[CANCER RESEARCH 63, 8108–8112, December 1, 2003]

Advances in Brief

FGFR3 and *TP53* Gene Mutations Define Two Distinct Pathways in Urothelial Cell Carcinoma of the Bladder

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Abstract

***FGFR3* and *TP53* mutations are frequent in superficial papillary and invasive disease, respectively. We used denaturing high-performance liquid chromatography and sequencing to screen for *FGFR3* and *TP53* mutations in 81 newly diagnosed urothelial cell carcinomas. Tumors were classified as follows: 31 pTa, 1 carcinoma *in situ*, 30 pT1, and 19 pT2-T4. Tumor grades were as follows: 10 G1, 29 G2, and 42 G3. *FGFR3* mutations were associated with low-stage ($P < 0.0001$), low-grade ($P < 0.008$) tumors, whereas *TP53* mutations were associated with high-stage ($P < 0.003$), high-grade ($P < 0.02$) tumors. Mutations in these two genes were almost mutually exclusive. Our results suggest that *FGFR3* and *TP53* mutations define separate pathways at initial diagnosis of urothelial cell carcinoma.**

Introduction

UCC[4] of the bladder is a spectrum disease characterized by unpredictability in its biological behavior and response to treatment. UCCs are classified into two broad types: superficial and invasive tumors. Most UCCs present as superficial bladder cancer, confined in 75% of cases to the epithelium (CIS, pTa) or lamina propria (pT1). The remaining (25%) tumors present as muscle invasive disease (≥pT2), with no history of superficial disease (1). However, superficial bladder cancer covers many lesions with different biological potentials, and morphologically similar tumors of the same stage may display completely different patterns of behavior (1). It has been suggested that different genetic changes account for these different forms of UCC and for the observed differences in biological behavior (2). Losses of chromosome 9 have been described as the most common finding in superficial papillary tumors, but such losses are in fact observed at all stages and grades. Alterations of chromosome 9q occur very early in the disease and seem to be involved in the development of bladder cancer. Regions of interest (at 9p21, 9q12–31, 9q32–33, and 9q34) harboring candidate tumor suppressor genes have been identified on both arms of chromosome 9 (reviewed in Ref. 2). However, the prognostic significance of these findings remains unclear. *TP53* mutations are frequent in bladder tumors of high stage and grade, and have been associated with invasiveness (3). It has also been shown that loss of heterozygosity on chromosome *17p*, to which *TP53* maps, is more frequent in high-grade than in low-grade tumors. The p53 protein functions as a transcription factor, regulating the expression of several downstream genes. Two major functions of p53 are cell cycle arrest and apoptosis (reviewed in Ref. 4).

Spruck *et al.* (5) suggested that UCCs progress via two molecular pathways, with *TP53* mutations and loss of heterozygosity on chromosome 17 more frequent in CIS and invasive tumors. In contrast, selective deletions of chromosome 9 are more common in superficial papillary tumors. It was suggested that CIS is the most likely precursor of invasive tumors (5). However, the role and the position of *FGFR3* mutations in current models of bladder carcinogenesis still need to be clarified. *FGFR3* receptors belong to a family of highly conserved, structurally related genes, which is classified into four subtypes (FGFR1, 2, 3, and 4) that bind fibroblast growth factors with different affinity (6). These tyrosine kinase receptors regulate several cellular processes including cell growth, differentiation, migration, wound healing, and angiogenesis, and this depends on the target cell type and the developmental stage (7, 8). *FGFR3* is located at *4P16.3* and comprises 19 exons spanning 16.5 Kb (9). *FGFR3* activating mutations identified in thanatophoric dysphasia were also found in cervix and bladder cancer (10). Currently, it is believed that these mutations result in constitutive activation of the receptor (11). An oncogenic role has been attributed to these mutations in bladder neoplasms, whereas they have an inhibitory role in skeletal diseases (10, 12). The coding sequence of *FGFR3* spanning exons 2–19 was investigated previously, and somatic mutations in bladder tumors were localized in exons 7, 10, and 15 (10, 13). *FGFR3* somatic mutations were shown to be the most frequent gene mutation in low-stage bladder tumors, which underlie their importance in bladder cancer (10, 13) The high frequency of *FGFR3* and *TP53* mutations in superficial papillary and invasive bladder cancers, respectively, led us to compare these two genetic alteration in 81 bladder cancer tumors of all stages and grades at initial diagnosis, to determine whether they correspond to different disease pathways and could be used for molecular classification of these tumors. In this study, we screened for *FGFR3* (exons 7, 10, and 15) and *TP53* (exons 2–11) mutations by DHPLC analysis and sequencing in consecutive primary tumors. We found that mutations in *FGFR3* and *TP53* were almost mutually exclusive, and defined several groups of tumors consistent with the TNM classification in terms of tumor aggressiveness: (*a*) superficial papillary UCCs with *FGFR3* mutations and no *TP53* mutations; (*b*) superficial papillary UCCs with no mutation in either gene; (*c*) superficial papillary UCCs with *TP53* mutations but no *FGFR3* mutations; and (*d*) invasive UCCs with and without *TP53* mutations. This observation provides a framework for the molecular classification of bladder cancer.

Received 7/18/03; revised 9/18/03; accepted 10/16/03.

Grant support: This research was funded by: INSERM, Université Paris 12-BQR, Association de la recherche contre le Cancer (ARC), Délégation à la recherche Clinique, Assistance Publique Hôpitaux de Paris (AP-HP; PHRC AOA 94015), L'association Claude Bernard, le ministère du Travail, UMR 144-Laboratoire Associé, Ligue Nationale Contre Le Cancer, Comité de Paris.

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[4] The abbreviations used are: UCC, urothelial cell carcinoma; CIS, carcinoma *in situ; FGFR3*, fibroblast growth factor receptor 3; DHPLC, denaturing high-performance liquid chromatography; TNM, Tumor-Node-Metastasis; mut, mutated; wt, wild type.

Table 1 *Primer sequences, fragment sizes, gradient ranges, and mobile phase temperatures used in this study*

Exon		Primer sequence 5′ → 3′	Fragment size	%B[a]	Time (min)	Predicted[b] temperature °C	DHPLC temperature °C
TP53							
2+3	F[c]	GATCCCCACTTTTCCTCTTG	287 bp	53–68	4.30	62	60 and
	R[d]	GTC CCA GCCC AACCCTTGT		49–64	4.30		62.5
4	F	CTGGTCCTCTGACTGCTCTT	358 bp	46–64	5.30	62	62 and
	R	AGGCATTGAAGTCTCATGGA		46–64	5.30		64
5	F	TGTTTGTTTCTTTGCTGCCGTGT	310 bp	49–67	5.30	58 and	60 and
	R	CAACCAGCCCTGTCGTCTCT		46–64	5.30	63	65 and
				44–62	5.30		67
6	F	TCCGCGCCATGGCCATCTAC	331 bp	50–68	5.30	62	60 and
	R	AACCACCCTTAACCCCTCCT		47–65	5.30		62
7	F	AACCACCCTTAACCCCTCCT	214 bp	47–65	5.30	62	62
	R	GCGCACTGGCCTCATCTTG					
8	F	CTGCCTCTTGCTTCTCTTTT	255 bp	51–66	4.30	61	58 and
	R	GAGGCAAGGAAAGGTGATAA		48–63	4.30		61 and
				45–60	4.30		63.5
9	F	GGAGGAGACCAAGGGTGCAGTT	235 bp	50–65	4.30	59	57.5
	R	ATGCCCCAATTGCAGGTAAAACA					
10	F	TTACTTCTCCCCCTCCTCTGTTG	193 bp	47–62	4.30	62	62.5
	R	GCTTTCCAACCTAGGAAGGCAG					
11	F	TCA TCT CTC CTC CCT GCT TCT GTC	229 bp	52–67	4.30	60	59.5 and
	R	TGC TTC TGA CGC ACA CCT ATT G					60.5
FGFR3							
7	F	AGT GGC GGT GGT GGT GAG GGA G	116 bp	43–58	4.30	65	67.5
	R	CAG CAC CGC CGT CTG GTT GG					
10	F	CAA CGC CCA TGT CTT TGC AG	165 bp	46–61	4.30	63	65
	R	GAG CCC AGG CCT TTC TTG G					
15	F	AGG ACA ACG TGA TGA AGA TCG	154 bp	46–61	4.30	63	65
	R	GTG TGG GAA GGC GGT GTT G					

[a] DHPLC gradient (eluent B).
[b] According to the DNA melt program.[5]
[c] F, forward.
[d] R, reverse.

Materials and Methods

Characteristics of the Patients and Tissue Samples. All 81 patients of the cohort studied here had newly diagnosed bladder tumors and were admitted for transurethral resection or radical cystectomy. None of the patients had received treatment before the analysis. Median age at time of diagnosis was 64 years (range, 38–86).

We collected matched tumors and blood samples after the patients had given written informed consent. Tumors were graded according to the WHO classification (14) and staged according to the 1997 TNM classification guidelines (15) as follows: 31 pTa, 1 CIS, 30 pT1, and 19 pT2 to pT4 (muscle invasive lesions). Ten of the tumors were classified as G1, 29 as G2, and 42 as G3.

DNA Extraction. Tumors were snap-frozen in liquid nitrogen and stored at −80°C. Venous blood, used as a source of reference DNA, was collected in EDTA-containing tubes and stored at −20°C until used for DNA extraction. DNA and RNA were extracted simultaneously with the cesium chloride cushion method, as described previously (16). DNA was extracted from blood with the QIAamp system (Qiagen S.A. France).

PCR. For *TP53*, we screened for mutations in exons 2–11 by DHPLC. PCR was performed in a final volume of 50 μl containing 100 ng genomic DNA, 1 × amplification buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.15 μM forward primer, 0.15 μM reverse primer, and 2.5 units of TaqDNA polymerase (HotStarTaq; Qiagen S.A. France).

PCR was carried out as follows: an initial denaturation step (95°C for 15 min) was followed by 40 cycles consisting of denaturation at 94°C for 30 s, primer annealing at 58°C (exons 2 to 11) or 59°C (exon 8) for 30 s, and extension at 72°C for 30 s. The mixture was then heated at 72°C for 10 min as a final extension step. The primers used for *TP53* amplification are shown in Table 1.

For *FGFR3* we amplified exons 7, 10, and 15 by PCR in a final volume of 50 μl containing 50 ng genomic DNA, 1 × amplification buffer, 1.5 mM $MgCl_2$, 80 μM of each dNTP, 0.3 μM forward primer, 0.3 μM reverse primer, and 2.5 units TaqDNA polymerase (HotStarTaq; Qiagen S.A. France). We used a touchdown PCR technique, with 35 cycles. Reaction mixtures were first heated for 15 min at 95°C. Annealing temperatures were lowered by 0.5°C/cycle from 72°C to 62°C over the course of 20 cycles (exon 7), from 71°C to 62°C over 18 cycles (exon 10), and from 70°C to 61°C over 18 cycles (exon 15) with each annealing step lasting 1 min. For the remaining of the 35 cycles, annealing temperatures were maintained at 62°C, 62°C, and 61°C for exons 7, 10, and 15, respectively, with each annealing step lasting 1 min. In each cycle, the denaturation step consisted of heating at 94°C for 1 min and the extension step of heating at 72°C for 1.20 min. At the end of the last cycle, samples were incubated for an additional 15 min at 72°C. The primers used for *FGFR3* amplification are shown in Table 1.

DHPLC. We carried out DHPLC analysis with the Varian (Prostar) Helix system, using the helix analysis column (the helix DNA^R column set). The formation of heteroduplexes and homoduplexes was encouraged by first denaturing PCR products by heating at 95°C for 10 min and then allowing them to reanneal at 62°C (*FGFR3*) and 58°C (*TP53*) for 1 h. We applied a 5-μl DNA sample to the column and eluted within a linear acetonitrile gradient as the eluent. The acetonitrile gradient was created by mixing buffer A [100 mM triethylammonium acetate (pH 7.0) and 0.1 mM EDTA] and buffer B [100 mM triethylammonium acetate (pH 7.0), 0.1 mM EDTA, and 25% (v/v) acetonitrile]. The flow rate was 0.45 ml/min, and we increased the proportion of buffer B by 3.3% per min for 4.5–5.5 min. The optimal melting temperature for each amplicon was first roughly determined by the analysis of wild-type sequence using an annealing algorithm at the Stanford DHPLC website (17).[5] The melting behavior of the specific DNA was established by repeatedly injecting the sample at temperature steps beginning at 50°C until complete denaturation is reached. A melting curve, retention time *versus* temperature, is made, and the temperature at which 25–50% denaturation was observed was selected (retention time is 0.75–1 min shorter than that under nondenaturing conditions), using the universal gradient (Varian). The acetonitrile gradient was then adjusted such that the peaks were eluted between 3 and 6.30 min. Whenever possible, mutation detection was checked by analyzing a sample known to harbor a specific sequence variation. DNA fragments were monitored by UV absorbency at 260 nm. Specific values for the gradient ranges and mobile-phase temperatures used are shown in Table 1.

DNA Sequencing. PCR products with elution profiles different from that of the corresponding wild-type DNA were sequenced. Sequencing was performed with the Big Dye Terminator kit, following the Applied Biosystems protocol. Samples were sequenced in an ABIPRISM 377 sequencer (Perkin-Elmer Applied Biosystems). If a mutation was identified, matched constitu-

[5] Internet address: http://insertion.stanford.edu/melt.html.

Table 2 *FGFR3 mutations in newly diagnosed bladder tumors*

Codon and mutated nucleotide positions are numbered according to the cDNA open reading frame corresponding to the FGFR3b isoform, which is produced in epithelial cells.

Exon	Codon	Nucleotide position and base change	Amino acid change	Number of tumors	%[a]
7	248	742C>T	Arg/Cys (R248C)	3	9%
7	249	746C>G	Ser/Cys (S249C)	19	60%
10	372	1114G>T	Gly/Cys (G372C)	1	3%
10	375	1124A>G	Tyr/Cys (Y375C)	6	19%
15	652	1954A>G	Lys/Glu (K652E)	2	6%
15	652	1955A>T	Lys/Met (K652M)	1	3%

[a] %: calculated as a percentage of the total number of *FGFR3* mutations found in this study.

tional DNA samples were also sequenced to confirm the somatic nature of the mutation. Results were confirmed by sequencing on both strands.

Statistical Analysis. The data are expressed as percentages. We carried out χ^2 analysis, using contingency tables and InStat 2.01 software. We used Fisher's exact test (two-sided) when appropriate. Values of $P \leq 0.05$ were considered significant.

Results

The various *FGFR3* mutations identified are listed in Table 2. Overall, these mutations were found in 40% (32 of 81) of the tumors. Six different mutations were detected, five of which (R248C, S249C, G372C, Y375C, and K652E) are identical to those found in thanatophoric dysplasia. The sixth mutation, K652M, has been reported in cases of severe achondroplasia with developmental delay and acanthosis nigricans.

The various *TP53* mutations identified are listed in Table 3. We identified 19 mutations in 17 of the 81 patients studied (23%; 2 patients had a double mutation). Mutations were found in all of the exons from 4 to 9, but no mutations were found in exons 2, 3, 10, or 11. Nine mutations were transitions (4 G>A, 3 A>G, and 2 C>T), and 8 were transversions (5 G>T, 1 A>C, 1 C>A, and 1 C>G). The C>G transversion was found in intron 6, at a splice acceptor site (patient 30). Two deletions were also found in exon 5 (patients 19 and 20). Two patients had a double mutation. Patient 27 had a double mutation in exon 7, with the first mutation at codon 244 and the second at codon 245. The other patient (number 41) had one mutation in exon 5, codon 175, and the second at exon 8, codon 306.

Sixty-one of the 81 bladder tumors studied (76%) were superficial papillary tumors; these tumors included 31 pTa, 30 pT1, and 1 (1%) flat CIS. Nineteen tumors (24%) were muscle invasive tumors (pT2-pT4), comprised of 16 pT2, 2 pT3, and 1 pT4. The frequency of superficial and muscle invasive tumors in this study was consistent with previous epidemiological studies on the stage at initial diagnosis, demonstrating the validity of our cohort of patients.

FGFR3 and *TP53* mutations identified as a function of stage and grade are shown in Fig. 1, *A* and *B*, respectively. These data clearly show opposite patterns for *FGFR3* and *TP53* mutations, in terms of distribution according to stage and grade, and a significant linear trend was observed for the distribution of mutations according to stage and grade for both genes (Fig. 1, *A* and *B*).

We found that *FGFR3* and *TP53* mutations were almost mutually exclusive (Fig. 1*C*). The frequency of the tumor group containing both mutations (*FGFR 3*mut/*TP53*mut) at various stages was not significant ($P < 0.9$).

In pTa tumors, *FGFR3* mut/*TP53*wt was the most prevalent genotype, accounting for 68% of tumors (21 of 31). The next most prevalent genotype was *FGFR3*wt/*TP53*wt, found in 9 of 31 tumors (29%). No *FGFR3*wt/*TP53*mut genotype was found. The *FGFR3*mut/*TP53*mut genotype was found in only 1 of 31 tumors (3%), a pTaG2 tumor (Fig. 1*C*).

In pT1 tumors, *FGFR3*wt/*TP53*wt was the most frequent genotype, found in 15 of 30 tumors (50%), followed by *FGFR3*mut/*TP53*wt, which was found in 8 of 30 tumors (27%), and *FGFR3*wt/*TP53*mut,

Table 3 *TP53 mutations and tumor characteristics in newly diagnosed bladder tumors*

Accession number for the genomic reference sequence: U94788.1.

Tumor number	Stage	Grade	Exon	Codon	Nucleotide position and base change	Type of mutation	Amino acid change
27[a]	pTa	G2	7	244	14058G>T[b]	transversion	Gly244Val[c]
				245	14060G>T	transversion	Gly245Cys
80	pT1	G3	5	136	13086A>C	transversion	Gln136Pro
58	pT1	G3	5	152	13134C>T	transition	Pro152Leu
70	pT1	G3	6	220	13419A>G	transition	Tyr220Cys
30	pT1	G2	Intron 6		IVS-5C>G[d]	transversion	
71	pT1	G3	7	249	14072A>G	transition	Arg249Gly
41[e]	pT1	G3	5	175	13203G>A	transition	Arg175His
			8	306	14585C>T	transition	Arg306X[f]
9	pT1	G3	9	331	14755G>A	transition	Gln331Gln
16	pT2	G2	4	110	12253G>T	transversion	Arg110Leu
19	pT2	G3	5	181	13221delG[g]	frameshift	
4	pT2	G3	6	213	13398G>T	transversion	Arg213Leu
59	pT2	G3	6	220	13419A>G	transition	Tyr220Cys
63	pT2	G3	7	241	14049C>A	transversion	Ser241Tyr
46	pT2	G3	7	248	14070G>A	transition	Arg248Gln
35	pT2	G3	8	294	14549G>T	transversion	Glu294X[f]
20	pT3	G3	5	136	13085delC[g]	frameshift	
65	pT3	G3	7	248	14070G>A	transition	Arg248Gln

[a] Tumor 27 has a double mutation.
[b] Tumor 27 has a base substitution G for T at nucleotide 14058.
[c] Deduced amino acid change: glycine to valine at codon 244.
[d] A mutation in intron 6 (splice acceptor site) denotes the C to G substitution at nucleotide −5 of the intron.
[e] Tumor 41 has a double mutation.
[f] X-stop codon.
[g] A deletion of one base (G) at nucleotide 13221 or (C) at nucleotide 13085.

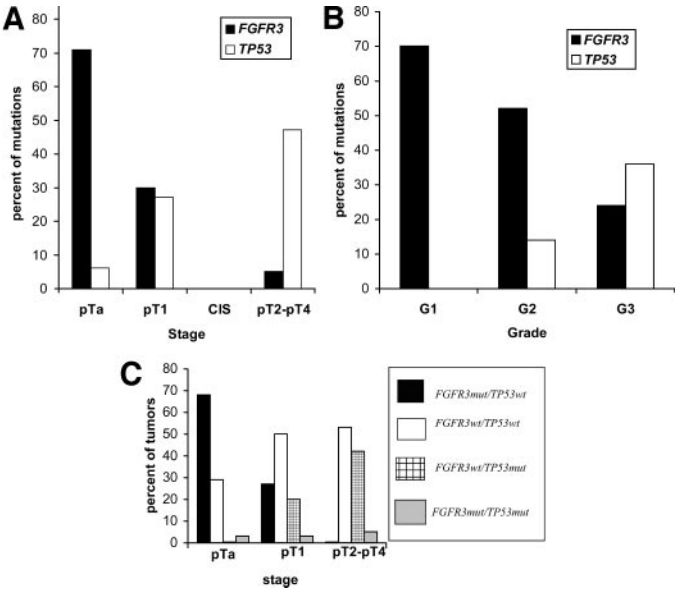

Fig. 1. *A*, *FGFR3* and *TP53* mutations according to stage. The *P* was calculated by a χ^2 test. $P < 0.05$ was considered significant. *FGFR3* mutations were detected in 71% pTa, in 30% pT1, and 5% pT2-pT4 tumors. *FGFR3* mutations are associated with low stage ($P < 0.0001$). *TP53* mutations were identified in 6% pTa, in 27% pT1, and in 47% pT2-pT4 tumors. *TP53* mutations are associated with high stage ($P < 0.003$). No mutations in *TP53* or *FGFR3* were found in the 1 CIS studied here. *B*, *FGFR3* and *TP53* mutations according to grade. The *P* was calculated by χ^2 test. $P < 0.05$ was considered significant. *FGFR3* mutations were found in 70% G1, 52% G2, and 24% G3 tumors. *FGFR3* mutations are associated with low grade ($P < 0.008$). *TP53* mutations were not found in the G1 tumors, and were identified in 14% G2 tumors and in 36% G3 tumors. *TP53* mutations are associated with high grade ($P < 0.02$). *C*, tumor genotypes according to mutations in both *FGFR3* and *TP53* at various stages. *P* was calculated using a two-sided Fisher's exact test. The frequency of the tumor group containing both mutations (*FGFR3*mut/*TP53*mut) at various stages was not significant ($P < 0.9$).

which was found in 6 of 30 tumors (20%). The *FGFR3*mut/*TP53*mut genotype was found in only 1 tumor (3%), a (pT1G3; Fig. 1*C*).

In pT2-pT4 tumors, the *FGFR3*wt/*TP53*wt genotype accounted for 10 of 19 (53%) cases. The next most frequent genotype was *FGFR33*wt/*TP53*mut, which was observed in 8 of 19 (42%) tumors. The *FGFR3*mut/*TP53*wt genotype was not found. A single *FGFR3*mut/*TP53*mut tumor (pT3G3; 5%) was identified (Fig. 1*C*).

Discussion

Elucidation of the molecular pathways involved in UCC is essential for our understanding of the etiopathogenesis of the disease, and, hence, for more precise diagnosis, accurate prognosis and better management of patients. To our knowledge, this is the first study investigating mutations in both *FGFR3* and *TP53* in bladder cancer. This analysis of genotypes in newly diagnosed bladder cancer patients, who had not received treatment previously, provides insight into the molecular basis of bladder cancer. Our results clearly show that *FGFR3* and *TP53* mutations are almost mutually exclusive at initial diagnosis (Fig. 1*C*) and more likely define separate pathways in the development of this disease.

Spruck *et al.* (5) observed that alterations of chromosome 9 were more frequent in superficial papillary tumors, whereas *TP53* mutations were more frequent in CIS and invasive tumors. It was suggested that these genetic differences might account for the markedly different clinical behavior and prognosis of superficial papillary noninvasive tumors and CIS. Indeed, they highlighted that UCCs progress via two distinct pathways. Our data concerning the frequency of *FGFR3* mutations provide additional evidence that *FGFR3* mutations are associated with low-stage, low-grade superficial papillary tumors, as reported previously in retrospective studies (18). It should also be noted that, in the same study, we found no *FGFR3* mutations in the 20 CIS studied (18). Studies on the prognostic value of *FGFR3* mutations have generated promising results, indicating that these mutations are associated with favorable disease characteristics, lower rates of recurrence, and progression (19). These mutations may correspond to the so-called "primary abnormalities" (20) involved in the production of low-grade/well-differentiated neoplasms. Such abnormalities alter cellular proliferation, but have little effect on cellular differentiation. *FGFR3* mutations might give a growth advantage for cancer cells, but, on the other hand, cell cycle and/or apoptosis mechanisms or genomic stability may still be maintained. Most of the *FGFR3* mutations we identified were in tumors that did not harbor *TP53* mutations (Fig. 1*C*). Therefore, genomic stability may have been maintained, because it has been suggested that *TP53* mutations destabilize the genome (21). Our results on *TP53* mutations, which were most frequent in high-stage, high-grade tumors, are consistent with those of previous investigators (3, 5).

We analyzed the mutations in the two genes in individual tumors and found that *FGFR3*mut/*TP53*wt was the most prevalent genotype in pTa (68%) tumors. Thus, *FGFR3* mutations are probably key genetic alterations in these lesions and may occur early in their development. *FGFR3* mutations occur with high frequency (75%) in urothelial papilloma (22), and it is still to be determined whether some pTa tumors might have been evolved from these lesions. In pT1 tumors, this genotype accounts for 27% of cases and was not observed in muscle invasive cancer. The presence of this genotype in both pTa and pT1 tumors indicates that a subgroup of pT1 is, from a molecular point of view, related to pTa. However, the clearly higher frequency of this genotype in pTa than in pT1 tumors ($P < 0.01$) and the presence of other genotypes in pT1 tumors that are not found in pTa tumors (*FGFR3*wt/*TP53*mut; 20%) are consistent with previous data suggesting that these two lesions harbor different genetic changes (23) and that their grouping together as superficial bladder cancer is imprecise and misleading.

The *FGFR3*wt/*TP53*wt genotype accounted for a considerable proportion of tumors, and was found in 29% of pTa, 50% of pT1, and 53% of pT2 tumors. The search for key genetic and/or epigenetic alterations should focus on these lesions, as other pathways may also be at work. Other alterations described in bladder cancer should perhaps also be studied in light of *FGFR3* and *TP53* mutations.

The *FGFR3*wt/*TP53*mut genotype was not observed in pTa tumors, but was found in 20% of pT1 and 42% of pT2 tumors. The increasing frequency of this genotype from pT1 to pT2-pT4 suggests that this genotype may be associated with more chromosomal alterations. This possibility is consistent with data showing a higher level of allelic imbalances in pT2-pT4 than in pT1 tumors and that tumors in which both *TP53* alleles are inactivated have many imbalances (24). Because *TP53* is involved in checkpoint control after DNA damage, changes in this gatekeeper gene may lead to the accumulation of additional genetic changes (25).

Our data suggest that pT1 tumors may be seen as an intermediate group, resembling both pTa and pT2-pT4 tumors, as they included the three main genotypes: *FGFR3*mut/*TP53*wt (27%), *FGFR3*wt/*TP53*wt (50%), and *FGFR3*wt/*TP53*mut (20%). These findings reaffirm the heterogeneity of this group of tumors. Given the clear variability in the biological potential of these lesions, grouping them according to common molecular changes may improve predictions of prognosis. Tumors with *TP53* mutations may not be grouped with those harboring *FGFR3* mutations.

The *FGFR3*mut */Tp53*mut genotype was found in only a few tumors: a pTaG2(3%), a pT1G3 (3%), and a pT2G3 (5%) tumor. Mutations in both genes may result from different oncogenic mechanisms occurring in the same tumor, which is not the general rule, at

least with respect to these two gene mutations. It is possible that these mutations define distinctive, separate pathways that are occasionally interrelated. The identification of this genotype in pTa, pT1, and pT3 tumors suggests that there is an intricate interplay between the effects of the two mutations, according to the surrounding molecular microenvironment, which would finally determine tumor phenotype.

The data presented herein suggest that mutations in the *FGFR3* and *TP53* genes at initial diagnosis probably define separate molecular pathways in UCCs, possibly leading to two different major clinical phenotypes. Moreover, molecular classification according to *FGFR3* and *TP53* mutational status might parallel that of TNM and grading classification, constituting an initial step toward a simple practical molecular classification of tumors facilitating the identification of patients with low and high risks of progression. *FGFR3* mutations might prove to be a powerful diagnostic tool and an important therapeutic target in UCC. Furthermore, tumor classification according to both gene mutations might serve as landmarks for future searches for other genetic or epigenetic alterations in bladder cancer.

Acknowledgments

We thank Dr. Brigitte Bressac-de Paillerets (Institute Gustav Roussy) for kindly providing us with *TP53* primers and useful discussions on DHPLC conditions.

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