FGFR3 and *P53* Characterize Alternative Genetic Pathways in the Pathogenesis of Urothelial Cell Carcinoma

Bas W. G. van Rhijn,[1,2] Theo H. van der Kwast,[1] André N. Vis,[1,2] Wim J. Kirkels,[2] Egbert R. Boevé,[3] Adriaan C. Jöbsis,[4] and Ellen C. Zwarthoff[1]

[1]Department of Pathology, Josephine Nefkens Institute, and [2]Department of Urology, Erasmus MC, Rotterdam, and [3]Departments of Urology, and [4]Pathology, Sint Franciscus Gasthuis, Rotterdam, the Netherlands

Abstract

***Fibroblast growth factor receptor 3* (*FGFR3*) and *P53* mutations are frequently observed in bladder cancer. We here describe the distribution of *FGFR3* mutations and *P53* overexpression in 260 primary urothelial cell carcinomas. *FGFR3* mutations were observed in 59% and *P53* overexpression in 25%. Interestingly, *FGFR3* and *P53* alterations were mutually exclusive, because they coincided in only 5.7% of tumors. Consequently, we propose that they characterize two alternative genetic pathways in urothelial cell carcinoma pathogenesis. The genetic alterations were reflected in the pathology and the clinical outcome, *i.e.*, *FGFR3* mutations were found in low-stage/-grade tumors and were associated with a favorable disease course, whereas *P53* alterations were tied to adverse disease parameters.**

Introduction

Urothelial cell carcinoma (UCC), of which bladder cancer is the major representative, is the fifth most common malignancy in Western society (1). Because of its frequent recurrence and the relatively long life span of patients, UCC is the most expensive cancer in health care (2). Approximately 70% of the patients initially present with superficial tumors (stages pT_a, pT_1, or pT_{is}). UCCs fall into two major groups with a substantially different natural behavior, *i.e.*, superficial and invasive UCC. More than 80% of superficial UCCs remain confined to the submucosa throughout their clinical course, whereas most of the invasive UCCs exhibit their invasive property at first presentation, and these tumors are associated with a high propensity to metastasize (3). In the last decade, many efforts were undertaken to find a molecular basis for this divergent disease pathogenesis of UCC. Mutations in the *P53* gene were frequently found in invasive UCC as well as in high-grade superficial UCC including carcinoma *in situ*, the putative precursor of invasive UCC, whereas these mutations were rare in well-differentiated superficial UCC (4, 5). On the other hand, loss(es) of heterozygosity (LOH) on the chromosomal arms 9p and 9q was the most frequent genetic alteration in the low-grade papillary lesions (4, 5). However, subsequent studies showed that LOH on 9p/q was also a very common finding in high-grade superficial and invasive UCC (6–9). Consequently, LOH on 9p/q did not provide a sufficient molecular explanation for the clinically divergent disease pathogenesis of UCC. Recently, *fibroblast growth factor receptor 3* (*FGFR3*) mutations, identical to the mutations responsible for several skeletal anomalies associated with dwarfism in most cases, were reported at a high frequency in UCC and at a low frequency in multiple myeloma and cervical cancer (10–12). Surprisingly, the oncogenic *FGFR3* mutations were particularly related to favorable UCCs in three pilot studies (13–15). The *FGFR3* mutations occur predominantly in UCC (11, 12), whereas *P53* mutations are found in over 50% of human cancers. In the present study, we investigated the distribution of *FGFR3* and *P53* alterations in 260 primary (first diagnosis) UCCs. We here report that *FGFR3* and *P53* characterize almost 80% of UCCs and that these mutations seem to exclude each other. These distinct molecular features were also reflected in the different pathological parameters and the clinical follow-up of the patients. We, therefore, propose that *FGFR3* and *P53* characterize different pathogenesis pathways for UCC.

Materials and Methods

Patients and Tumor Samples. We analyzed the tumors of 260 patients (196 males) with first diagnosis UCC. The median age of the patients was 67.2 years. No patient had a hereditary skeletal anomaly. A paraffin-embedded, formalin-fixed tissue block was obtained from the archives of two pathology departments (Erasmus MC and Sint Franciscus Gasthuis, Rotterdam, the Netherlands) and was classified according to the TNM and WHO guidelines. A single pathologist (T.H. v. d. K.) reviewed the slides using the 1998 WHO/International Society of Urological Pathology classification system for grading. In case of multifocality ($n = 67$), the lesion with the highest grade/stage was taken. The largest tumor was taken if grade/stage were the same for multiple UCCs.

P53 Analysis. Four-μm-thick sections were freshly cut from each tissue block and mounted on amino alkylsilane-coated slides. Incubation with primary antibody P53 (clone DO-7; DAKO, Glostrup, Denmark; dilution, 1:200) was 30 min in PBS/BSA 5%. Positive and negative controls were included. The conventional avidin-biotin complex method was applied for all immunostainings. Two persons (B. W. G. v. R. and A. N. V.) independently assessed the slides without knowledge of clinical data. In case of heterogeneity, the parts within the tumor that showed the highest positive:total ratio were particularly assessed. This was performed if these regions comprised at least 10% of the tumor load in the examined tissue section. P53 overexpression was scored if >10% stained positive. In case of discrepancy between the observers, the slides were reassessed in a combined session without the information of the previous scores.

FGFR3 Analysis. Standard H&E slides served as templates for manual microdissection. The dissected samples contained a minimum of 70% tumor cells. DNA was extracted using the DNeasy Tissue kit (Qiagen GmbH, Hilden, Germany). *FGFR3* mutation analysis was by PCR single-strand conformation polymorphism analysis (14), followed by sequencing in case of a shift (T7 Sequenase v2.0; Amersham life Science, Inc., Cleveland, OH). DNA extracted from venous blood was available from 139 patients. Mutation analyses were performed without knowledge of clinical data.

Clinical Follow-Up and Statistical Analysis. The follow-up data were collected by chart review. Disease-specific survival was determined. The patients were censored at their last clinical visit or at the time of their death. The statistical package for social sciences 9.0 (SPSS Inc., Chicago, IL) computer software was used for the data documentation and analysis. The two-sided Fisher's exact test was used to analyze the relationships between the molecular variables and their correlation with pathological stage and grade.

Received 8/5/03; revised 1/26/04; accepted 1/30/04.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Ellen C. Zwarthoff, Department of Pathology, Josephine Nefkens Institute, Erasmus MC, P. O. Box 1738, 3000 DR Rotterdam, the Netherlands. Phone: 3110-408-7929; Fax: 3110-408-9487; E-mail: e.zwarthoff@erasmusmc.nl.

The clinical outcome was analyzed by applying the Kaplan-Meier method. Statistical significance was assumed if $P < 0.05$.

Results

Mutations in the *P53* and *FGFR3* genes were studied in 260 primary UCCs. Activating *FGFR3* mutations were found in 153 (59%) of these tumors. We found 117, 32, and 4 mutations in the exons 7 (R248C and S249C), 10 (G372C, Y375C, and A393E), and 15 (K652T, K652E, and K652M), respectively. No mutations were detected in exon 19. No mutations were seen in the matched blood samples. P53 overexpression, presumably reflecting missense mutations in the *P53* gene, was noted in 66 (25%) cases. Table 1 shows a highly significant inverse relation between the presence of a *FGFR3* mutation and P53 overexpression ($P < 0.0001$). Only 5.7% of the 260 primary UCCs were positive for both markers, whereas 72.7% UCCs were either positive for *FGFR3* or positive for P53.

We subsequently determined the correlation of *FGFR3* and *P53* with pathological stage and grade for the 260 UCCs. *FGFR3* mutations were found in 77% of pT_a, 31% pT_1, and 15% $\geq pT_2$ tumors. Conversely, P53 overexpression was found in 11% pTa, 51% pT1, and 56% $\geq$pT2 tumors, respectively ($P < 0.0001$). The numbers for pathological grade showed the same trend. *FGFR3* mutations were found in 85% of urothelial neoplasia of low malignant potential (LMP), in 71% of low-grade (LG) and in 26% of high-grade (HG) cases. On the other hand, P53 overexpression was found in only 1% of LMP, in 15% of LG and in 54% of HG cases, respectively ($P < 0.0001$). The distributions of the stages and grades for the *FGFR3*/P53 subgroups are given in Fig. 1. In general, the *FGFR3* mutation related to favorable, *i.e.*, pTa and LMP/LG disease, whereas P53 overexpression indicated unfavorable, *i.e.*, invasive and HG disease.

The UCCs, which were wild type for both genes, the so-called double negatives, were a substantial subgroup encompassing 21% of the 260 UCCs (Table 1). When compared with stage and grade, these UCCs apparently included tumors of all grades and stages, with a higher percentage of pT_a tumors than the P53-positive subgroup, *i.e.*, 51% *versus* 22%, but also a higher percentage high-grade tumors than the *FGFR3*-mutation subgroup, *i.e.*, 48% *versus* 12% (Fig. 1).

Besides histopathological parameters, the clinical outcome of the 260 patients was also determined. The mean follow-up was 5.6 years (SD, 3.7 years). Twenty-one patients died of UCC. The Kaplan-Meier analyses in Fig. 2 clearly show that patients with a *FGFR3* mutation in their UCC have a favorable prognosis, whereas patients with P53 overexpression have a worse prognosis than patients with a normal P53 expression pattern.

Discussion

UCCs are genetically characterized by frequent LOH; mutations and deletions in the *P53*, *RB* and *p16* tumor suppressor genes; and activating point mutations in the gene for the FGFR3 receptor, LOH of chromosomal arms 9p and 9q is observed in over 50% of UCC. Homozygous deletions of the p16 cyclin-dependent kinase inhibitor are found in 20–30% of high-grade and -stage tumors (16). Bladder tumors with pRB alterations are significantly more prone to metastasize (Ref. 17, and references therein). Mutations in the *P53* gene are observed in poorly differentiated carcinomas, and mutations in this gene apparently characterize tumors with a worse prognosis. On the basis of these observations Spruck *et al.* (5), proposed a two-pathway model for UCC pathogenesis, in which *P53* mutations delineated one arm of the pathway. LOH on chromosome 9 was supposed to characterize the other arm. However, later work revealed that chromosome 9 loss is found in all grades and stages and, therefore, can no longer serve as a marker for the noninvasive pathway (6–9). A recent update

Table 1 *Inverse relationship between FGFR3*[a] *mutations and P53 overexpression in urothelial cell carcinoma ($P < 0.0001$)*

A *P53* mutation was assumed if >10% of the cells stained positive.

	FGFR3 wt	*FGFR3* mt
P53 wt	56	138
P53 mt	51	15
Total	107	153

[a] FGFR3, fibroblast growth factor receptor 3; wt, wild type; mt, mutated type.

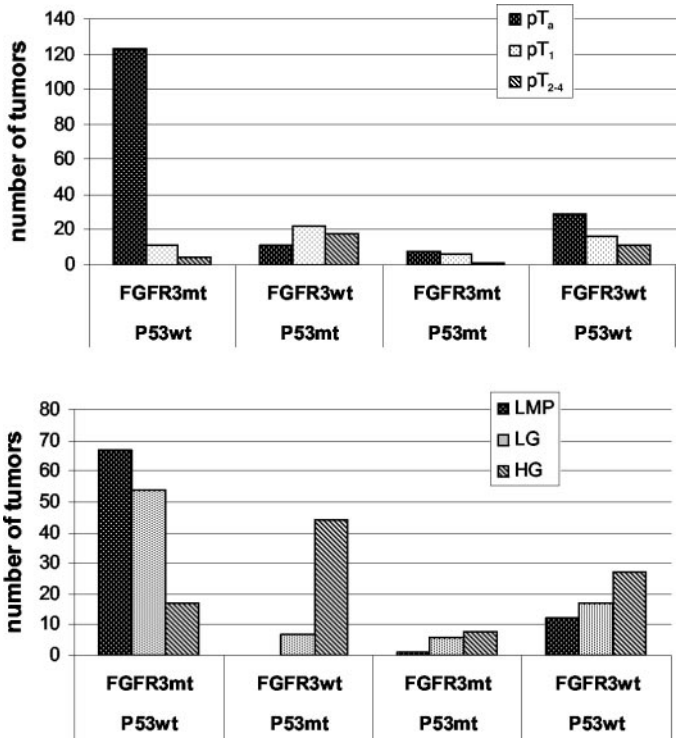

Fig. 1. The relationship for *FGFR3/P53* subgroups with pathological stage (*top panel*) and grade (*bottom panel*). The stage distribution was 171 pT_a, 55 pT_1, and 27 pT_2 , and 5 pT_3, and 2 pT_4 lesions. The correlation between mutations in the *FGFR3* gene with pT_a and low-grade [low malignant potential (LMP) and low-grade urothelial carcinoma (LG)] tumors is notable. Conversely, *P53* overexpression, presumably caused by missense mutations, was associated with invasive ($\geq pT_1$) and high-grade (HG) disease. The tumors that were wild type (*wt*) for both genes represent a group in which all stages and grades are found. *FGFR3-mt*, *FGFR3*-mutant tumors; *FGFR3wt*, *FGFR3*-wild-type tumors; *P53wt*, *P53*-wild-type tumors; *P53mt*, *P53*-mutant tumors

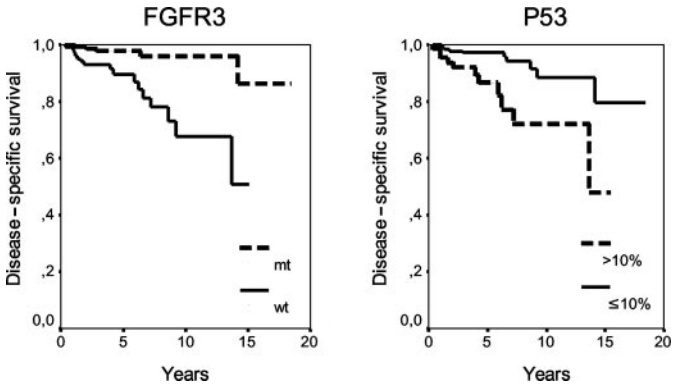

Fig. 2. Kaplan-Meier analyses for disease specific-survival. The survival plots (log-rank) for FGFR3 ($P = 0.0001$) and P53 ($P = 0.0011$) are shown. The *dashed lines*, the follow-up of patients with a *FGFR3* mutation and P53 overexpression, respectively. *mt*, mutant; *wt*, wild-type.

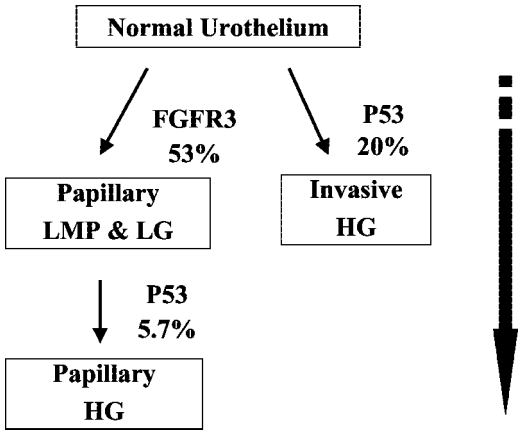

Fig. 3. Model for urothelial cell carcinoma (UCC) pathogenesis. *FGFR3* and *P53* indicate two different pathways for UCC pathogenesis, and, together, they classify 79% of primary UCCs. These mutations clearly result in distinct pathological parameters. The small group of tumors with mutations in both genes is presumably derived from the *FGFR3* group (see "Results"). The 21% tumors that lack alterations in *P53* or *FGFR3* are not included in the figure. *The arrow on the right* signifies increasing loss of heterozygosity and mutations in other genes. *LMP*, low malignant potential; *LG*, low-grade urothelial carcinoma; *HG*, high-grade urothelial carcinoma.

of the UCC pathogenesis model, including more markers, was published by Cote and Datar (17). However, although many genes may play a role in UCC pathogenesis, most of them are only mutated in a limited percentage of tumors and, at present, their mutual dependencies, when present, are not entirely clear.

We have recently shown that *FGFR3* mutations are generally associated with favorable disease characteristics such as low stage and grade, low recurrence rate, and a better prognosis (13, 18). In the present study, we intended to place this new genetic marker in the genetic pathogenesis model for UCC. To this end, we determined the *FGFR3* and *P53* status in 260 primary tumors. To our surprise, we observed that alterations in *FGFR3* and *P53* were almost always mutually exclusive. Together these two markers described 79% of the primary UCC. However, they concurred in only 5.7%. This suggested that *FGFR3* and *P53* characterize two distinct genetic pathways in UCC pathogenesis. While this paper was being reviewed, Bakkar *et al.* (19) reported similar findings based on a study of 81 patients. In our patients, the *FGFR3* mutation was associated with pT_a and LMP/LG tumors, whereas the P53 marked invasive and HG carcinomas. In addition, the clinical outcome of the two investigated molecular markers confirmed this suggestion of two genetic pathways. A model for UCC pathogenesis based on our observations is given in Fig. 3. This model resembles Spruck's original model [Spruck *et al.* 5)], but clearly designates two distinct pathways with genetic markers for a noninvasive and papillary, FGFR3-associated pathway and an invasive P53-associated pathway. Together, these two markers provided a genetic framework for the majority of UCCs. The addition of the FGFR3 marker significantly expands Spruck's original model [most recently reviewed in Cote and Datar (17)]. We feel that, at the moment, it is not completely clear where to put LOH, especially of chromosome 9q, in this pathway. In *P53*-positive tumors, this event may have occurred before the *P53* mutation. However, in a considerable percentage of *FGFR3*-mutant tumors, LOH may occur after the *FGFR3* mutation, because *FGFR3* mutations are more frequent than LOH of 9q. On the other hand, we previously showed, in evolutionary tree models of multiple UCC recurrences, that tumors with LOH of 9q can precede recurrences with a mutation in the *FGFR3* gene (7). Thus, the *FGFR3* mutation is not necessarily the first genetic event in superficial tumor formation. In the model displayed here, we also suggest that UCC with alterations in both *FGFR3* and *P53* derive from *FGFR3*-mutant tumors. We argued that it would be more difficult for a *P53*-positive, invasive, HG carcinoma to become a lower-grade superficial tumor than that a pT_a, LMP/LG tumor would transform into a more invasive, higher-grade descendant. This small group of UCCs apparently displays an intermediate phenotype when compared with the tumors with single alterations (Ref. 18 and present study). Thus, it appears that the effect of the *FGFR3* mutation mitigates the effect of the *P53* mutation. The remainder of UCCs, 21% in our series, lacking *P53* or *FGFR3* alterations, forms an interesting subset. The fact these tumors include all possible grades and stages suggests that they may also harbor different genotypes. Some of these tumors may in the future, perhaps, be placed in a *FGFR3*-like group or a *P53*-like group. However, it is also possible that yet a third genetic pathway is responsible for a portion of these tumors. Previous work reviewed by Cote and Datar (17) suggests that RB and p16 and/or pARF alterations are mostly associated with the P53-mutant UCCs and, thereby, are expected to be part of this pathway. However, it is not yet clear whether these mutations occur before or after the *P53* event. The same holds true for other less frequently occurring alterations. Thus far, no mutations, besides LOH for 9p/9q and other chromosomes have been described to concur with *FGFR3* mutations. Therefore, additional investigations are required to place all markers in the pathway model. Consequently, the *arrow on the right* in Fig. 3 signifies an increasing frequency of LOH and other genetic aberrations from the top to the bottom.

The colorectal cancer model proposed by Fearon and Vogelstein (20) is the prototype for the molecular evolution of cancer. The model is presented as a linear model, in which inactivation of *APC*, mutation of *KRAS*, inactivation of a gene on 18q, and inactivation of *P53* occur as sequential steps in the development from low-grade adenomas to carcinoma. However, Smith *et al.* (21) recently showed that co-occurrence of mutations in both *KRAS* and *P53* in colorectal cancers is extremely rare, and they suggest that these mutations, in fact, lie on alternate pathways of colorectal tumor development. Thus, the route to colorectal cancer seems to bifurcate after the initial *APC* step into at least two alternative genetic pathways. This situation is similar to the one that we describe for UCC in Fig. 3. In colorectal cancer, *KRAS* mutations are overrepresented in Dukes C tumors, and, thereby, *KRAS* may be a marker for tumor progression. The added value of the model for UCC pathogenesis is that *FGFR3* and *P53* represent makers for favorable and unfavorable disease, respectively. Together, these markers characterize almost 80% of all primary tumors. The search for mutations in other genes of both pathways may increase the percentage of molecularly characterized tumors further and may facilitate a more complete molecular description of UCC as a possible alternative for classical pathology (18). Moreover, the favorable *FGFR3* marker is a potential candidate to identify patients for whom a less frequent follow-up is required, and this may help reduce the costs of management for patients with UCC.

Acknowledgments

We thank the histotechnology and the immunohistochemical laboratories of the University Hospital Rotterdam for the preparation of slides and the immunostaining procedures. The medical-ethical committee of the Erasmus University and the University Hospital Rotterdam approved the study (MEC 168.922/1998/55).

References

1. Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003. CA Cancer J Clin 2003;53:5–26.
2. Riley GF, Potosky AL, Lubitz JD, Kessler LG. Medicare payments from diagnosis to death for elderly cancer patients by stage at diagnosis. Med Care 1995;33:828–41.
3. Raghavan D, Shipley WU, Garnick MB, Russell PJ, Richie JP. Biology and management of bladder cancer. N. Engl J Med 1990;322:1129–38.

4. Dalbagni G, Presti J, Reuter V, Fair WR, Cordon-Cardo C. Genetic alterations in bladder cancer. Lancet 1993;342:469–71.
5. Spruck, C. H. III, Ohneseit PF, Gonzalez-Zulueta M, et al. Two molecular pathways to transitional cell carcinoma of the bladder. Cancer Res 1994;54:784–8.
6. Primdahl H, von der Maase H, Christensen M, Wolf H, Orntoft TF. Allelic deletions of cell growth regulators during progression of bladder cancer. Cancer Res 2000;60: 6623–9.
7. van Tilborg AA, de Vries A, de Bont M, Groenfeld LE, van der Kwast TH, Zwarthoff EC. Molecular evolution of multiple recurrent cancers of the bladder. Hum Mol Genet 2000;9:2973–80.
8. Hartmann A, Schlake G, Zaak D, et al. Occurrence of chromosome 9 and p53 alterations in multifocal dysplasia and carcinoma *in situ* of human urinary bladder. Cancer Res 2002;62:809–18.
9. van Rhijn BW, Lurkin I, Chopin DK, et al. Combined microsatellite and FGFR3 mutation analysis enables a highly sensitive detection of urothelial cell carcinoma in voided urine. Clin Cancer Res 2003;9:257–63.
10. Capellen D, De Oliveira C, Ricol D, et al. Frequent activating mutations of FGFR3 in human bladder and cervix carcinomas. Nat Genet 1999;23:18–20.
11. Sibley K, Stern P, Knowles MA. Frequency of fibroblast growth factor receptor 3 mutations in sporadic tumours. Oncogene 2001;20:4416–8.
12. Karoui M, Hofmann-Radvanyi H, Zimmermann U, et al. No evidence of somatic FGFR3 mutation in various types of carcinoma. Oncogene 2001;20:5059–61.
13. van Rhijn BW, Lurkin I, Radvanyi F, Kirkels WJ, van der Kwast TH, Zwarthoff EC. The fibroblast growth factor receptor 3 (FGFR3) mutation is a strong indicator of superficial bladder cancer with low recurrence rate. Cancer Res 2001;61:1265–8.
14. Billerey C, Chopin D, Aubriot-Lorton, M-H, et al. Frequent FGFR3 mutations in papillary non-invasive bladder (pTa) tumors. Am J Pathol 2001;158:1955–9.
15. Kimura T, Suzuki H, Ohashi T, Asano K, Kiyota H, Eto Y. The incidence of thanatophoric dysplasia mutations in FGFR3 gene is higher in low-grade or superficial bladder carcinomas. Cancer (Phila.) 2001;92:2555–61.
16. Orlow I, Lianes P, Lacombe L, Dalbagni G, Reuter V, Cordon-Cardo C. Chromosome 9 allelic losses and microsatellite alterations in human bladder tumors. Cancer Res 1994;54:2848–51.
17. Cote RJ, Datar RH. Therapeutic approaches to bladder cancer: identifying targets and mechanisms. Crit Rev Oncol/Hematol 2003;46 Suppl:S67–83.
18. van Rhijn BW, Vis AN, van der Kwast TH, et al. Molecular grading of urothelial cell carcinoma with fibroblast growth factor receptor 3 and MIB-1 is superior to pathologic grade for the prediction of clinical outcome. J Clin Oncol 2003;21:1912–21.
19. Bakkar AA, Wallerand H, Radvanyi F, et al. *FGFR3* and *TP53* gene mutations define two distinct pathways in urothelial cell carcinoma of the bladder. Cancer Res 2003;63:8108–12.
20. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990; 61:759–67.
21. Smith G, Carey FA, Beattie J, Wilkie MJ, Lightfoot TJ, Coxhead J, Garner RC, Steele RJ, Wolf CR. Mutations in APC, Kirsten-ras, and p53–alternative genetic pathways to colorectal cancer. Proc Natl Acad Sci USA 2002;99:9433–8.