Role of Activating *Fibroblast Growth Factor Receptor 3* Mutations in the Development of Bladder Tumors

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Abstract

Purpose: Bladder tumors develop through different molecular pathways. Recent reports suggest activating mutations of the *fibroblast growth factor receptor 3* (*FGFR3*) gene as marker for the "papillary" pathway with good prognosis, in contrast to the more malignant "carcinoma *in situ*" (CIS) pathway. The aim of this clinical follow-up study was to investigate the role of *FGFR3* mutations in bladder cancer development in a longitudinal study.

Experimental Design: We selected 85 patients with superficial bladder tumors, stratified into early (stage T_a/grade 1-2, $n = 35$) and more advanced (either stage T_1 or grade 3, $n = 50$) developmental stages. The patients were followed prospectively, and metachronous tumors were included. We did screening for *FGFR3* and *TP53* mutations by direct bidirectional sequencing and for genome-wide molecular changes with microarray technology.

Results: A total of 43 of 85 cases (51%) showed activating mutations of *FGFR3*. The mutations were associated with papillary tumors of early developmental stage. However, after stratifying for developmental stage, *FGFR3*-mutated tumors showed the same malignant potential as wild-type tumors. Tumors with concomitant CIS were generally *FGFR3* wild type. They were characterized by different patterns of chromosomal changes and gene expression signatures compared with *FGFR3*-mutated tumors, indicating different molecular pathways.

Conclusions: *FGFR3* mutations seem to have a central role in the early development of papillary bladder tumors. These tumors follow a common molecular pathway, which is different from tumors with concomitant CIS. *FGFR3* mutations do not seem to play a role in bladder cancer progression.

Bladder tumors are the fifth most common neoplasms in developed countries. They come in two different forms: superficial and invasive. The most superficial tumors have a noninvasive papillary phenotype (stage T_a) and tend to have a benign clinical course; however, up to 25% will eventually develop an aggressive, invasive phenotype. The individual course is difficult to predict; markers of advanced malignant development, as the invasion of the lamina propria, high grade of dysplasia, or the presence of high-grade intraurothelial neoplasia (HGIUN)/carcinoma *in situ* (CIS) are risk factors for cancer progression to the muscle-invasive phenotype (1–5). Prognostic factors that reliably predict whether the disease is going to progress or not would be of great value.

Superficial bladder tumor development follows at least two different molecular pathways; one pathway comprises papillary tumors, and one includes the occurrence of CIS (6, 7). Recently, activating mutations of the *fibroblast growth factor receptor 3* (*FGFR3*) gene have been shown in bladder tumors with high frequency (8). They are associated with early papillary lesions with low malignant potential (9, 10). In fact, the mutations have been found in 75% of nondysplastic genuine urothelial papillomas (11), indicating that they are very early events in the papillary tumor development. FGFR3 is a receptor tyrosine kinase activating the extracellular signal-regulated kinase/mitogen-activated protein kinase and the protein kinase B/Akt pathways that both promote cell survival (12). Constitutively activating mutations might enhance proliferation and provide growth advantage (13). *FGFR3*-mutated tumors have some malignant potential, because around 25% of the advanced bladder cancers show a *FGFR3* mutation. On the other hand, it seems that the frequency of *FGFR3* mutations decreases with increasing tumor stage and grade of dysplasia (9, 10). The most probable explanation for this finding is that the majority of *FGFR3*-mutated tumors never progress. Most of the advanced cancers would then derive from nonmutated precursors following a different pathway of tumor development. Subsequent publications substantiate this hypothesis. Two independent studies found changes of both *FGFR3* and *TP53* almost mutually exclusive,

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as only 5% and 5.7%, respectively, showed this pattern (13, 14). *FGFR3* mutations are rarely found in CIS (9), which is known to have a high frequency of *TP53* mutations (7) and a high probability of progression. Consequently, *FGFR3*-mutated tumors are regarded as tumors with low malignant potential and a favorable prognosis (10, 15). However, the development of *FGFR3*-mutated and wild-type tumors has never been shown in longitudinal studies.

We established in 1994 a consecutive tissue bank combined with a clinical database with comprehensive prospective follow-up data for studying molecular aspects of bladder cancer development. It now contains material from almost 2,000 patients. The aim of the present study was to examine the development of *FGFR3*-mutated and wild-type bladder tumors, with emphasis on the development of the muscle-invasive phenotype. Furthermore, we looked for evidence for different molecular pathways and investigated the correlation of the development of the different tumors with *TP53* mutations. For this, we selected patients with superficial bladder tumors and stratified them by developmental stage into early (stage T_a/grade 1-2 tumors, no previous T_1 or grade 3 disease) and more advanced (either stage T_1 or grade 3 disease, previous or present). The disease courses were followed prospectively. We determined genome-wide chromosomal instability patterns using 10K single nucleotide polymorphism (SNP) microarrays and gene expression changes using expression microarrays and screened the tumors for *FGFR3* and *TP53* mutations in parallel. Metachronous tumors were examined in a subgroup of cases to show changes in developing tumors.

Materials and Methods

Study design, sample selection, clinical follow-up, and tissue sampling

This study was based on a tissue bank holding bladder tumors from almost 2,000 patients. The patients were followed prospectively. The study was approved by the scientific ethical committee of the county of Aarhus, and all patients were informed and gave their written consent. Samples were collected prospectively but selected retrospectively from the cohort data.

The bladder tumor specimens were cleaved immediately after transurethral resection. Appropriate amounts of tissue were sent to conventional histopathologic diagnosis, the rest snap-frozen in liquid nitrogen and stored at −80°C. Tissue for RNA extraction was stored in a guadinium thiocyanate solution as described (16). Tumors were staged according to tumor-node-metastasis and graded according to the Bergkvist classification.

A total number of 85 patients with initial superficial bladder tumors (histopathologic stage pT_a and pT_1) were selected based on the disease course and availability of material. Our aim was to study superficial lesions (stage T_a/T_1) to obtain information on the biological difference between those that progress and those that do not. We therefore included a relatively high proportion of potentially malignant (either stage T_1 or grade 3, previous or present) tumors. In total, we examined 115 tumors: for 22 patients, we examined at least one subsequent tumor; for five patients, we examined two subsequent tumors; and for one patient, we examined three subsequent tumors.

Patients were followed prospectively with control cystoscopies every 4 months in the first year, every 6 months in the second, and hereafter with yearly controls until a recurrence-free period of 5 years. Disease recurrences were treated in a standardized fashion. Eleven patients received intravesical instillations of Bacillus Calmette-Guerin during the course.

The clinical disease courses were allocated to the following groups:

1. Low risk, tumors with benign biological behavior [exclusively noninvasive stage T_a, low-grade (Bergkvist 1-2) tumors with no CIS; n = 26: 6 females and 20 males; median age, 64 years; median follow-up time, 56 months],
2. Medium risk, a single occurrence of either stage T_1 or grade 3 tumor or CIS but no such recurrences during the entire course (n = 18: 2 females and 16 males; median age, 70 years; median follow-up time, 63 months),
3. High risk, either recurrent stage T_1 or grade 3 tumors or widespread CIS but no progression (n = 15: 5 females and 10 males; median age, 70 years; median follow up time, 49 months),
4. Progressing, defined as developing at least muscle-invasive bladder cancer (T_2-T_4) or metastases (N_1^+ or M_1; n = 26: 6 females and 20 males; median age, 72 years; median time to progression, 13 months).

All patients, tumors, the previous history, stage, grade, concomitant intraurothelial neoplasia [CIS/HGIUN, low-grade intraurothelial dysplasia (LGIUN)], the mutation status, and the microarray analyses done are detailed in Table 1. For further clinical characteristics of the individual disease courses, see Supplementary Information.

DNA purification and mutation analysis

Genomic DNA for SNP analysis was extracted and purified, and the microarray analysis (Affymetrix GeneChip Mapping 10K Early Access Arrays, Affymetrix, Santa Clara, CA) was done as described earlier (17).

DNA for *FGFR3* and *TP53* mutation screening was extracted from the corresponding formalin-fixed, paraffin-embedded tissue sections, using a standard DNA extraction kit (Gentra, Minneapolis, MN). Tumor tissue was trimmed in the microscope to ensure at least 75% tumor cells and digested with proteinase K before DNA extraction. PCR of parts of exons 7, 10, and 15 of *FGFR3* and exons 5 to 8 of *TP53* was done using primer sequences and PCR procedures as reported by Bakkar et al. (13) and Koed et al. (17), respectively. Mutation analysis was done by direct bidirectional sequencing using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Single nucleotide polymorphism array data transformation

Loss of heterozygosity analysis. The arrays were normalized and scored using the Microarray Suite 5.0 and Genotyping Tools software supplied by the array manufacturer (Affymetrix) to obtain allelic calls for tumor DNA and corresponding normal DNA extracted from blood. Scoring of allelic imbalance was conducted as in Koed et al. (17).

Copy number analysis. Normalization: The arrays were normalized, and signal values for the individual SNPs were extracted according to a procedure described previously (18). Briefly, an invariant set normalization method was applied to normalize all arrays at the probe intensity level to a baseline array. After normalization, a model-based method (the perfect match/mismatch difference model) was used to extract a single signal value (the "observed signal") for each SNP in each array. Subsequently, data was normalized SNP-wise to have mean = 0 and SD = 1, using the empirical mean and SD in 113 normal DNA samples extracted from blood: $z_{ij} = (\gamma_{ij} - m_j)/SD_J$, where y_{ij} is the "observed signal" for array i and SNP_j, m_i is the mean of SNP_j, and SD_J the SD of SNP_j. To reduce noise, data z_{ij} was further smoothened over K SNPs to obtain $x_{ij} = \Sigma z_{ik}$, where the sum is over $k = j - K,\ldots, j + K$. Here, $K = 5$.

Test for difference between *FGFR3* wild-type and *FGFR3*-mutated tumors (relative copy number changes): The average of x_{ij} was calculated for *FGFR3* wild-type (group 1) and *FGFR3*-mutated (group 2) tumors for each SNP, and the difference d_j between the two averages was found. High and low values of d_j were indicative of

Table 1. Clinical data, mutation status and array analyses performed on 115 bladder tumors from 85 patients, sorted by clinical course

Risk at sampling	Clinical course	Patient no.	Visit no.	Highest previous Tstage	Actual Tstage	Actual grade	IUN in the course	*FGFR3* status	*TP53* status	Global gene expression analysis	SNP chip analysis	CIS classifier
EBDC	1	368	2	T_a	T_a	2	LGIUN	WT				
			5	T_a	T_a	2		WT		+		+
			6	T_a	T_a	2		WT				
			7	T_a	T_a	2		WT		+		
EBDC	1	576	4	T_a	T_a	2	No	Mut		+		+
EBDC	1	669	1	primary	T_a	2	LGIUN	Mut	WT	+		
EBDC	1	692	1	primary	T_a	2	No	Mut	WT	+		+
EBDC	1	703	1	primary	T_a	1	No	WT	WT	+		+
EBDC	1	746	1	primary	T_a	2	No	Mut	WT	+		+
EBDC	1	763	1	primary	T_a	2	No	Mut	WT	+		+
EBDC	1	815	1	primary	T_a	2	No	Mut	WT	+		+
EBDC	1	829	1	primary	T_a	2	No	Mut				+
EBDC	1	898	1	T_a	T_a	2	No	Mut	WT	+		+
EBDC	1	942	1	primary	T_a	2	LGIUN	Mut				+
EBDC	1	968	1	primary	T_a	2	No	Mut	WT	+		
EBDC	1	969	1	T_a	T_a	2	No	WT				
EBDC	1	972	1	primary	T_a	1	ND	Mut	WT	+		+
EBDC	1	997	1	primary	T_a	2	No	Mut	WT	+		+
EBDC	1	1060	1	primary	T_a	2	No	Mut	WT	+		+
EBDC	1	1086	1	primary	T_a	2	No	WT				+
EBDC	1	1105	1	primary	T_a	2	No	Mut				+
EBDC	1	1131	1	primary	T_a	1	No	WT	WT	+		+
EBDC	1	1146	1	primary	T_a	2	No	Mut	WT	+		+
EBDC	1	1206	1	primary	T_a	1	No	WT	WT	+		+
EBDC	1	1250	1	primary	T_a	1	ND	Mut	WT	+		+
EBDC	1	1294	1	primary	T_a	1	No	Mut	WT	+		
EBDC	1	1303	1	primary	T_a	2	No	Mut	WT	+		+
EBDC	1	1342	1	primary	T_a	1	LGIUN	Mut	WT	+		+
EBDC	1	1352	1	Primary	T_a	2	No	Mut	WT			+
gr 3	2	150	6	T_a	T_1	3	HGIUN	(Mut)*	WT	+		
EBDC	2	154	5	T_a	T_a	2	HGIUN	Mut	WT		+	
			6	T_a	T_a	2		Mut	WT		+	
			10	T_a	T_1	3		WT	WT		+	
T_1/gr 3	2	602	1	T_1	T_a	2	No	WT	WT			
			8	T_1	T_a	3		WT		+		
EBDC	2	747	3	T_a	T_a	2	HGIUN	WT	WT	+	+	+
			5	T_a	T_1	3		WT	WT	+	+	+
gr 3	2	775	1	primary	T_a	3	LGIUN	WT	WT	+		+
T_1/gr 3	2	780	1	T_a	T_1	3	HGIUN	Mut	WT			
T_1	2	794	1	T_1	T_1	2	No	Mut	WT	+		
gr 3	2	797	1	primary	T_a	3	HGIUN	WT	WT	+		+
gr 3	2	833	2	T_a	T_a	3	No	Mut		+		
EBDC	2	876	1	primary	T_a	2	No					+
			5	T_a	T_a	3		Mut		+		
T_1/gr 3	2	880	1	primary	T_1	3	LGIUN	Mut	WT	+		+
EBDC	2	925	1	T_a	T_a	2	No	Mut				+
gr 3	2	1066	1	primary	T_a	3	No	WT	WT	+		+
gr 3	2	1093	1	primary	T_a	3	HGIUN	Mut	Mut	+		+
EBDC	2	1145	1	primary	T_a	2	LGIUN	Mut				+
gr 3	2	1264	1	primary	T_a	3	No	Mut	WT	+		
gr 3	2	1330	1	primary	T_a	3	HGIUN	WT	WT	+		+

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Table 1. Clinical data, mutation status and array analyses performed on 115 bladder tumors from 85 patients, sorted by clinical course (Cont'd)

Risk at sampling	Clinical course	Patient no.	Visit no.	Highest previous Tstage	Actual Tstage	Actual grade	IUN in the course	*FGFR3* status	*TP53* status	Global gene expression analysis	SNP chip analysis	CIS classifier
gr 3	2	1350	1	primary	T_a	3	No	WT	WT	+		
T_1/gr 3	3	112	2	T_1	T_a	3	HGIUN	WT	WT	+	+	
			12	T_1	T_1	3		WT	WT	+	+	+
gr 3	3	140	8	T_a	T_a	2	No	WT	WT		+	
			12	T_a	T_a	3		WT	WT		+	
			16	T_a	T_1	3		WT	WT		+	
gr 3	3	166	5	T_a	T_a	3	LGIUN	Mut	WT		+	
			9	T_a	T_a	3		Mut	WT		+	
			14	T_a	T_1	3		Mut	WT		+	
T_1/gr 3	3	625	1	primary	T_1	3	No	WT	Mut	+		
T_1	3	686	3	T_1	T_a	2	No	WT	WT	+		
T_1	3	795	13	T_1	T_a	2	HGIUN	Mut	WT	+		+
gr 3	3	825	3	T_a	T_a	3	HGIUN	Mut	WT	+	+	+
			5	T_a	T_1	3		Mut	WT	+	+	
EBDC	3	865	1	T_a	T_a	2	LGIUN	Mut	WT	+	+	+
			2	T_a	T_1	3		Mut	WT	+	+	
gr 3	3	956	2	T_a	T_a	3	No	WT	WT	+		
T_1/gr 3	3	1024	1	primary	T_1	3	HGIUN	WT	Mut	+		+
gr 3	3	1070	1	primary	T_a	3	HGIUN	WT	WT	+		
T_1/gr 3	3	1182	1	primary	T_1	3	HGIUN	Mut		+		+
T_1/gr 3	3	1252	1	primary	T_1	3	HGIUN	WT				+
EBDC	3	1327	1	primary	T_a	2	No	WT				
T_1/gr 3	3	1337	1	primary	T_1	3	HGIUN	WT	WT	+		
T_1/gr 3	4	172	3	T_1	T_1	3	HGIUN	WT	WT		+	
			4	T_1	T_2-T_4	3		WT	WT		+	
T_1/gr 3	4	245	1	primary	T_1	3	HGIUN	WT	WT			
			3	T_1	T_1 †	3		WT		+		
			5	T_1	T_2-T_4	3		WT	WT	+		
T_1/gr 3	4	320	1	T_1	T_a	3	HGIUN	WT	WT			
			7	T_1	T_2-T_4	3		WT	WT	+		
T_1/gr 3	4	347	5	T_1	T_1	3	No	Mut	WT	+		
T_1/gr 3	4	365	1	primary	T_1	3	No	Mut	WT		+	
			2	T_1	T_2-T_4	3		Mut	WT		+	
T_1/gr 3	4	501	1	primary	T_1	3	LGIUN	Mut	WT	+	+	
			5	T_1	T_2-T_4	3		Mut	WT	+	+	
T_1/gr 3	4	607	1	primary	T_1	3	HGIUN	WT	WT	+	+	
EBDC	4	679	2	T_a	T_a	2	HGIUN	WT	WT	+		+
T_1/gr 3	4	744	1	primary	T_1	3	HGIUN	WT	Mut	+	+	
T_1/gr 3	4	839	1	primary	T_1	3	HGIUN	WT	Mut	+	+	+
			2	T_1	T_2-T_4	3		WT	Mut	+	+	
T_1/gr 3	4	941	4	T_1	T_a	3	No	Mut	WT	+	+	+
			6	T_1	T_2-T_4	3		Mut	WT	+	+	
T_1	4	962	10	T_1	T_a	2	LGIUN	WT		+		+
T_1/gr 3	4	966	2	T_1	T_2-T_4	3	HGIUN	WT	Mut	+		
T_1/gr 3	4	1013	1	primary	T_1	3	No	WT	Mut		+	
			3	T_1	T_2-T_4	3		WT	Mut		+	
T_1/gr 3	4	1017	1	primary	T_1	3	HGIUN	WT	WT	+	+	+
			3	T_1	T_2-T_4	3		WT	WT	+	+	
T_1/gr 3	4	1033	1	primary	T_1	3	ND	Mut	Mut		+	
			2	T_1	T_2-T_4	3		Mut	Mut		+	
EBDC	4	1058	3	T_a	T_a	2	No	Mut	WT		+	

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Table 1. Clinical data, mutation status and array analyses performed on 115 bladder tumors from 85 patients, sorted by clinical course (Cont'd)

Risk at sampling	Clinical course	Patient no.	Visit no.	Highest previous Tstage	Actual Tstage	Actual grade	IUN in the course	*FGFR3* status	*TP53* status	Global gene expression analysis	SNP chip analysis	CIS classifier
			10	T_a	T_1	3		Mut	Mut		+	
gr 3	4	1062	2	T_a	T_a	3	HGIUN	WT	WT	+		
			3	T_a	T_1	3		WT				
T_1/gr 3	4	1078	1	T_1 †	T_2-T_4	3	ND	WT	Mut	+		+
T_1	4	1083	1	T_a	T_1 †	2	No	WT	WT	+		+
T_1/gr 3	4	1276	1	primary	T_1	3	ND	WT	WT	+	+	
			2	T_1	T_1 †	3		WT				
			3	T_1	T_1 †	3		WT	WT	+	+	
T_1/gr 3	4	1321	1	T_1	T_2-T_4	4	No	WT		+		
T_1/gr 3	4	1336	1	primary	T_1	3	LGIUN	Mut	Mut	+		
T_1/gr 3	4	1345	1	primary	T_1	3	HGIUN	WT	WT	+		+
gr 3	4	1354	1	primary	T_a	3	No	WT	Mut	+		+
gr 3	4	1586	1	primary	T_a	3	No	Mut	WT	+		

Abbreviations: EBDC, expected benign disease course (stage T_a/grade 1-2); IUN, intraurothelial neoplasia; ND, not determined; WT, wild type; Mut, mutant; gr, grade.
*This tumor harbored as the only change the F386L mutation, whose biological significance is not clear. The tumor was therefore excluded in the statistical evaluation.
†Advanced cancer by clinical examination (bimanual palpation).

group differences. Low values indicated that group 1 had higher mean than group 2 (corresponding to gains in *FGFR3* wild-type tumors or losses in *FGFR3*-mutated tumors), and high values indicated that group 2 had higher mean (gains in *FGFR3*-mutated tumors or losses in *FGFR3* wild-type tumors). Long segments of SNPs with high (or low) values of d_j were more likely to be due to true differences; thus, the number of segments with length exceeding a fixed threshold (here, 10) and the maximum segment length were also calculated. Significance of the observed statistics was calculated using a permutation test. New groups of the same sizes were formed by permuting group labels 50,000 times, and the number of times (in %) a higher value (or lower) was observed was calculated.

Loss and gains: The pattern of "losses" and "gains" in the two groups were derived for each SNP in each array using the x_{ij} values. The empirical distribution of all SNPs in 113 normal samples was calculated. If x_{ij} is in the upper 1% tail of the distribution, "gain" is reported; if x_{ij} is in the lower 1% tail of the distribution, "loss" is reported. Whether the number of losses (gains) in the two groups for a particular SNP deviates from random was assessed, assuming losses (gains) were drawn without replacement from the pooled samples of the two groups (i.e., a test statistic based on the hypergeometric distribution was applied).

Gene expression array data

Preparation of cRNA from total RNA and subsequent hybridization and scanning of the Affymetrix GeneChip arrays was done as described previously (19). The biotinylated targets were hybridized to Eos Hu03 – customized Affymetrix GeneChip oligonucleotide arrays and to U133A Affymetrix GeneChip oligonucleotide arrays. For further details on the Eos Hu03 array design, expression measure, and normalization procedure, see Dyrskjot et al. (20). The U133A array expression measures were generated and normalized using the GC-RMA procedure implemented in the ArrayAssist software (Stratagene, La Jolla, CA).

Filtered gene sets were generated for each array type. Before analyzing the expression data from the Eos Hu03 GeneChips, all control probes were removed. Furthermore, we added 300 to all values to avoid negative values. Values were log 2 transformed, and only probes with expression levels above 8.64 ($\log_2$ 400) in at least three experiments and with max-min of $\geq$0.8 were selected. This filtering generated a gene set consisting of 9,045 probes. For the U133A expression data, we also removed the Affymetrix control probes before analysis. Values were log 2 transformed, and only probes with expression levels of >7 in at least three experiments and with max-min of $\geq$2 were selected. This filtering generated a gene set consisting of 8,858 probes.

The average linkage hierarchical cluster analysis of the tumor samples was carried out using a modified Pearson correlation as similarity metric. Genes and arrays were median centered and normalized to the magnitude of 1 before clustering using the Cluster software (21). Data were visualized using the Treeview software (21).

We linked the filtered probe sets from both microarray platforms by gene IDs (LocusLink), resulting in 6,257 linked probes. Then, we compared the sample groups in both data sets by computing common t values for all linked probes. For further details, see Supplementary Table 6.

Carcinoma *in situ* classifier

To investigate relations of *FGFR3* mutations to the "CIS pathway," we used a 76-gene molecular "CIS classifier" based on the 100 significant genes, which we published previously (16). We applied an in-house fabricated microarray platform as described in Dyrskjot et al. (20).

Results

FGFR3 mutations

A total of 55 *FGFR3* mutations were found in 115 tumors (46%; Table 1). At least one tumor showed an activating mutation in 43 of the 85 patients (51%). See Supplementary Information for details of the identified *FGFR3* mutations.

The analysis of metachronous tumors in 22 patients showed identical mutation pattern in 21 patients. In a single patient (#154), no mutation was found in the subsequent tumor, in contrast to two previous tumors harboring an activating mutation. We did not observe any patient who acquired a *FGFR3* mutation during the disease course (Supplementary Table 3).

At analysis time, 35 tumors were early tumors with expected benign disease course (stage T_a/grade 1-2 tumors, no previous T_1 or grade 3 disease). Twenty-six of these tumors showed an

activating *FGFR3* mutation (74%). Among tumors with either previous or present stage T_1 or grade 3 disease, *FGFR3* mutations were less frequent, as only 17 of 50 (34%) presented this feature. This difference was highly significant ($P = 0.0003$, χ^2).

Association between *FGFR3* mutations and the development of bladder cancer disease

Recurrence frequency. Five of nine *FGFR3* wild-type tumors with expected benign disease course developed frequent recurrences and so did a similar fraction (15 of 26) of *FGFR3*-mutated tumors (Fig. 1). Of the tumors from patients with T_1 or grade 3 disease, 12 of 24 (50%) *FGFR3* wild-type tumors recurred frequently, and in mutated tumors, 13 of 15 (87%). This difference was significant ($P = 0.02$, Mann-Whitney; Fig. 1A).

***Intraurothelial neoplasia/carcinoma* in situ.** HGIUN (CIS or dysplasia Bergkvist grade 3) are recognized as tumor precursors with a high risk of progression (1–5). The significance of LGIUN (dysplasia Bergkvist grade 1 and 2) is less pronounced (5, 22). During follow-up, flat intraurothelial neoplasias were observed in 37 of 79 patients (47%). HGIUN was more frequently observed with *FGFR3* wild-type tumors than with mutated tumors (19 wild type versus 6 mutated; $P = 0.0013$). LGIUN was observed in 9 of 40 patients (22.5%) with *FGFR3*-mutated tumors and in only 3 of 39 patients with wild-type tumors (7.7%). The difference in mutation status between HGIUN and LGIUN was highly significant ($P = 0.003$; Fig. 1B).

In the 37 disease courses where intraurothelial neoplasias were observed, 12 of 22 patients (55%; one LGIUN) progressed when the tumors were *FGFR3* wild type, and only 2 of 15 (13%, both LGIUN) progressed when the tumors were mutated ($P = 0.01$).

Clinical course/progression. After initial analysis, most of the early tumors followed the benign courses 1 and 2 (31 of 35, 89%); two developed a high risk course (recurrent T_1 or grade 3 disease), and two progressed (stage $\geq T_2$-T_4). Importantly, there was no difference in outcome between *FGFR3*-mutated and wild-type tumors ($P = 0.46$, Mann-Whitney; Fig. 1C). Among tumors with previous or actual T_1 or grade 3 disease, 13 followed the benign course 2 (no stage T_1 or grade 3 recurrences), 13 followed the high-risk course 3 (recurrent stage T_1 or grade 3 disease), and 24 followed the progressive course 4 (stage $\geq T_2$-T_4). Again, we observed no difference in outcome between *FGFR3*-mutated and wild-type tumors ($P = 0.35$, Mann-Whitney; Fig. 1C).

Of the 16 muscle-invasive cancers analyzed (four at initial analysis and 12 during the disease course; see Table 1), four (25%) had a *FGFR3* mutation. All these tumors already had the same mutations at the superficial stage; the four tumors that were progressed at initial analysis were wild type.

Correlation with *TP53* mutation

We screened tumors from 69 patients for both *FGFR3* and *TP53* mutations. Bidirectional sequencing of exons 5 to 8 of *TP53* was done, as *TP53* mutations are most frequently reported in these exons (23). We observed *TP53* mutations in tumors from 12 patients (17%), *FGFR3* mutations in tumors from 35 patients (51%), and both *TP53* and *FGFR3* mutations in tumors from four patients (5.8%); no mutations were found in tumors from 27 patients (39%; Table 1). The mutations are listed in detail in Supplementary Table 1.

As expected, *TP53* mutations were associated with a progressive disease course, because nine of the 12 patients (75%) progressed. Tumors with expected benign disease course (stage T_a/grade 1-2) at analysis had no *TP53* mutations. However, we observed an acquired *TP53* mutation in one of the later tumors of this group, with subsequent progression (#1058). In the group of stage T_1 or grade 3 tumors, *TP53* mutations were found in 3 of 17 (18%) *FGFR3*-mutated tumors and 8 of 32 (25%) *FGFR3* wild-type tumors. This difference was not significant and did not support previous publications (13, 14).

Correlation with chromosomal instability pattern

Tumors from a total of 19 patients were analyzed using 10K SNP arrays and screened for *FGFR3* and *TP53* mutations. Four tumors with expected benign disease course at the time of the first analysis all developed at least T_1 tumor during the course; thus, no tumors with the low-risk course 1 were included. For detailed information, see Table 1 and Koed et al. (17).

The results of the loss of heterozygosity (LOH) study were published previously (17); however, the addition of *FGFR3*

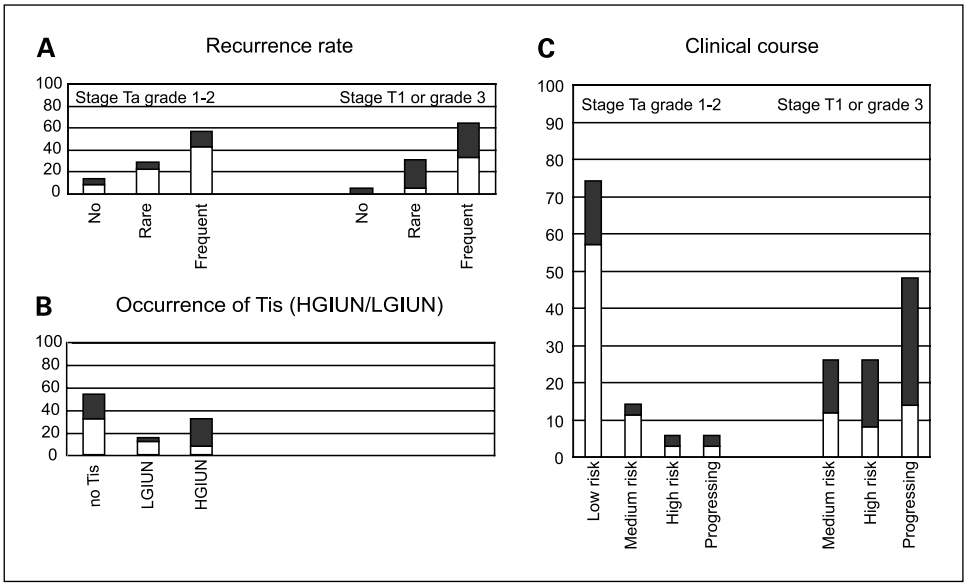

Fig. 1. *FGFR3* mutation status and outcome variables of 85 patients with superficial bladder tumors. White columns, *FGFR3*-mutated tumors; black columns, *FGFR3* wild-type tumors. All numbers are percentages. *A,* recurrence rate: rare, ≤1 recurrence/year; frequent, ≥2 recurrences/year. Recurrence was assessed from 74 patients, 35 with stage T_a/grade 1-2 and no previous T_1 or grade 3 disease (*left*) and 39 with previous or present stage T_1 or grade 3 tumors (*right*). The missing cases experienced progression within 1 year; accordingly, the recurrence frequency could not be assessed. *B,* occurrence of concomitant LGIUN or HGIUN. This was assessed from 80 patients; in the missing cases, no "preselected site" biopsies were taken. *C,* clinical disease courses: assessed from 35 patients with stage T_a/grade 1-2 tumors and no previous T_1 or grade 3 disease (*left*) and 50 tumors with previous or present T_1 or grade 3 tumors (*right*).

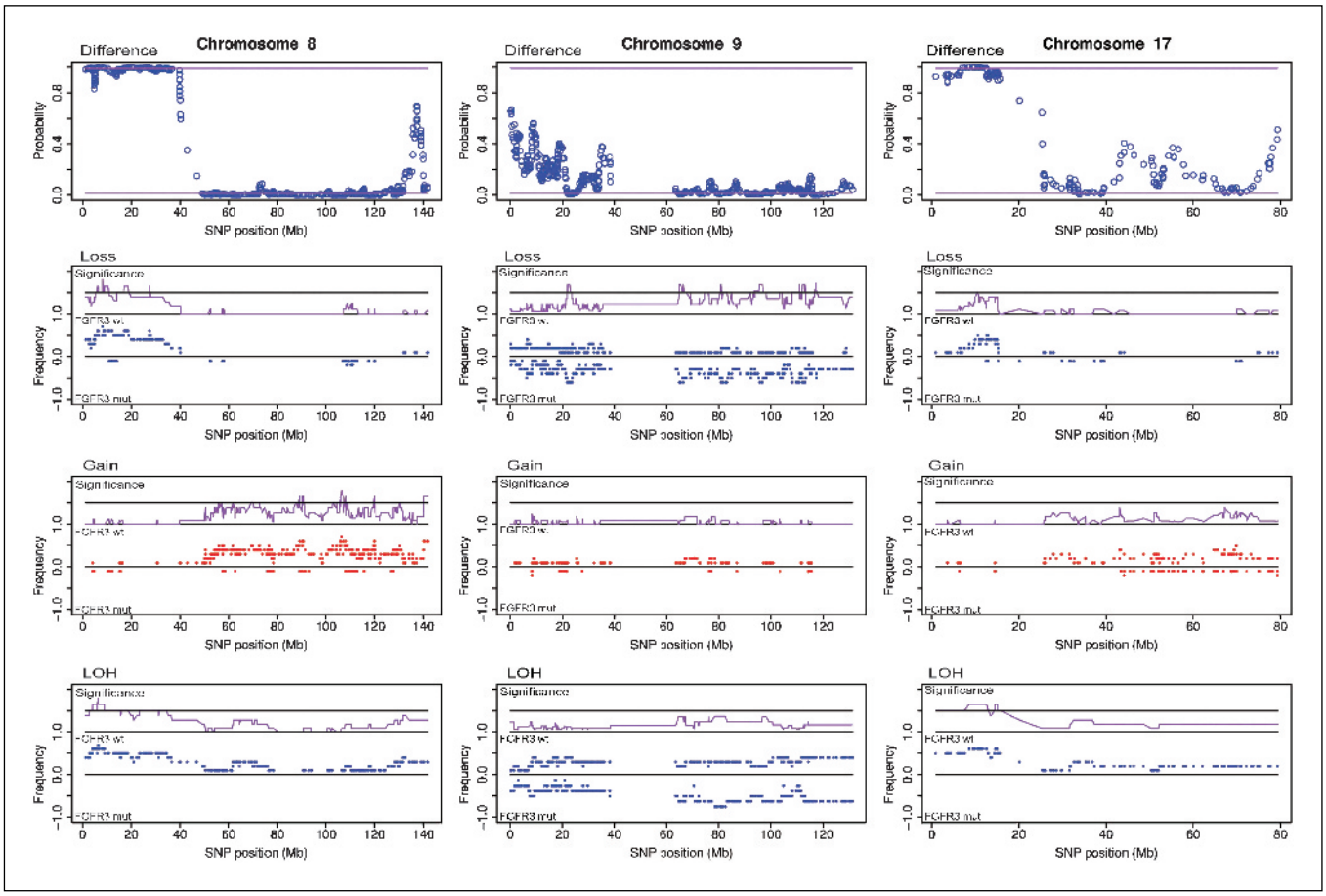

Fig. 2. LOH and DNA copy number changes at chromosomes 8 (*left*), 9 (*middle*), and 17 (*right*). Difference between the *FGFR3* mutant and wild-type groups; the difference due to either LOH and/or gain and/or loss of chromosomal material. Top, test for difference between *FGFR3* mutant and wild-type groups. Probability for each SNP to obtain a d_i higher than observed. Values close to 0 indicate that the average in *FGFR3* wild-type tumors is higher than the average in mutated tumors; values close to 1 indicate the reverse. Values close to 0.5 are insignificant changes. Inserted lines, 1% and 99% percentiles, respectively. Top middle, frequency of losses in the mutant and wild-type groups (*blue dots*). Bottom, *FGFR3*-mutated tumors (0 to −1); top, *FGFR3* wild-type tumors (0 to 1.0). Top, probability of obtaining the observed distribution of losses in the two groups by chance alone in logarithmic scale. Inserted black line, 0.01 significance level; when the probability is above this line, the finding is significant (as in chromosome 8 at 10 Mb). Bottom middle, frequency of gains in the two groups (*red dots*) in top middle. Bottom, frequency of LOH in the two groups as in top middle. Derived using the method described in Koed et al. (17).

mutation analysis and computation of DNA copy number changes are novel. The LOH study revealed few regions of LOH in most nonprogressing tumors, predominantly on chromosome 9q (66% LOH). LOH of >33% of the informative SNPs throughout the genome, which indicated general chromosomal instability, was seen in progressing tumors. This pattern was characterized by LOH at distinct areas of chromosome 17p13.1 (the *TP53* locus) and at two loci at chromosomes 8p12 and 8p23.1. The tumors from three patients (30%) retained chromosomal stability throughout the cancer development [i.e., also in the progressed tumors (stage T_2-T_4)]. This observation illustrated the complexity of the bladder cancer development.

We computed DNA copy number changes in tumors from 17 patients. The data transformation procedure (see Materials and Methods) enabled an impression of gained and reduced DNA copy number regions in addition to the LOH analysis. See Supplementary Fig. 1 for a survey of all observed copy number changes at late superficial stages.

We found no correlation between *FGFR3* mutation status and general chromosomal instability (defined as LOH of >33% of the informative SNPs). We did, however, observe a distinct pattern of chromosomal changes that correlated with the *FGFR3* mutation status. This was shown for the three most significantly affected chromosomes in bladder cancer: chromosomes 8, 9, and 17 (Fig. 2). Chromosome 8 is known to exhibit complex chromosomal instability in invasive bladder cancer, typically with losses of 8pter-8p12 and gains of the rest of the chromosome. Changes at chromosome 17 typically include loss of 17p13 (the *TP53* locus), also preferentially found in invasive tumors. Figure 2 shows that these patterns also were found in superficial, *FGFR3* wild-type tumors, whereas mutant tumors had only few and random changes at chromosomes 8 and 17 ($P < 0.01$). In contrast, losses on chromosome 9, the most frequent chromosomal change observed in bladder cancer, were more frequent in *FGFR3* mutant than wild-type tumors. This was significant ($P < 0.01$) at 9p21 (the *MTS1/INK4* locus) and at broad regions of the 9q chromosomal arm.

The LOH analysis of the same tumors (Fig. 2, *bottom*) and Supplementary Fig. 2 illustrate the complexity of the chromosomal changes. For example, both LOH and copy number gains on chromosome 8q were present in some *FGFR3* wild-type tumors, probably due to loss of one allele with consecutive amplification of the remaining allele. The significant decrease in copy number at 9p21 in *FGFR3* mutant tumors was not

reflected by a similar increase in frequency of LOH. This is explained by homozygous deletions (no LOH); the corresponding tumors had extremely low copy number signals (<0.01 percentile; see Supplementary Fig. 2). The complex chromosomal pattern remained virtually unchanged in the progressed tumors of the same patients (Supplementary Fig. 2, *right*).

In general, significant copy number gains were only seen in *FGFR3* wild-type tumors. A survey of the significantly ($P < 0.01$) gained regions is provided in Supplementary Table 4. Supplementary Data also includes figures of differences in copy numbers, gains, losses, and LOH between *FGFR3* wild-type and *FGFR3*-mutated tumors for all chromosomes (Supplementary Figs. 3 and 4). Furthermore, the number of 10 SNP segments that change in each chromosome and the length of these changes are listed together with the associated probabilities (Supplementary Table 5). The reported results were not tested for relation to tumor stage, because only late superficial tumors (mostly stage T_1) were included in the analysis.

Correlation with gene expression pattern

We did genome-wide expression profiling using tumors from 64 patients and two different microarray platforms: Customized Affymetrix GeneChips (34 tumors) and Affymetrix U133A GeneChips (33 tumors). Three tumors were analyzed on both

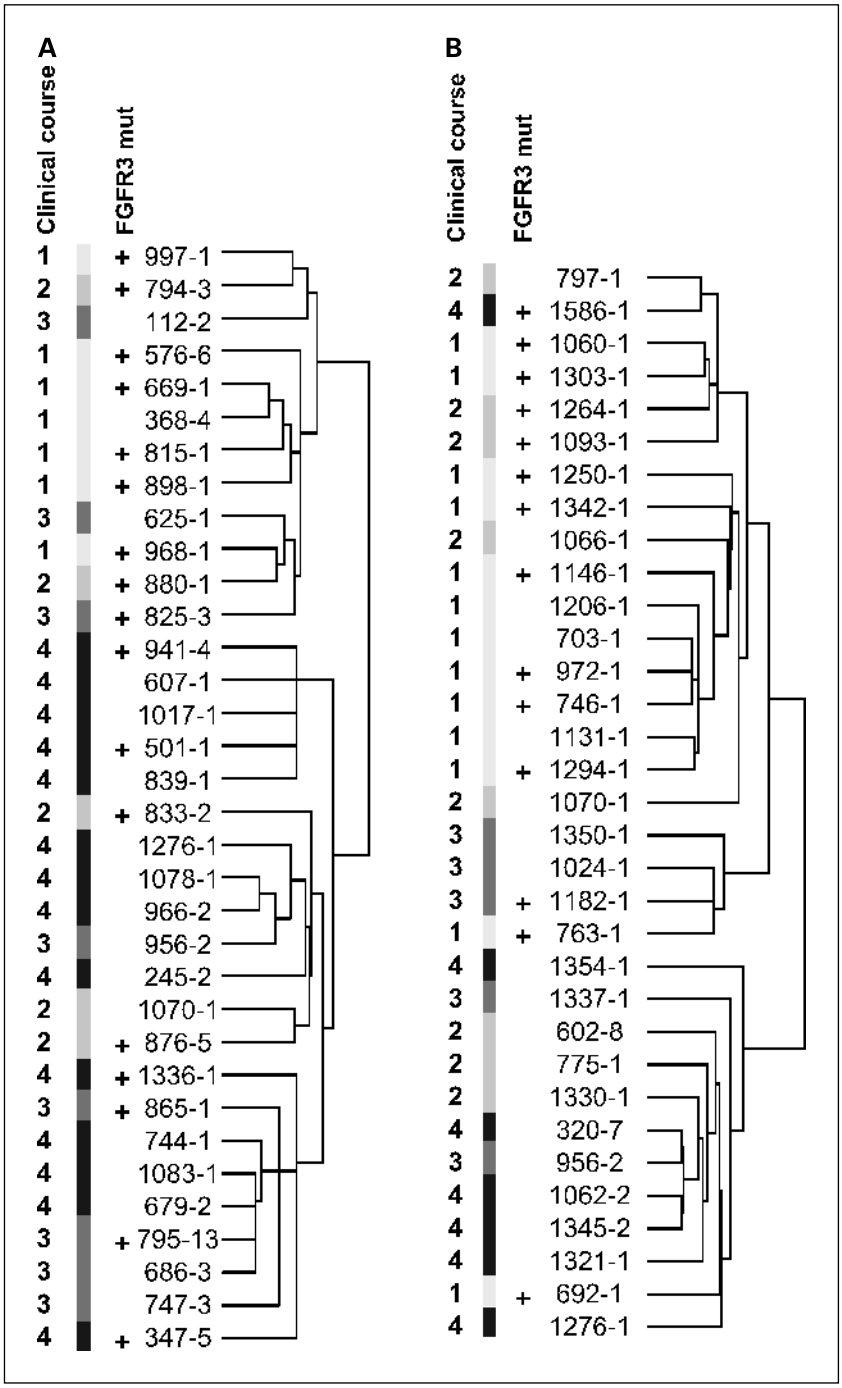

Fig. 3. Hierarchical clustering of 64 bladder tumors screened for gene expression by oligonucleotide microarrays. *A,* 34 tumors analyzed using customized GeneChip microarrays. *B,* 33 tumors analyzed using U133A GeneChip microarrays. Three tumors were analyzed on both platforms (#956, #1070, and #1276). +, *FGFR3* mutations. Numbers and the colored bars refer to the clinical disease course groups: 1, low risk; 2, medium risk; 3, high risk; 4, progressing.

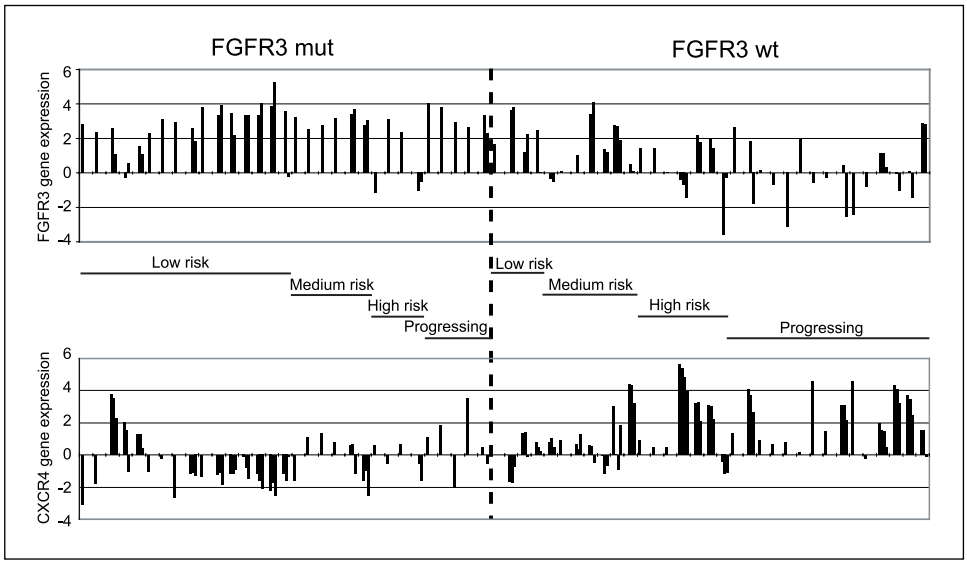

Fig. 4. Gene expression (log 2 fold change) of the *FGFR3* and the *CXCR4* gene transcripts in individual superficial tumors. More than one bar per patient indicate various probe sets. *FGFR3*-mutated tumors (*left*) and *FGFR3* wild-type tumors (*right*). Furthermore, tumors are sorted according to clinical courses as indicated.

arrays. *FGFR3* mutations were found in 31 tumors, whereas 33 were wild type. Twenty tumors progressed to muscle-invasive stages; five (25%) had *FGFR3* mutations. The *FGFR3*-mutated tumors were about equally distributed on the two microarray platforms (17 versus 14; Fig. 3).

We filtered out the genes that showed minimal variation across all samples and did hierarchical cluster analysis based on these two filtered gene sets. As expected from previous work (19), the two principal clusters generated with both microarray types divided tumors with benign (low/medium risk) from those with malignant (high risk/progressing) clinical courses with only few exceptions. The "benign" cluster contained most of the mutated tumors; the "malignant" cluster contained most of the wild-type tumors (Fig. 3). There was no obvious subclustering beyond the "benign-malignant" division.

We identified differentially expressed genes on both platforms in a *FGFR3* mutation–dependent manner. We found 135 of 6,257 probe sets being differentially expressed with a significance threshold of 0.005 (*t* test; expected number: 31). One of the most significant differentially expressed genes was the *FGFR3* gene itself, whose expression was increased in mutated tumors but also in benign, wild-type tumors (Fig. 4). Most of the differentially expressed genes were down-regulated in *FGFR3*-mutated tumors compared with *FGFR3* wild-type tumors. For example, Fig. 4 shows the differential expression of the *CXCR4* gene. The differentially expressed genes were involved in cell-matrix interaction, invasion, angiogenesis, apoptosis, and metastasis and thus seemed related to the oncogenic process associated with tumor development. However, our results did not provide evidence that *FGFR3* mutations are driving this process. A complete list of significantly altered genes is found in Supplementary Table 6.

Correlation with carcinoma *in situ* classifier

Seventy tumors were classified using the CIS classifier; 50 of these were also analyzed for *FGFR3* mutations (Table 1). We found a highly significant ($P = 0.0008$, χ^2) correlation between the tumors with *FGFR3* mutations and the tumors classified as "no CIS." The performance of the classifier and the correlation with *FGFR3* mutations is illustrated in Table 2. Notably, none of the benign *FGFR3* wild-type tumors showed the molecular "CIS" signature.

Discussion

It has become widely accepted that *FGFR3* mutations are very early markers of papillary bladder tumor development. This does not a priori mean a good prognosis. In fact, in this longitudinal study, we found that *FGFR3*-mutated superficial tumors actually do progress and retain their mutation and chromosomal instability patterns during progression. Our results provide evidence that the decreasing frequency of *FGFR3* mutations in later stages of tumor development is caused by the emergence of tumors following a different molecular pathway with no *FGFR3* mutations. The tumors of this latter pathway are associated with CIS. We show here that the tumors within each pathway are molecularly related in terms of chromosomal instability and expression patterns.

FGFR3-mutated tumors have malignant potential, although *FGFR3* mutations apparently are not directly involved in cancer progression. It was speculated that the mutation may alter cellular proliferation and provide a growth advantage, but that cell cycle and/or apoptosis mechanisms and genomic stability may still be maintained (13). Recent results support this by suggesting that *FGFR3* mutations may be involved in bypassing the G_1 checkpoint of the cell cycle, leading to replicative stress and activation of the DNA damage response mechanisms (24). This would result in precursor lesions of benign appearance. However, as a key event of malignant development due to sustained replicative stress, the DNA damage response mechanisms may become impaired, leading to genomic instability and cancer progression (24). To investigate the relative malignant potential of *FGFR3* mutations, we stratified the tumors in this study into early stage (T_a) well-differentiated (grade 1-2) tumors (with an expected benign disease course) and tumors presenting features of malignant development (stage T_1 or grade 3).

Table 2. Performance of a 76-gene molecular CIS classifier and correlation with *FGFR3* mutations

CIS classifier result	Concomitant HGIUN	Concomitant LGIUN	No IUN in the course	Total	*FGFR3* wild type	*FGFR3* mutated	Total
CIS	13	10	6	29	15	5	20
No CIS	5	9	27	41	8	22	30
	18	19	33	70	23	27	50

Abbreviation: IUN, intraurothelial neoplasia.

After stratification, neither the recurrence rate, the frequency of progression, nor *TP53* mutation frequency differed between *FGFR3*-mutated and *FGFR3* wild-type tumors, in contrast to previous reports (10, 13–15). This suggested that precursor lesions harboring a *FGFR3* mutation have about the same malignant potential as wild-type lesions at comparable developmental stages. However, the *FGFR3* mutations are observed very early in development of papillary tumors and could be a driving force in the formation of these tumors. As papillary tumors are diagnosed at early stages due to bleeding and other symptoms, it may seem that FGFR3-mutated tumors have a low malignant potential. A closer examination of these early lesions, however, shows that there is no difference in the likelihood of progression between FGFR3-mutated and non-mutated cases.

We further investigated differences in the papillary/*FGFR3*-mutated and CIS pathways, using a selection of tumors previously screened for genome-wide molecular changes by SNP and gene expression microarrays and screened the tumor samples for *TP53* and *FGFR3* mutations in parallel. In fact, we found differences both in the patterns of chromosomal imbalance and in gene expression profiles. Only advanced developmental stages (stage T_1) were analyzed with SNP microarrays. *FGFR3* mutations were correlated with LOH and homozygous deletions of the well-known regions on chromosome 9 (7), whereas the wild-type tumors had chromosomal features usually related to CIS, including the well-documented patterns of gains and losses on chromosome 8 with the characteristic breakpoints at fragile sites (25–27), LOH of 17p13 (17), and extensive copy number gains at characteristic regions of the genome (26). Because the progression frequencies were the same for both groups, the chromosomal patterns were characteristic for pathways rather than developmental stage. In addition, the different chromosomal patterns remained unchanged in the corresponding progressed tumors, indicating molecular relationship within the pathways. However, the extent of chromosomal instability and the frequency of *TP53* mutations increased in progressing/progressed tumors of both groups (*FGFR3* mutated and wild type). General chromosomal instability has previously been associated with *TP53* changes (28). This is consistent with the above-cited model (24) and the assumption that both chromosomal instability and *TP53* changes, in contrast to *FGFR3* mutations, are markers of advanced cancer development.

We also found differences in gene expression profiles between *FGFR3*-mutated and *FGFR3* wild-type tumors. However, the analysis was biased by different developmental stages (20). This is illustrated in Fig. 3. To further validate the different pathways, we applied a recently published molecular CIS classifier (16). This classifier showed good classification results for *FGFR3*-mutated tumors, which further supported the existence of different molecular pathways. Interestingly, early-stage *FGFR3* wild-type tumors did not express the CIS signature. Moreover, the *FGFR3* gene was up-regulated in these tumors as observed in *FGFR3*-mutated tumors but in contrast to tumors of the CIS pathway (Fig. 4). This confirmed an earlier reverse transcription-PCR–based report (8). Thus, *FGFR3* wild-type tumors with expected benign disease course probably belong to a different pathway.

In conclusion, this study addressed the role of *FGFR3* mutations in bladder cancer development and the correlation with specific molecular pathways. Our results suggest a central role of *FGFR3* mutations in the early development of papillary bladder tumors. We present evidence of different molecular pathways of *FGFR3*-mutated tumors and CIS-associated tumors by showing different chromosomal and gene expression changes. However, the progression of advanced bladder cancer was unaffected by *FGFR3* mutations.

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