Assessment of the Transcriptional Activity of p53 Improves the Prediction of Recurrence in Superficial Transitional Cell Carcinoma of the Bladder

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Abstract

Purpose: To investigate the value of p53 functional analysis of separated alleles in yeast (FASAY) as a witness of p53/p21 pathway alteration and as a predictor of recurrence in superficial transitional cell carcinomas.

Experimental Design: p53 transcriptional activity was prospectively analyzed in 52 newly diagnosed transitional cell carcinoma using FASAY competent for the transactivation of *p21* and *bax* promoters. *TP53* and *p21* gene expression was quantified by real-time PCR, and expression of corresponding proteins was assessed by immunohistochemistry. In addition to tumor stage and grade, the predictive value of FASAY, real-time PCR, and immunohistochemistry for tumor recurrence was assessed by Cox survival analysis.

Results: A total (*p21* and *bax*) or partial (*bax* only) loss of transcriptional activity was observed in 15 of 52 (29%) and 4 of 52 (7.7%) cases, respectively, a partial loss being consistently associated with R283H mutation. p53 nuclear overexpression grossly overestimated (~40%) or underestimated (~10%) the true incidence of p53 transcriptional abnormalities, especially in T_a-T_1 grade 1 to 2 tumors. Loss of *p21* transactivation significantly correlated with decreased *p21* gene expression and lack of expression of p21 ($P = 0.001$). FASAY had a better predictive value for recurrence than p53 immunohistochemistry (Cox hazard ratio, 6.57 versus 3.95; $P = 0.0002$ versus 0.019, respectively), whereas neither p21 immunohistochemistry (hazard ratio, 1.9; $P = 0.29$) nor *TP53* or *p21* gene expression were significant predictors of recurrence. The prognostic difference between FASAY and p53 immunohistochemistry was maintained in the subgroup of T_a-T_1 grade 3 tumors.

Conclusions: FASAY is a valuable surrogate marker for assessing p53/p21 pathway alteration and predicts transitional cell carcinoma recurrence better than p53 immunohistochemistry.

Transitional cell carcinoma (TCC) of the bladder is the fourth most common malignancy in men and the eight most common cancer in women. Eighty percent of TCCs are initially superficial (TMN97: T_a-T_1) and easily treated by transurethral resection of bladder tumor. Despite initial complete resection of the tumor, recurrence occurs in 30% to 90% of the cases (1, 2). Progression to muscle invasive and/or metastatic stages requires radical and/or systemic therapies in 15% to 20% of the cases. To decrease recurrence and progression rates, intravesical administration of chemotherapy and/or Bacillus Calmette-Guerin is considered as standard therapy (2, 3). Because these therapies are not devoid of local and systemic toxicity, their use should better be guided by individual risks for recurrence and progression (4).

Over the years, many attempts have been made to identify molecular markers that would supersede clinical features, such as tumor stage, grade, and multifocality (5, 6). Expression of *TP53* gene has been one of the most frequently assessed genetic alterations in bladder TCC. In an overwhelming majority of studies, the prognostic significance of *TP53* alterations in bladder TCC is relying on the identification of p53 nuclear overexpression by immunohistochemistry (7–15). Considering that the carcinogenic effect of *TP53* mutations in tumor cells is mainly mediated by inactivation of p53 transcriptional activity, including loss of *p21* expression, routine evaluation of this functional activity should be substituted to immunohistochemistry (16). The number of discordant results reported between immunohistochemistry and DNA analyses shows indeed that p53 nuclear protein accumulation cannot be considered as a reliable surrogate marker of *p53* mutations and still less of its transcriptional activity (10, 13, 17–21). The routine use of a functional assay in yeast to selectively identify inactivating p53

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Received 1/19/05; revised 4/8/05; accepted 4/12/05.

Grant support: Communauté Française de Belgique Action de Recherche Concertée grant 04/09-317, Loterie Nationale de Belgique, and Fonds National de la Recherche Scientifique grant 7.4536.04.

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Table 1. Primers and probes used in the real-time PCR

Target	Primers/probes	Sequence (5′→3′)	Position* (exon)	Size (bp)	Concentration (nmol/L)
TP53	Forward	CGTCTGGGCTTCTTGCATTC	328-347 (4)	106	300
	Reverse	AAGACCTGCCCTGTGCAGC	415-433 (5)		
	Probe	FAM-CTGTGACTTGCACGTACTCCCCTGCC-VIC	362-387		100
p21	Forward	CTGGAGACTCTCAGGGTCGAAA	401-422 (2)	101	300
	Reverse	GAGGAAGCCTAATCCGCC	483-501 (3)		
	Probe	FAM-CGGCAGACCAGCATGACAGATTTCTACCA-VIC	427-455		100
Abl	Forward	AGCCATGGAGTACCTGGAGAAG	1,047-1,068 (6)	70	300
	Reverse	TTCTCCCCTACCAGGCAGTTT	1,101-1,121 (7)		
	Probe	FAM-AAACTTCATCCACAGAGATCTTGCTGCCC-VIC	1,071-1,098		75

**p53* Genbank accession no. AY838896, *p21* Genbank accession no. AF497972, and *Abl* Genbank accession no. U07563.

mutations in bladder tumors seems therefore as a better approach but still needed confirmation of its predictive value on the outcome in superficial TCCs of the bladder (21, 22).

Here, the *TP53* gene status of the tumor was determined by a functional analysis of separated alleles in yeast (FASAY) competent for the transactivation of *p21* and *bax* promoters (22–24). Quantification of *TP53* and *p21* gene expression was carried out by real-time PCR, and quantification of p53 and p21 protein expression was carried out by immunohistochemistry. Data were compared and correlated with clinicopathologic features and clinical outcome.

Materials and Methods

Patients. After informed consent, 52 patients newly diagnosed with a bladder tumor were enrolled in the study. Patients with previous history of urinary infection, prostatic carcinoma, or *in situ* carcinoma of the bladder were not included. All patients underwent a complete transurethral resection of bladder tumor. In each case, a fragment of the tumor was obtained and processed for p53 transcriptional activity (see below). The remaining material tumor was formalin fixed and paraffin embedded for pathologic evaluation. In addition, a biopsy of the normal-looking surrounding bladder mucosa was obtained in 26 of 52 patients and processed for p53 transcriptional activity. These samples were used as paired controls in the quantification of gene expression by real-time PCR.

Anatomopathologic evaluation of the paraffin-embedded tumor specimens was done by a single pathologist (J.P.C.) according to TMN97 staging system for disease extension and WHO/International Society of Urological Pathology consensus for grade (25, 26).

All the patients received a single immediate postoperative instillation of epirubicin according to the European Organization for Research and Treatment of Cancer recommendation (3). All the patients, but 6 patients with muscle invasive disease, were then followed every 3 months by cytoscopy and urinary cytology. Bladder biopsy or transurethral resection of bladder tumor was done in the case of abnormal cystoscopy or class V cytology (27). A recurrence was defined as the diagnosis of at least one new tumor further classified T_a-T_1 of any grade and a progression as a new lesion classified $\geq T_2$.

Immunohistochemistry for p53 and p21 and scoring. Immunohistochemistry techniques were adapted from a previous report (28). Briefly, paraffin-embedded slides were dewaxed with Histosafe (Yvsolab, Beerse, Belgium), rehydrated, treated in citrate solution, and immunostained using the anti-p53 mouse monoclonal antibody DO-7 (Neomarkers, Fremont, CA) at a dilution of 1:300 or the anti-p21 mouse monoclonal antibody NCL-L-WAF-1 (Novocastra, Newcastle, United Kingdom) at a dilution of 1:30 for 16 hours at 4°C. The second antibody used was a ready-for-use anti-mouse EnVision-Peroxidase system (DAKO, Glostrup, Denmark) for p53 and PowerVision (ImmunoLogic, Duiven, the Netherlands) for p21 according to the manufacturer's protocols. A normal goat serum was used as negative control. Regarding p21 staining, a normal tissue biopsy was used as positive control. For p53 staining, cell lines showing a positive (DU145) and negative (LNCaP) p53 staining were used as positive and negative controls, respectively (29). Slides were examined under a light microscope. For p53, samples were stratified into three groups according to the percentage of positive nuclei [<10% (negative staining), ≥10% to <50% (intermediate staining), and ≥50% (positive staining); refs. 13, 21] and to the intensity of p53 nuclear staining stratified as weak or intense in the intermediate group (13). For p21, tumors with ≥10% nuclear staining were defined as positive (14).

Functional analysis of separated alleles in yeast. To detect inactivating mutations in *TP53* gene, a FASAY assay was used as described (30, 31). In brief, the transcriptional activity of human p53 in tumor cells is assessed in *Saccharomyces cerevisine* where it activates a p53 target gene (*Ade2*). This reporter gene is under the control of a promoter that contains the p53-binding site from either ribosomal gene cluster (*RGC*), *p21*, or *bax* genes. Bladder biopsies and tumor samples stored at −80°C until mRNA extraction were thawed and thoroughly homogenized. The mRNA was extracted using a Dynabeads mRNA DIRECT kit (Dynal, Oslo, Norway) according to the manufacturer's recommendations. *TP53* mRNA was reverse transcribed and part of the *TP53* open reading frame comprising exons 4 to 11 was amplified by PCR. The reporter strains YIG-397, YPH-p21, and YPH-bax were grown and cotransformed with unpurified *TP53* amplicons and a linearized yeast expression vector carrying the 5′ and 3′ ends of the *TP53* open reading frame. Activation of the reporter by wild-type p53 results in white colonies, whereas mutant p53 produces pink or red colonies. Knowing that samples containing wild-type p53 can give a background of 5% to

Table 2. Distribution of stage and grade in superficial TCCs of the bladder

Stage	Grade 1	Grade 2	Grade 3	Total
T_a	14	10	13	37
T_1		1	8	9
T_2			4	4
T_3			1	1
T_4			1	1
Total	14	11	27	52

Table 3. Patient characteristics

No.	FASAY	Age	Stage	Grade	Recurrence	Follow-up (mo)
1	Nonfunctional	81	T_a	3	Yes	29
2	Nonfunctional	77	T_a	3	Yes	7
3	Nonfunctional	62	T_1	3	Yes	3
4	Nonfunctional	56	T_1	3	Yes	6
5	Nonfunctional	67	T_2	3	Cystectomy	
6	Nonfunctional	76	T_a	3	No	28
7	Nonfunctional	73	T_a	3	Yes	7
8	Nonfunctional	88	T_a	3	Yes	16
9	Nonfunctional	82	T_2	3	Cystectomy	
10	Nonfunctional	77	T_a	3	Yes	13
11	Nonfunctional	67	T_a	3	No	11
12	Nonfunctional	66	T_3	3	Cystectomy	
13	Nonfunctional	81	T_1	3	No	11
14	Nonfunctional	69	T_1	2	No	6
15	Nonfunctional	72	T_1	3	No	6
16	Nonfunctional/partially functional	63	T_4	3	Cystectomy	
17	Partially functional	73	T_a	2	No	19
18	Partially functional	68	T_1	3	No	18
19	Partially functional	76	T_2	3	Cystectomy	
20	Functional	58	T_a	1	No	33
21	Functional	75	T_a	3	Yes	5
22	Functional	81	T_a	2	No	33
23	Functional	67	T_a	1	No	33
24	Functional	77	T_1	3	Yes	5
25	Functional	68	T_a	1	No	32
26	Functional	78	T_a	2	Yes	11
27	Functional	68	T_a	1	No	32
28	Functional	86	T_a	2	Yes	15
29	Functional	81	T_a	1	No	32
30	Functional	67	T_a	1	No	31
31	Functional	73	T_a	1	No	31
32	Functional	75	T_1	3	No	31
33	Functional	73	T_a	3	No	30
34	Functional	74	T_a	3	No	29
35	Functional	79	T_a	2	No	29
36	Functional	55	T_a	2	No	29
37	Functional	76	T_a	3	No	28
38	Functional	64	T_a	1	No	28
39	Functional	71	T_a	1	No	28
40	Functional	78	T_a	1	No	28
41	Functional	77	T_a	2	Yes	19
42	Functional	78	T_2	3	Cystectomy	
43	Functional	70	T_a	2	No	11
44	Functional	75	T_a	1	No	11
45	Functional	76	T_a	2	No	11
46	Functional	43	T_a	1	No	6
47	Functional	89	T_a	1	No	6
48	Functional	81	T_a	1	No	6
49	Functional	52	T_a	2	No	6
50	Functional	77	T_a	3	No	6
51	Functional	82	T_1	3	No	6
52	Functional	79	T_a	3	No	6

Abbreviation: ND, not determined.

*Percent of gene expression in tumoral tissue compared with normal tissue. Each value is the mean ± SD calculated on three independent experiments.

Table 3. Patient characteristics (Cont'd)

p53 immunohistochemistry (%)	p21 immunohistochemistry (%)	Real-time PCR	
		TP53*	p21*
≥50	<10	1,147 ± 152	960 ± 151
10-50 (strong intensity)	≥10	ND	ND
≥50	<10	471 ± 93	53 ± 2
≥50	<10	173 ± 8	77 ± 4
ND	ND	78 ± 7	37 ± 2
≥50	<10	12 ± 3	27 ± 1
≥50	≥10	356 ± 77	2,100 ± 255
≥50	≥10	ND	ND
ND	ND	ND	ND
≥50	ND	ND	ND
<10	ND	ND	ND
≥50	<10	ND	ND
≥50	ND	ND	ND
≥50	ND	ND	ND
>50	ND	ND	ND
<10	≥10	ND	ND
10-50 (strong intensity)	ND	ND	ND
10-50 (strong intensity)	ND	ND	ND
≥50	<10	ND	ND
<10	ND	ND	ND
<10	ND	ND	ND
10-50 (weak intensity)	ND	ND	ND
<10	≥10	23 ± 2	250 ± 13
10-50 (strong intensity)	≥10	120 ± 16	240 ± 34
<10	≥10	ND	ND
10-50 (weak intensity)	≥10	15,979 ± 783	4,900 ± 359
10-50 (weak intensity)	≥10	4,064 ± 459	1,060 ± 192
<10	≥10	25 ± 8	33 ± 3
<10	≥10	ND	ND
10-50 (weak intensity)	≥10	1,645 ± 156	4,570 ± 441
10-50 (strong intensity)	≥10	2,075 ± 81	445 ± 17
10-50 (weak intensity)	<10	340 ± 15	3,000 ± 221
<10	≥10	270 ± 29	260 ± 11
<10	≥10	335 ± 38	1,760 ± 146
<10	≥10	140 ± 22	1,030 ± 118
<10	≥10	611 ± 71	440 ± 53
10-50 (weak intensity)	≥10	99 ± 10	83 ± 11
≥50	≥10	116 ± 17	9 ± 1
ND	ND	50 ± 2	170 ± 16
10-50 (weak intensity)	≥10	25,689 ± 2,641	1,500 ± 195
<10	≥10	ND	ND
<10	≥10	6,334 ± 279	3,000 ± 191
10-50 (weak intensity)	≥10	ND	ND
<10	≥10	ND	ND
ND	ND	ND	ND
10-50 (strong intensity)	≥10	ND	ND
<10	≥10	ND	ND
<10	≥10	ND	ND
<10	≥10	ND	ND
<10	≥10	ND	ND
≥50	≥10	ND	ND
10-50 (strong intensity)	≥10	ND	ND

10% red colonies due to PCR-induced errors and to the presence of an alternatively spliced *TP53* mRNA, *TP53* was considered as wild-type when <10% of red or pink colonies were detected (negative FASAY). Above this cutoff value, the FASAY was considered positive. The activity of the p53 mutant was determined by the color of at least 100 colonies per strain. A tissue sample carrying a well-characterized mutation and a blood sample from a Li-Fraumeni patient were both used as a positive control (32).

Sequence analysis. To characterize the inactivating mutations at the molecular level, the p53 plasmids were then recovered from red or pink yeast. Sequence analysis was done on an automated ABI 377 A apparatus (Applied Biosystems, Foster City, CA) using the Taq Dye Deoxy Terminator Cycle Sequencing kit from the same manufacturer and according to its instructions. The mutation was identified on both strands of plasmids from individual colonies. For each patient sample, 15 colonies (i.e., 5 per yeast promoter) were sequenced.

Quantification by real-time PCR Taqman assay. Gene-specific PCR primers and Taqman probes for human *TP53*, *p21*, and housekeeping gene Abelson (*Abl*) were designed using Primer Express Software version 1.5 (Applied Biosystems; Table 1) and purchased from Eurogentec (Ougrée, Belgium). Each probe was labeled with a fluorescent 5′ reporter dye [6-carboxy-fluorescein (FAM)] and a 3′ quencher [6-carboxy-tetramethyl-rhodamine (TAMRA)]. mRNA was extracted from frozen tissue samples using a Dynabeads kit according to the manufacturer's protocol and reverse transcribed by using random hexamers (Eurogentec) and SuperScript RNase H^- reverse transcriptase (Invitrogen, Merelbeke, Belgium). Amplification was done using 2.5 μL cDNA, 12.5 μL Universal PCR Master Mix 2× (Applied Biosystems) in a total reaction volume of 25 μL. The *Abl* housekeeping gene and the genes of interest were amplified in parallel. The reaction was initiated at 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute.

Data were recorded as cycle threshold (Ct) on a Taqman 7700 Sequence Detection System (Applied Biosystems) using analytic software from the same manufacturer. Each cDNA sample was amplified in triplicate and Ct values were averaged. The average Ct value for *Abl* was subtracted from the average Ct value for a gene of interest. This ΔCt value obtained in pathologic tissues was then subtracted from a ΔCt value obtained in normal tissues, giving a ΔΔCt value. As amplification efficiencies of the genes of interest and *Abl* were comparable, the amount of mRNA of each gene, normalized to *Abl*, was given by the relation $2^{-\Delta\Delta Ct}$. Positive (DU145) and negative (LNCaP) controls were those used for immunohistochemistry.

Statistical methods. The correlations among p53 FASAY, p53 immunohistochemistry, *TP53* expression, p21 immunohistochemistry, *p21* expression, and tumor stage and grade were tested by cross-tabulating data and applying the Spearman correlation test. Survival curves were built using a Kaplan-Meier test. Predictors of survival were identified using the Cox proportional hazard regression method. Statistical significance was set at $P < 0.05$. All analyses were done with the SPSS Statistical Package version 12.0 for Windows (SPSS, Inc., Chicago, IL).

Results

Patient characteristics. There were 37 males and 16 females ages 43 to 89 years (median, 73 years). Diagnosis of TCC was confirmed in all patients but one with adenocarcinoma of the urachus. This patient was subsequently withdrawn from further evaluation. Tumor stages and grades are summarized in Table 2. The six patients diagnosed with muscle-invading tumor were scheduled for radical cystectomy.

Correlation between functional analysis of separated alleles in yeast and tumor stage and grade. A total or partial transcriptional inactivation of p53 (i.e., FASAY-positive) was found in 19 of 52 (36.5%) tumors, including 6 of 15 (40%) females and 13 of 37 (35%) males (Table 3). All the paired normal samples were FASAY negative. Fifteen p53-inactive mutants failing to transactivate the three yeast promoters and producing only red colonies were defined as nonfunctional. Four mutants presenting a transcriptional activity that was partial on *RGC* promoter (pink colonies), normal on *p21* promoter (white colonies), and abolished on *bax* promoter (red colonies) were defined as partially functional (Tables 3 and 4). Sequence analysis of 15 yeast colonies obtained with a particular tumor consistently showed the same mutation (Table 4). A missense mutation was found in 17 (89%) mutants, all but one (G105D; patient 5) being already reported in the p53 database (IARC p53 mutation database version R9, December 2004: http://www-p53.iarc.fr/index.html; ref. 33). A compound heterozygous mutation was found in one of the two remaining cases (patient 16) and a 3-bp insertion in the other (patient 12). The R283H mutation was identified in all four mutants exhibiting partial binding activity (i.e., *p21* transcribed but not *bax*). Considering the polymorphism at codon 72, a proline was found in 3 of 19 *TP53* mutants (Table 4: patients 4, 6, and 15).

There was a strong correlation among FASAY, stage (Spearman $R = 0.514$; $P = 0.000$), and grade (Spearman $R = 0.561$; $P = 0.000$) of the tumors. The distribution of p53 FASAY mutation frequency by stage/grade is detailed in Table 5. Noteworthy, only 2 of 25 (8%) of T_a-T_1 grade 1 to 2 carried a nonfunctional p53 versus 12 of 21 (57%) of the T_a-T_1 grade 3.

Correlation between tumor stage and grade and p53/p21 immunohistochemistry. Immunostaining of p53 and p21 was done in 48 of 52 (92%) of the samples (Table 3). A positive p53 nuclear staining was found in 29 of 48 (60%) of the tumors. Staining distribution and intensity are detailed in Table 5. There was a strong correlation among p53 immunohistochemistry, stage (Spearman $R = 0.418$; $P = 0.003$), and grade (Spearman $R = 0.386$; $P = 0.007$) of the tumors. The rate of positive staining in T_a-T_1 grade 1 to 2 tumors was 11 of 23 (47.8%).

Immunostaining of p21 was positive in 35 of 48 (72.9%) of the tumors. Repartition of p21 staining by stage/grade is detailed in Table 5. There was a strong correlation among p21 immunohistochemistry, stage (Spearman $R = 0.555$; $P = 0.000$), and grade (Spearman $R = 0.480$; $P = 0.001$) of the tumors. In T_a-T_1 grade 1 to 2 tumors, the rate of positive staining was 22 of 23 (95.7%).

Correlation between functional analysis of separated alleles in yeast and p53/p21 immunohistochemistry. Fourteen of 31 (45%) samples with a functional protein showed a p53 nuclear immunohistochemistry staining, whereas 1 of 13 (7.7%) with a totally nonfunctional p53 was p53 immunohistochemistry negative. One partially functional p53 mutant (1 of 4, 25%) was also p53 immunohistochemistry negative. Among both falsely immunohistochemistry-negative p53 mutants, one (patient 16) carried a compound heterozygous mutation in exon 5 (P153T) and exon 8 (R283H) and the other one (patient 6) a mutation in exon 5 (L130F). In spite of these discrepant results, there was a good correlation between FASAY and p53 immunohistochemistry (Spearman $R = 0.692$; $P = 0.000$; Table 6).

Thirty of 31 (96.8%) samples with a functional p53 were p21 immunohistochemistry positive, whereas 10 of 13 (76.9%) samples with a nonfunctional p53 were p21 immunohistochemistry negative. Among the four partially functional p53

Table 4. Activities in yeast of bladder tumor p53 mutations (p53 FASAY) and p53 mutant identification by sequence analysis

Patient no.	p53	Pink and/or red yeast colonies (%)*			Exon	Codon	Mutation
		YIG-397 (*RGC*)	YPH-p21	YPH-bax			
1	Nonfunctional	73	71	77	8	317	TAG (stop)
2		63	65	68	5	175	CAC (H)
3		100	100	100	4	110	CTT (L)
4		60	62	66	8	273	CTT (L)†
5		96	96	97	4	105	GAC (D)
6		81	79	80	5	130	TTC (F)†
7		60	61	64	7	234	TGC (C)
8		100	100	100	7	236	TGC (C)
9		85	80	90	8	271	AAG (K)
10		55	54	52	7	245	GTC (V)
11		86	87	82	5	127	TTC (F)
12		56	56	53	7-8	261-262	3-bp insertion
13		76	74	77	5	175	CAC (H)
14		97.5	96	97	4	111	CCG (P)
15		45	46	44	8	285	AAG (K)†
16‡		11	12	13	5	153	ACC (T)
16‡	Partially functional	**14**	—	12	8	283	CAC (H)
17		**23**	5	28	8	283	CAC (H)
18		**25**	5	30	8	283	CAC (H)
19		**43**	9	37	8	283	CAC (H)

*All the colonies were red, except those labeled in bold that appeared pink and corresponded to a partially functional p53 mutant.
†Polymorphism 72P found at codon 72 (all the other samples at the same codon being 72R).
‡Mixture of red and pink colonies obtained from the same tumor sample; this sample was characterized by the presence of a compound heterozygous mutation showing a partially (codon 283) and a totally nonfunctional (codon 153) p53 mutants. In this tumor sample, the red colonies obtained with YPH-p21 contained only the mutant at codon 153.

mutants, two were p21 immunohistochemistry positive and two were immunohistochemistry negative. There was an excellent correlation between status of p53 determined by FASAY and p21 immunohistochemistry (Spearman $R = 0.738$; $P = 0.000$).

Correlation between functional analysis of separated alleles in yeast and quantitative PCR. *TP53* and *p21* quantitative gene expression was carried out in 23 paired tumor and normal tissue samples. For *TP53*, no correlation was found between FASAY and *TP53* quantitative gene expression (Spearman $R =$

Table 5. Correlation between tumor and grade and p53 FASAY/p53 immunohistochemistry/p21 immunohistochemistry

Tumor		FASAY			p53 immunohistochemistry				p21 immunohistochemistry	
Stage	Grade	Functional	Partially functional	Nonfunctional	<10%	≥10% - <50% (weak intensity)	≥10% - <50% (strong intensity)	≥50%	<10%	≥10%
T_a	1	14			7	3	2	1		13
	2	9	1		5	3	1			9
	3	6		7	5	1	2	5	4	9
	Total	29	1	7	17	7	5	6	4	31
T_1	1									
	2			1				1	1	
	3	3	1	4		1	2	5	6	2
	Total	3	1	5		1	2	6	7	2
>T_1	1									
	2									
	3	1	2	3	2			2	2	2
	Total	1	2	3	2			2	2	2

Table 6. Correlation between p53 FASAY and p53 immunohistochemistry/p21 immunohistochemistry

p53 FASAY	*n*	p53 immunohistochemistry				p21 immunohistochemistry	
		<10%	≥10% - <50%		≥50%	<10%	≥10%
			(weak intensity)	(strong intensity)			
Negative (functional)	31	17	8	4	2	1	30
Positive (partially functional)	4	1		2	1	2	2
Positive (nonfunctional)	13	1		1	11	10	3

0.108; $P = 0.659$). Compared with control samples, *TP53* gene expression in tumor samples was increased by a factor of 52.2 ± 82.4 (mean ± SD) in 13 of 17 (76.5%) patients with a functional p53, whereas it was also increased by a factor of 5.4 ± 4.3 (mean ± SD) in 4 of 6 (66.7%) patients with a nonfunctional p53. In the remaining patients with functional and nonfunctional p53, the *TP53* gene expression in the tumor samples was lower than in the paired control samples (Table 3).

A significant correlation was found between FASAY and *p21* gene expression (Spearman $R = 0.468$; $P = 0.024$): in comparison with paired control tissues, *p21* gene expression was decreased by a factor of 2.4 ± 1.04 (mean ± SD) in 4 of 6 (66.7%) patients with a nonfunctional p53 and increased by a factor 16.4 ± 16.1 (mean ± SD) in 14 of 17 (82.4%) patients with a functional p53.

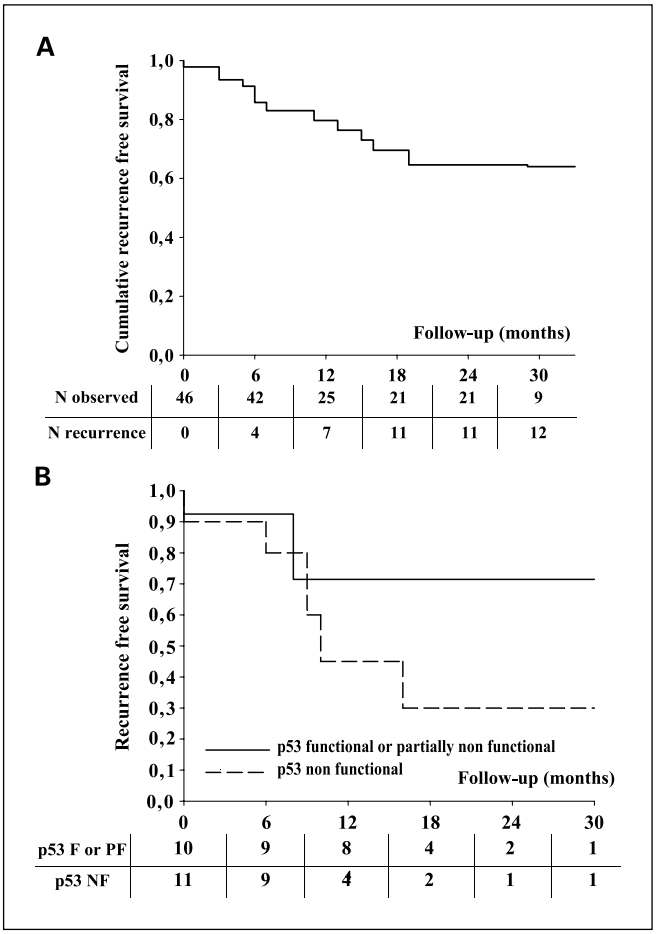

Fig. 1. *A*, Kaplan-Meier survival analysis for recurrence. Number of patients still in observation. *B*, Kaplan-Meier survival analysis for recurrence in the group of patients with T_a-T_1 grade 3 tumors. Solid line, tumors with a functional (*F*) or partially functional (*PF*) p53; dashed line, tumors with a nonfunctional (*NF*) p53. Number of patients still in observation.

Prediction of tumor recurrence. Median follow-up was 27 months (lower and upper 95% confidence intervals, 24 and 30 months, respectively). Twelve recurrences were diagnosed and no progression (Table 3). Recurrence-free survival rates of 1 and 2 years are 83% and 69%, respectively (Fig. 1A).

Cox regression analysis models were built to assess the predictive value of the following variables on tumor recurrence: grade 3, stage T_a versus T_1, p21 immunohistochemistry (≥10%), p53 immunohistochemistry (≥10% or ≥50% positive), and p53 FASAY (totally functional or totally nonfunctional). The results are detailed in Table 7. The presence of a nonfunctional p53 was the best predictor of recurrence in univariate analysis followed by grade 3. On the other hand, p21 immunohistochemistry was not a predictor of tumor recurrence. In the subgroup of 21 patients with a T_a-T_1 grade 3 tumor, there was still a quasi-significant correlation ($P = 0.094$) between the rate of recurrence and a positive FASAY (Cox hazard ratio, 3.95; 95% confidence interval, 0.8-19.7). Recurrence was indeed observed in 7 of 11 (63%) of the patients with a nonfunctional p53 and in 2 of 10 (20%) of the patients with a normal or partially functional p53 (Fig. 1B). In contrast, p53 immunohistochemistry, with or without enforced cutoff values, did not allow such discrimination in the same subgroup of patients.

Discussion

Despite significant advances in the understanding of tumor biology, evaluation of the risk of recurrence of bladder TCC still relies on the interpretation of clinical features, including tumor's stage and grade, multifocality, and presence of carcinoma *in situ* (34). Consequently, there is a major interest in identifying molecular features indicating a higher risk of recurrence or progression (6, 35, 36). In this respect, p53 has been one of the most widely studied predictor of outcome (9, 10, 15). However, unlike the many observations with other tumors, the prognostic significance of nuclear expression of p53 in bladder TCC remains controversial (7, 8, 12, 14, 37–39). To some extent, this uncertainty may result from known limitations of immunohistochemistry. Although immunohistochemistry is ubiquitously used to detect p53 mutations (7–15), the technique carries several known interferences, including nonsense mutations and activation of wild-type p53 by xenobiotics or activated oncogenes as well as from methodologic biases resulting from variations in sample preparation, technique of fixation, anti-p53 antibody specificity, or variable positive cutoff (7, 8, 10, 13, 17–21). To improve

Table 7. Cox regression analysis for recurrence: univariate models

Predicting factors	Odds ratios	95% Confidence interval (lower-upper)	*P*
p53 nonfunctional ($n = 15$) vs partially functional/functional ($n = 37$)	6.57	2.01-21.44	0.0002
Grade 3 ($n = 27$) vs 1/2 ($n = 25$)	5.07	1.36-18.89	0.015
p53 nonfunctional or partially functional ($n = 19$) vs functional ($n = 33$)	4.63	1.42-15.09	0.011
Immunohistochemistry p53 staining ≥50% ($n = 14$ of 48)	3.95	1.24-12.55	0.019
Stage T_1 ($n = 9$) vs T_a ($n = 37$)	2.68	0.70-10.20	0.147
Immunohistochemistry p53 staining ≥10% ($n = 29$ of 48)	2.18	0.58-8.12	0.243
Immunohistochemistry p21 staining ≥10% ($n = 35$ of 48)	1.90	0.53-6.36	0.296

immunohistochemistry results, several methods have been developed, such as quantitative fluorescence image analysis (40). Despite being not yet routinely carried out in clinical laboratory, they should be considered in forthcoming comparisons with the p53 functional assay.

The critical biochemical function of p53 in carcinogenesis and tumor progression relies on alterations of the transcription of target genes controlling cell growth and apoptosis (16). Therefore, the use of a functional assay in yeast to selectively identify inactivating p53 mutations in bladder tumors, first reported by Pfister et al. in 1999, seems to be a more valuable alternative to immunohistochemistry (16, 21, 22). In this study, the transcriptional activity of p53 was compared with p53 and p21 at the protein and gene expression levels. There was a significant correlation among p53 mutations, lack of *p21* transcriptional activity, and lack of p21 staining, but our data did not confirm the prognostic value of p21 staining for bladder cancer progression as reported for more invasive TCCs (15, 39). There were significant correlations between p53 immunohistochemistry and tumor stage and grade as well as between p53 immunohistochemistry and p53 transcriptional abnormalities. However, there was no correlation between p53 mutations and *TP53* gene expression.

The proportion of p53 alterations detected by FASAY in T_a-T_1 tumors in the present series was similar to the proportion reported by Pfister et al., 36.5% (19 of 52) versus 34% (16 of 47), respectively (22), as was the percentage of positive FASAY in invasive TCCs [83% (5 of 6) versus 100% (4 of 4), respectively], grade 2 tumors (17% versus 18%, respectively), and grade 3 tumors (79% versus 61%, respectively). However, we also compared FASAY and p53 immunohistochemistry. The rate of p53-positive immunohistochemistry without detectable mutation and p53-negative immunohistochemistry in tumors with p53 mutation confirmed the superiority of FASAY for detecting the p53 alterations (10, 13, 15). It is of note that discrepant results were mostly found in the group of T_a-T_1 grade 1 to 2 tumors, p53 mutation being suspected based on a positive p53 immunohistochemistry in 13 patients but confirmed by FASAY in only 2 patients, including 1 with a partially functional p53. Discrepancy between immunohistochemistry and FASAY was striking in a specimen carrying a single premature stop codon in exon 8 but intense p53 staining (20, 21). Nevertheless, we confirmed a strong p53 staining in 4 of the 6 (66.7%) assessable tumors with a mutation in exon 8 (13).

In the current study, the mutational spectrum of p53, assessed on the nearly entire *TP53* open reading frame, confirmed that the mutation rate is age independent (13). All the mutations, except the G105D, were already listed in the p53 mutation database (IARC p53 mutation database version R9, December 2004: http://www-p53.iarc.fr/index.html; ref. 33). Interestingly, four partially functional mutants were characterized by the same R283H mutation, giving a partial transcription of the consensus *RGC* promoter (pink colonies), a normal transcription of *p21* promoter (white colonies), and a loss of transcription of the *bax* promoter (red colonies). Accordingly, the prevalence of R283H in superficial bladder TCCs and its prognostic value deserves further evaluation. In addition, assessment of the polymorphic codon 72 showed a proline (72P) and arginine (72R) alleles in 3 and 16 samples, respectively. The weak proportion of 72P mutants in our series of patients did not support a significant contribution of polymorphism 72P to recurrence, in opposition to an earlier report (41).

However, the most interesting and original result presented here is the added predictive value of FASAY test for tumor recurrence in a homogenous population of patients with superficial bladder TCCs. Compared with other classic predictors of recurrence, a positive FASAY test seemed indeed to be the strongest predictor of recurrence and superseded tumor grading and stage as well as p53 immunohistochemistry staining (for cutoff 50%). As already mentioned, p21 immunohistochemistry and p53 immunohistochemistry (for cutoff 10%; ref. 8) or *p21* and *TP53* gene expression were not significant prognostic factors for recurrence. Whereas grade 3 was a strong predictor of relapse, a positive FASAY maintained also its ability to better identify patients at risk of recurrence in the T_a-T_1 grade 3 subgroup. However, unlike Lopez-Beltran et al. (7), we did not confirm the value of p53 immunohistochemistry, with or without enforced cutoff values, as a predictor of recurrence in the same subgroup of patients. Considering the unpredictable and highly variable natural history of T_a-T_1 grade 3 tumors, these results highlight the interest of FASAY test as an adjunct in this subset of patients.

Conclusions

FASAY seems as an optimal and robust screening assay that is readily done, requires only tiny amounts of tumor cells, and allows a sensitive and specific detection of p53 transcriptionally inactive mutants while ruling out biologically silent polymorphisms. The assay is rapid and reproducible. Mutations altering totally or partially the p53 transcriptional activity are easily recognized as illustrated with the recurrent R283H mutation. FASAY results are also significantly correlated to tumor stage and grade and p53/p21 immunohistochemistry. Altogether, these features compare favorably with *TP53*

sequence analysis and immunostaining, making FASAY an attractive surrogate marker for assessing p53/p21 pathway alterations. Finally, the FASAY significant predictive value for recurrence in T_a-T_1 tumor, including the group of grade 3 tumors, is an essential observation. Indeed, adequate predictions of recurrence after transurethral resection in noninvasive bladder TCCs are still lacking and would have practical therapeutic implications.

Acknowledgments

We thank Ph. Camby (Department of Pathology) and H. Delhez and M. Heusterspreute (Laboratory of Applied Molecular Technologies) for expert assistance, Prof. J. Lebacq (Department of Physiology) for careful reading of the article, Prof. P.J. Van Cangh (Chairman of the Division of Urology) for support during the study, and Th. Frebourg (University Hospital, Rouen, France) and R. Iggo (Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland) for providing the yeast.

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Clin Cancer Res 2005;11:4724-4732.