transcripts. All specimens that showed the presence of red colonies in yeast assay were confirmed to have mutation by sequence analysis. As shown in Table II, in exons 4-11 of *p53* gene we found fifteen missense mutations, three nonsense mutation, two deletions, and two insertions including splice site mutation. Furthermore in three cases we found multiple mutations in the *p53* gene with one complex mutation. In case of splice site mutation at the intron 7 – exon 8 border we observed inclusion of last 3 bp of intron 7 into the coding sequence resulting in the insertion of stop codon. It is worth noting that it is the first time when the mutation at codon 110 in TCC tissue is reported (Table III).

In our study, the status of the codon 72 polymorphism of 12 tumours was determined. The arginine residue at codon 72 was observed in 9 out of 12 (75%) tumours with the *p53* mutations. This may support the observation of Martin et al. that mutant alleles containing R72 are preferentially selected during carcinogenesis (18). They found that R72 *p53* mutants had enhanced ability to bind p73 protein (a homologue of the p53 protein) thus, neutralizing p73-induced apoptosis.

In most cases the genetic change in urine DNA was identical to that identified in the tumour. However in one case (BT34) the *p53* mutation found in the tumour biopsy (R273H) was different from *p53* mutation found in corresponding urine (G279E). Moreover in case BT25 we found one additional mutation at codon 181 in urine sediment comparing to tumour tissue with the only mutation at codon 174. In only one case with the mutation in tumour tissue we did not find any mutation in corresponding urine sediment.

Correlation of *p53* mutations with clinicopathological markers

A correlation analysis between the frequency of the *p53* mutations and (TNM/UICC) stage and grade (WHO) was performed. The association was found between the presence of *p53* mutation and stage ($P = 0,0077$, two-sided Fisher's exact test), grade ($P < 0,001$), and lymph node involvement ($P = 0,027$). There was no association between the *p53* mutation and patient sex, and age. Multivariate analysis using a logistic regression model demonstrated that grade was a dominant factor associated with the *p53* gene mutation (OR 6; 95% CI 1,55 – 23,6; $P = 0,01$).

Patient survival analysis

Survival data were available for all cases studied. The 3-year overall survival (OS) was 82% (50/61). At the end of analysis 15% of patients died of disease and 3% died disease-free. Kaplan-Meier overall survival curves for bladder cancer patients showed a statistically significant association between the presence of *p53* mutations and poor outcome ($P = 0,033$; log-rank test) (Figure 1). Univariate analysis revealed significant correlation for age ($P = 0,021$; log-rank test), grade ($P = 0,029$), stage ($P = 0,003$), and lymph node status ($P = 0,0002$). Furthermore, multivariate Cox hazard regression model was used to investigate several variables at time. In multivariate analysis, *p53* mutation ($P = 0,85$) was not an independent predictor of tumour survival when assessed with tumour grade, tumour stage, lymph node status and age.

Disease free survival from time of surgery was 70%. Of 61 patients, 18 (30%) developed local recurrence with median time of 5 months (ranging: 1-24). In Ta/T1 tumours the frequency of recurrence was 37% (11/30) comparing to 23% (7/31) of T2-T4 tumours.

There was no relationship between the *p53* mutations and decreased disease-free survival (DFS) ($P = 0,8$; log-rank test). On univariate analysis, sex ($P = 0,7$) grade ($P = 0,3$), and stage ($P = 0,17$) were not significant factors.

Discussion

In this study we focused on functional analysis of the *p53* mutations using yeast assay. Functional analysis is based on inability of the *p53* mutants to transactivate target sequence in yeast and specifically detects functionally important mutations thus, avoiding analysis of polymorphisms and silent mutations. Moreover, it overcomes the problem of contamination of tumour tissue with normal tissue making this assay particularly useful for large scale screening. This assay allowed us to detect the *p53* mutations not only in tumour tissue but also in urine sediment. We detected the *p53* mutations in 42% of T2-T4 tumour tissues and in 39% of urine sediments and demonstrated that *p53* mutation analysis based on yeast assay can be a useful tool for the detection of muscle-invasive bladder cancer. In contrast to study of Curigiliano et al. (19) who found *p53* mutation in 41% stage T1 tumour samples by denaturing gradient gel electrophoresis, we found mutations only in 10% stage Ta/T1 tumours, and the *p53* mutation pattern found in urine sediment was not always identical to those found in tumour tissue. It should be, however, noted that we determined only functionally important mutations, what can result in lower frequency of *p53* mutations in our study. The presence of additional mutations in two urine sediments comparing to tumour tissue can be a result of either (i) upper urinary track lesions or (ii) possible polyclonal expansion of tumour. This is not consistent with the concept of clonal origin of bladder cancer (20), however, recently some studies have provided evidence for polyclonal origin of recurrent bladder tumours and the existence of more than one tumour clone especially in early

stage bladder carcinoma (21, 22). One case in which the *p53* mutation could not be identified in the urine sediment had low stage, low grade tumour. Absence of *p53* mutation in this case may reflect (i) a lack of exfoliated cancer cells in the urine (ii) high ratio of normal cell to tumour cells in the specimen or (iii) a presence of heterogeneous clones in the tumour and urine specimens. The 80% (8/10) concordance between the *p53* mutation in tumour tissue sample and urine sediment, comparable to 84% (16/19) in study by Prescott et al. (2001) and higher comparing to 25% (2/8) agreement in study by Dahse et al. (2002) indicates that urine may be as efficient as tumour tissue analysis, however further evaluation is needed (23, 24).

In this study we identified eight *p53* mutations that have not been found in transitional cell carcinoma of the bladder based on the IARC database analysis (Table III). The most noteworthy of these mutations was splice site mutation at intron7/codon 261 that generated new stop codon which can induce nonsense-mediated mRNA decay (25). Premature stop codons were observed in three more cases what suggests that in our group of patients mRNA decay or/and translational repression of nonsense-containing mRNA may occur (26). Thus, providing new evidence of utility of yeast functional assay for *p53* mutations screening over immunohistochemical analysis.

Most of the mutations (20 out of 22) were located in the core domain of *p53* (Table III). We found predominantly missense mutation however, nonsense and splice site mutations were observed as well. Interestingly, in three cases we found very rare multiple mutations. In our group of patients endogenous mutations were very rare, we found only one C to T transition at the cytidine phosphate guanosine site at codon 248 what suggests spontaneous deamination of 5-methylcytosine. The prevalence of exogenous mutations is not surprising as cigarette smoking and occupational exposure to arylamines are thought to account for more than half of all cases of bladder cancer, with smoking being the more important risk factor. It is also suggested that there is a certain pattern of mutation in smoking patients (27, 28). There are also weak hot spots of transversion mutations at codon 241, 249, and 280, which may suggest the action of carcinogenic adducts. Interestingly in our study we found in two cases G>T transversion at codon 157 and 273 suggesting that tobacco carcinogen maybe responsible for these mutations (29).

Using functional assay in yeast we demonstrated that this method allows the identification of multiple mutations in a single gene in a single patient's cDNA sample. Three multiple mutations found in our group of patients were predominantly point mutations. In two cases (BT15 and BT9) second mutation always led to premature translation termination. The presence of nine multiple *p53* mutations in bladder cancer patients was reported by Taylor et al. (1996) who found thirty-two *p53* mutations out of 64 bladder tumours (30). One of these tumours had five mutations at four codons (89, 101, 132, 214). Although this tumour was from a patient exposed to arylamine, the frequency of multiple mutations was high for both exposed and unexposed patients.

In general *p53* mutation is associated with a higher frequency of progression to a more advanced stage and higher rate of death from bladder cancer. The unfavourable prognostic effect of *p53* mutations on bladder cancer patient survival has been shown by many authors. However, data from the literature concerning the prognostic relevance of the *p53* mutations on OS and DFS are contradictory. In our study we found association between the *p53* mutation and stage, high histological grade, and lymph node involvement. Patients with *p53* mutations showed statistically significant shorter survival ($P = 0,033$), but in a multivariate analysis *p53* mutation was not an independent predictor of OS when assessed with tumour grade, stage, lymph node status and age. There was no correlation of DFS with the *p53* status. Low incidence of *p53* mutations in Ta/T1 tumours may suggest that *p53* alterations in superficial bladder cancer are related to more aggressive phenotype and a higher risk of recurrence.

In conclusion our results provide support for the use of yeast-based assay for *p53* mutation screening in tumour tissue and indicate that urine may be as efficient as tumour tissue analysis. However further evaluation should be carried out as the voided urine is not an optimal source of RNA extraction. A key advantage of this type of analysis is detection of functionally important *p53* mutations and high specificity. Furthermore, this study assessed the prognostic value of *p53* mutations in relation to overall survival and disease-free survival in TCC. Though *p53* mutation can be found in urine specimens by yeast functional assay, the use of this type of analysis to aid the non-invasive detection of tumours needs further validation.

Acknowledgments

We gratefully thank T. Soussi for the yeast strain yIG397 and yeast expression vectors. We also thank J. Swierczynski for helpful comments and discussion on the manuscript.

The work was supported by the State Committee for Scientific Research Grant KBN 6 P05A 092 20.

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Table I. Clinicopathological characteristics of 61 bladder cancers.

Variable	No. Pts. (%)
Female	14 (23)
Male	47 (77)
Age	
≥ 65	27 (44)
<65	34 (56)
Grade	
G1	23 (38)
G2	25 (41)
G3	13 (21)
Pathological stage	
(pTa/T1)	30 (49)
(pT2-pT4)	31 (51)
Lymph node status	
Positive	13 (21)
negative	48 (79)

Table II. Characteristics of tumours containing *p53* mutations

No	Case	Age/ Gender	Tumour pathology	Exon	Codon number	Codon	Base change	AA change	Urine sample
1	BT26	68/M	G2pT3	4	94	TCA>TAA	C>A	Ser>Stop	the same
2	BT33	47/M	G3pT3b	4 5 7 ^a	96 163 248	TCT>TTT TAC>TGC CGG>TGG	C>T A>G C>T	Ser>Phe Tyr>Cys Arg>Trp	the same
3	BT18	64/F	G3pT3b	4 ^a	110	CGT>CTT	G>T	Arg>Leu	the same
4	BT55	59/M	G2pT3	5	130	Del C			the same
5	BT4	60/M	G3pT4	5	132	AAG>AAC	G>C	Lys>Asn	the same
6	BT51	57/M	G2pT1	5	135	TGC>TAC	G>A	Cys>Tyr	wt-p53
7	BT35	50/M	G3pT3b	5	146	TGG>TAG	G>A	Trp>Stop	NA
8	BT15	51/M	G3pT3b	5 7 ^b	157 Intron7/ 261	GTC>TTC 3bp ins.	G>T	Val>Phe ins 1 a.a.	NA
9	BT25	62/M	G3pT3b	5	174	Del G			174 del G 181 CGC>TGC Arg>Cys
10	BT12	61/M	G3pT3b	5 ^a	175	CGC>CAC	G>A	Arg>His	NA
11	BT39	62/M	G2pT3	5	180	GAG>AAG	G>A	Glu>Lys	NA
12	BT28	61/M	G2pT1	6	220	TAT>TGT	A>G	Tyr>Cys	the same
13	BT5	51/M	G2pT3	8	262	3bp ins		ins. 1 a.a.	the same
14	BT7	65/F	G3pT4	8	270	TTT>TGT	T>G	Phe>Cys	NA
15	BT34	75/M	G2pT1	8 ^a	273	CGT>CAT	G>A	Arg>His	279 GGG>GAG Gly>Glu
16	BT9	75/F	G2pT3a	8	280 286	AGA>AAA GAA>TAA	G>A G>T	Arg>Lys Gly>Stop	NA

NA- not available; pT: pathological tumour stage; G: histological grade

^a CpG

^b splice

Table III- Comparative analysis of the incidence of *p53* mutants isolated from patients with transitional cell carcinoma of the bladder with the IARC database

<i>p53</i> mutations	Location in the secondary structure^a	Mutant <i>p53</i> (%) occurrences in TCC IARC N=380	Mutant <i>p53</i> (%) occurrences in cancer IARC N=17 689
S94Stop	out	0	1 (0,0056)
S96F	out	1 (0,26)	1 (0,0056)
R110L	S1	0	10 (0,056)
130 del C		0	2 (0,011)
K132N	S2'	4 (1,05)	17 (0,096)
C135Y	S2'	1 (0,26)	55 (0,31)
W146Stop	S3	2 (0,53)	36 (0,20)
V157F	S4	2 (0,26)	122 (0,69)
Y163C	S4	3 (0,79)	91 (0,51)
174 del G	L2	1 (0,26)	7 (0,039)
R175H	L2	13 (3,42)	786 (4,44)
E180K	H1	1 (0,26)	6 (0,034)
R181C	H1	0	20 (0,11)
Y220C	S7-S8	10 (2,63)	217 (1,22)
R248W	L3 in LSH	9 (2,37)	478 (2,69)
intron 7/codon 261		0	5 (0,028)
262 - ins.	S9-S10	0	0
F270C	S10 in LSH	0	19 (0,107)
R273H	S10 in LSH	4 (1,05)	519 (2,93)
G279E		1 (0,26)	28 (0,16)
R280K	H2 in LSH	7 (1,84)	50 (0,28)
E286Stop	H2 in LSH	0	18 (0,102)

^a Structural features from Cho et al. (15), H= Helix; L= loop; S=Beta sheet ; TCC: transitional cell carcinoma; IARC database <http://www.iarc.fr/p53/> Hainaut et al. (16), Oliver at al. (17).

Figure legend

Fig. 1. Cancer specific overall survival for bladder cancer stratified by the *p53* status.
P = 0,033, log-rank test.

