Activation of the Fibroblast Growth Factor Receptor 3 in Bladder Cancer

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Activatie van de fibroblast groeifactor receptor 3 in blaaskanker

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CONTENTS

LIST OF ABBREVIATIONS

CIS	carcinoma *in situ*
CGH	comparative genomic hybridization
CK20	cytokeratin 20
ECM	extracellular matrix
EN	epidermal nevi
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FUH	flat urothelial hyperplasia
HNPCC	hereditary nonpolyposis colorectal cancer
IHC	immunohistochemistry
LOH	loss of heterozygosity
MA	microsatellite analysis
MI	methylation index
MM	multiple myeloma
MSI	microsatellite instability
SADDAN	severe achondroplasia with developmental delay and acanthosis nigricans
siRNA	small interfering RNA
SK	seborrheic keratosis
SSCP	single-strand conformation polymorphism
TD	thanatophoric dysplasia
TUR	*trans*-urethral resection
UCC	urothelial cell carcinoma
UTT	upper urinary tract tumor

CHAPTER 1

General introduction

INTRODUCTION

Bladder cancer is the fifth most common cancer in the western world with an incidence of 20 new cases per year per 100,000 people in the U.S. The majority (77%) of bladder tumors occur in men, leading to a male:female ratio of 3:1 [1]. Risk factors are old age, cigarette smoking, and work-related contact with cyclic organic chemicals. Most bladder tumors (>90%) are urothelial cell carcinomas (UCC). Approximately 80% of patients present with noninvasive (pTa, CIS) or superficially invasive (pT1) tumors at the time of initial diagnosis, whereas the remaining 20% present with muscle-invasive tumors (pT2-4) (for description of staging and grading, see [2]).

Patients with muscle-invasive disease are at risk for developing distant metastases, and therefore have to undergo cystectomy with adjuvant chemotherapy. Nevertheless, their 5-year survival is only 50%. Generally, treatment of pTa and pT1 tumors consists of surgical removal by *trans*-urethral resection, and patients are closely monitored by cystoscopy afterwards since these tumors recur in about 60% of patients and they may progress to invasive disease in 20-25% [3, 4]. The gold standard for the detection of recurrences currently remains cystoscopy, which is an uncomfortable and invasive procedure for the patient. The 5-year survival of patients with pTa/pT1 tumors is 90% [2]. Therefore, the prevalence of bladder cancer is high. Because many patients have to be monitored for a long time, bladder cancer is one of the most expensive cancers in health care [5].

Several genetic changes may occur in bladder cancer, but a mutation in the fibroblast growth factor receptor 3 (*FGFR3*) gene is the most common and most specific genetic abnormality in bladder cancer. Interestingly, these mutations were associated with bladder tumors of low stage and grade [6], which makes the *FGFR3* mutation the first marker that can be used for diagnosis of noninvasive bladder tumors.

FIBROBLAST GROWTH FACTOR RECEPTOR 3

The fibroblast growth factor receptor family

The fibroblast growth factor (FGF) receptor family consists of four transmembrane tyrosine kinase receptors (FGFR1-4), and 23 FGF ligands have been described [7]. The receptors are structurally similar to each other: they consist of an extracellular domain that includes an NH_2-terminal hydrophobic signal peptide followed by three immunoglobulin (Ig)-like domains, a hydrophobic transmembrane domain, and an intracellular split tyrosine kinase domain (Figure 1). FGFRs are highly conserved at the amino acid level: FGFR1 and FGFR2 are most closely related (72% aa identity), whereas FGFR1 and FGFR4 are the least closely related (55% identity) [8]. Another FGFR was discovered, FGFR5, that lacks the kinase domain [9, 10]. The mitogenic activity of FGFs is not restricted to fibroblasts, but stimulates many cell types, including endothelial cells and chondrocytes [11]. The diversity in binding specificity of FGFRs for the different FGFs and the formation of heterodimers between FGFRs leads to a large combinatorial set of possible interactions [12].

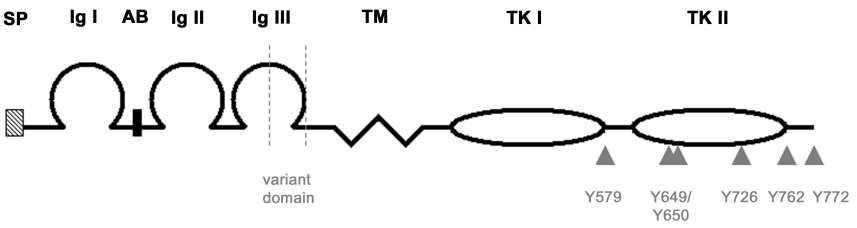

Figure 1. Structure of FGFR3 protein and tyrosine residues.
The second half of Ig III is a variant domain, coded either by exon 8 (variant IIIb) or exon 9 (variant IIIc). Y726 is the most important residue for downstream signalling; Y762 is the binding site for PLCγ; Y772 is not an autophosphorylation site. SP, signal peptide; Ig, immunoglobulin-like domain; AB, acid box; TM, transmembrane domain; TK, tyrosine kinase domain.

FGFR3 variants and tissue distribution

Alternative splicing

Alternative splicing of mRNA is responsible for generating multiple forms of FGFRs [8, 13, 14], which all have different ligand properties, and are cell- and tissue-specific, thus increasing the variety of the FGFR receptor family. Two forms of

FGFR3 exist as a result of splicing: FGFR3 IIIc, in which exon 8 is not present, and FGFR3 IIIb, where exon 9 is missing [13, 15, 16] (Figure 1). The IIIb splice variant appeared to be highly specific for FGF-1 [13], although it also binds FGF-9 [12], and its expression was shown to be restricted to epithelial cells and tissues [17]. In contrast, FGFR3 IIIc can be activated by FGF-1, -2, -4, -8, -9, -17, -18, and -23 [18], and is highly expressed in the developing brain, in the spinal chord, and in all bony structures [19]. The lineage-specific expression of the IIIb and IIIc isoforms of FGFRs enables interaction between the epithelial and mesenchymal layers during development in response to different FGFs [18]. Recently, a third splice variant, FGFR3 Δ8-10, was identified. This form is secreted by normal urothelial cells and acts as a dominant-negative regulator of the full-length receptor [20].

Expression in normal tissue

Biosynthesis of FGFR3 is characterized by three isoforms with various degrees of N-glycosylation: a 98 kDa unglycosylated protein, a 120 kDa intermediate membrane-associated glycoprotein, and a 130 kDa mature glycoprotein [15]. FGFR3 is activated upon ligand binding, which induces receptor dimerization, *trans*-phosphorylation and activation. This leads to activation of specific signal transduction pathways and expression of FGF target genes, critically required during embryogenesis, angiogenesis and tissue repair [11]. In the developing mouse embryo, Fgfr3 transcripts are expressed predominantly in brain, spinal chord, and cartilage rudiments of developing bone, and in adult animals Fgfr3 transcripts were detected in brain, kidney, skin, and lung [21]. In humans, FGFR3 is expressed in brain, kidney, and testis (www.expasy.org/uniprot/P22607#comments). FGFR3 probably plays a role in brain development by regulating its size through controlling proliferation and apoptosis of cortical progenitors [22]. Expression of FGFR3 has also been found in normal human bladder tissue [20, 23].

FGFR3 and disease

The *FGFR3* gene is located on chromosome region 4p16.3 [24]. It consists of 19 exons and 18 introns spanning 16.5 kb [16], and encodes a 4.4 kb mRNA [15]. There are two mechanisms that cause abnormal activation of FGFR3: translocation of chromosome 4 to chromosome 14 leading to overexpression, and activating point mutations in the *FGFR3* gene.

Skeletal dysplasias

Activating *FGFR3* germline mutations lead to short stature in humans due to growth retardation and skeletal dysplasia. The first mutation that was reported was a Gly → Arg missense mutation (G382R)[1] in achondroplasia, which is the most common form of dwarfism in humans [25, 26]. Other skeletal disorders that are caused by *FGFR3* mutations include the lethal thanatophoric dysplasias I (TDI; R248C, S249C, G372C, S373C, Y375C, stop codon) and II (TDII; K652E) [27, 28], hypochondroplasia (N542K) [29], Crouzon syndrome (A393E) [30], craniosynostosis (P252R) [31], and severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN; K652M) [32]. Recently, an *FGFR3* mutation (D515N) has also been reported in lacrimo-auriculo-dento-digital (LADD) syndrome [33].

Loss of FGFR3 function caused by a dominant negative missense mutation in the tyrosine kinase domain leads to tall structure and hearing loss in humans [34], which corresponds to the bone overgrowth and inner ear defect in mice that Colvin *et al.* found after targeted disruption of the *FGFR3* gene (*Fgfr3*$^{-/-}$) [35]. These results confirm that FGFR3 is a negative regulator of bone growth.

Cancer

Multiple myeloma (MM) is caused by several chromosomal translocations to the IgH locus (14q32), including the translocation t(4;14)(p16.3;q32) that leads to overexpression of wild type FGFR3 (generally isoform IIIc). This inappropriate expression leads to myeloma cell proliferation and prevents apoptosis [36]. However, in later stages of the disease process activating mutations in the selectively expressed translocated allele of the *FGFR3* gene occur [37, 38], which probably play a critical role in tumor progression of the subset of MM carrying the t(4,14) translocation. Strikingly, *FGFR3* and Ras mutations appear to be mutually exclusive in MM [37]. Decreased expression of FGFR3 in MM is associated with apoptosis, differentiation, and downregulation of VEGF. Inhibition of FGFR3 by either tyrosine kinase inhibitors [40, 41] or ribozyme-targeted downregulation [42] may therefore present a therapeutic opportunity for MM carrying the t(4;14) translocation.

Next to MM, activating somatic *FGFR3* mutations were also found in urothelial cell carcinomas (bladder) adenocarcinomas (colon) and squamous cell carcinomas (cervix, nasopharynx, mouth) (Table 1). *FGFR3* mutations are most frequent in bladder cancer, and are present in 50% of tumors. However, they only

[1] all amino acid numbers in this chapter refer to the FGFR3 IIIb isoform

sporadically occur in tumors of the colon [43, 44], cervix [44-49], and nasopharynx [48]. Cervical and nasopharyngeal carcinoma are the two major virus associated squamous cell carcinomas, and they exclusively harbor the S249C mutation. In oral squamous cell carcinoma (OSCC), a particular *FGFR3* mutation (G697C) was present in 62% of tumors, suggesting that FGFR3 may play an important role in oral tumorigenesis [50]. However, these data are now controversial since another study comprising 39 OSCC did not show any G697C mutations, which indicates that this mutation is unlikely to be a common oncogenic mutation in OSCC generally [51]. *FGFR3* mutations were not found in prostate, brain, renal cell, breast, ovarian, rectal, stomach, lung, and oesophageal tumors [44, 52, 53].

Benign skin lesions

FGFR3 mutations are also frequently found in benign skin lesions. When targeting the FGFR3-S249C transgene to the mouse epidermis with the keratin 5 promoter, Logie *et al.* found benign tumors with no malignant potential on the eyelids, snout, throat, and upper trunk. Since these lesions had several histological features in common with a benign skin lesion in humans called seborrheic keratosis (SK), they analyzed a series of 62 SKs for *FGFR3* mutations, and somatic activating *FGFR3* mutations identical to previously identified mutations were found in 39% of tumors [54]. A subgroup of SK, adenoid SK, seem to be characterized by a higher frequency of FGFR3 mutations (85%) [55]. Both groups showed seven different codon mutations with comparable frequencies, although R248C was the most frequent mutation in the adenoid SK series (38%). In contrast, mutations almost exclusively occur at codon 248 in a series of 39 epidermal nevi (EN), indicating that FGFR3 activation by the R248C mutation plays an important role in the development of this rare benign skin lesion. EN are benign skin lesions that are caused by a mosaicism which develops from a postzygotically mutated keratinocyte. The occurrence of R248C in multiple EN from one patient and the absence of mutations from adjacent normal tissue suggests that a mosaicism of the R248C mutation exists in these patients [56]. Considering the discovery of large monoclonal patches in urothelium [57], and the association of EN with urothelial tumors, mosaicisms of *FGFR3* mutations might also occur in the bladder; however this requires further study [58].

Table 1. Overview of literature on *FGFR3* mutations in cancer.

Cancer	Tumor features	*FGFR3* mutations	Percentage	Remarks	References
multiple myeloma	tumors, t unknown	0/80	0%		[59]
	tumors with t(4;14)	1/7	14%		[37, 60]
	tumors with t(4;14)	1/11	9%		[61]
	tumors with t(4;14)	1/24	5%		[62]
bladder	urothelial papillomas	9/12	75%		[63]
	pTaG1 with associated wt EN	0/2	0%		[58]
	pTaG1 tumors	68/79	86%		[63]
	pTa/T1 tumors	43/85	51%		[64]
	pTa/T1 tumors	46/75	61%		[65]
	pTa/T1 tumors	387/764	50%	exon 7/10 only	[66]
	pT1G3 tumors	20/119	17%		[67]
	primary UCCs	9/26	35%		[45]
	primary UCCs	172/286	60%		[68]
	primary UCCs	153/260	59%		[69]
	primary UCCs	43/107	40%		[70]
	primary UCCs	32/81	40%		[71]
	UCCs	26/63	41%		[72]
	UCCs	34/71	48%		[73]
	UCCs	48/132	36%		[6]
	UCCs	25/81	31%	TD mut only	[74]
	UCCs	181/297	61%		[75]
	UCCs	26/59	44%		[76]
	UCCs	54/98	55%		[77]
	UCCs	43/95	45%		[78]
	UCCs	69/127	54%	exon 7/10 only	[79]
	tumors from patients <20 y	0/10	0%		[80]
cervix	intraepithelial neoplasias	0/80	0%		[49]
	SCCs	3/12	25%		[45]
	SCCs	1/51	2%		[46]
	SCCs	0/75	0%	S249C only	[48]
	SCCs	4/75	5%		[49]
	SCCs	0/91	0%	S249C only	[47]
	SCCs	1/28	4%		[44]
colorectal	colorectal adenocarcinomas	2/40	5%	1 frameshift mt	[43]
	colon adenocarcinomas	0/14	0%		[44]
	rectal adenocarcinomas	0/5	0%		[44]
nasopharynx	SCCs	1/69	1%		[48]
mouth	SCCs	0/12	0%		[53]
	SCCs	0/39	0%	G697C only	[51]
	SCCs	44/71	62%		[50]
skin	adenoid seborrheic keratoses	23/27	85%		[55]
	seborrheic keratoses	24/62	39%		[54]
	epidermal nevi	16/39	41%		[56]
	epidermal nevi	6/23	26%		[58]
	SCCs	0/11	0%		[53]
	BCCs	0/10	0%		[53]
	BCCs	0/12	0%		[55]
prostate	adenocarcinomas	0/20	0%	exon 7 only	[52]
	adenocarcinomas	0/10	0%		[44]
stomach	adenocarcinomas	0/3	0%		[44]
	adenocarcinomas	0/10	0%		[53]
	Signet ring cell carcinomas	0/5	0%		[53]
ovarian	adenocarcinomas	0/6	0%		[44]
	Brenner tumors	0/21	0%		[63]
breast	adenocarcinomas	0/6	0%		[44]
kidney	renal cell carcinomas	0/27	0%		[44]
brain	glioblastomas	0/5	0%		[44]
	meningiomas	0/8	0%		[44]
	astrocytomas	0/13	0%		[44]
oesophagus	SCCs	0/24	0%		[53]
	adenocarcinomas of Barrett's	0/8	0%		[53]

lung	SCCs	0/11	0%	[53]
	adenocarcinomas	0/5	0%	[53]
	small cell carcinomas	0/5	0%	[53]
oropharynx	SCCs	0/3	0%	[53]
hypopharynx	SCCs	0/12	0%	[53]

UCC, urothelial cell carcinoma; SCC, squamous cell carcinoma; BCC, basal cell carcinoma

FGFR3 mutations: genotype-phenotype relations

Mutations in *FGFR3* have been shown to be dominant and to result in constitutive activation of the receptor by increasing the stability of the receptor [81, 82]. It therefore seems likely that they contribute to the malignant phenotype.

Mutations in the extracellular and transmembrane domain that lead to cysteine substitution are suggested to induce disulfide-mediated receptor dimerization and ligand-independent constitutive activation. These mutations, which all induce the lethal chondrodysplasia TD1, have differential activities: studies by Monsonego-Ornan *et al.* showed that the intensity of FGFR3 activation may be dependent on the location of the cysteine [83], and that some mutant receptors are still predominantly ligand-dependent but enhance their signalling by accumulation of the receptor, which is no longer downregulated [84, 85].

Similarly, distinct mutations in the tyrosine kinase domain, which lead to constitutive activation by altering receptor conformation, cause either severe (TDI, TDII, and SADDAN, K652E/M) or milder (hypochondroplasia, K652N/Q) skeletal dysplasia [86-88]. Lievens *et al.* found that the mutations responsible for TDII (K652E) and SADDAN (K652M) cause receptor signaling from intracellular compartments such as the endoplasmic reticulum (ER). In contrast, the hypochondroplasia mutation K652N can only signal from the cellular membrane and is also phosphorylated to a lesser extent [89]. Different mutations within *FGFR3* are therefore associated with distinct phenotypes.

FGFR3 mutations were reported in bladder cancer for the first time in 1999 by the group of François Radvanyi [45]. All identified mutations were identical to the germline mutations that cause TD. Several other mutations were found in bladder tumors that had already been identified in skeletal dysplasias or were similar to these mutations [6, 73, 75, 77]. However, the frequency between the mutations differs (Table 2): the most frequent mutations in bladder cancer are mutations that create a cysteine residue in the extracellular domain or the first part of the transmembrane domain (S249C, Y375C, R248C, G372C), and they are identical

to mutations causing lethal TDI. In contrast, mutations in the transmembrane (A393E, G382R) and kinase domain (K652E/M/Q), which account for the non-lethal dysplasias, are rare in bladder tumors. Especially the G382R mutation, which causes achondroplasia and may be the most common germline mutation in humans [90] has only been found once in a bladder tumor until now [75]. It appears therefore that only the strongly activating mutations occur in bladder cancer. This is consistent with the observation that dwarfism as a consequence of nonlethal germline mutations is not associated with a greatly increased risk of cancer in later life [91]. In bladder cancer, no differences in tumor phenotype or behavior have been found with respect to different *FGFR3* mutations, although recently Hernandez *et al.* found a preferential association of the rare A393 mutation with bladder tumors of low malignant potential in a large study of 772 bladder cancer patients [66].

FGFR3 and signal transduction

FGFR3 signaling pathways

Activation of FGFR3 results in autophosphorylation at multiple tyrosine residues. Six conserved tyrosine residues have been identified in FGFR3 (Figure 1), of which Y726 seems to be the most important residue for downstream signalling [96]. Y649/Y650 are activation loop tyrosine residues and they are required for kinase activity of both wild type and mutant FGFR3 [81]. Signaling via FGFRs is mediated via direct recruitment of signaling proteins to these phosphorylated tyrosines, and via closely linked docking proteins that form a complex with signaling proteins via their SH2-domain (Figure 2A). An important docking protein in FGFR signaling is FGF Receptor Substrate 2α (FRS2α). It contains four binding sites for the adaptor protein Grb2 and two binding sites for the protein tyrosine phosphatase Shp2, which recruits additional Grb2 molecules. Grb2, which forms a complex with Sos, links the receptor with the Ras/MAPK signaling pathway [97]. Shp2 modulates activation of the Ras/MAPK and PI3K pathways [98], and can also bind directly to the receptor [99]. Next to this recruitment of positive regulators, FRS2α alsocontrols a negative feedback mechanism by mediating ubiquitination and subsequent rapid degradation of the activated receptor [100, 101].

Table 2. Frequency of the 11 bladder cancer associated *FGFR3* mutations in disease.

Disease	Frequency of different *FGFR3* mutations												Phenotype	Refs
	R248C	S249C	G372C	S373C	Y375C	G382R	A393E	K652E	K652M	K652Q	K652T	Other		
Skeletal dysplasias														
achondroplasia						100%							mild	[90, 92]
thanatophoric dysplasia I	65%	4%	1	1	15%				4%			10%	lethal	[27, 28, 32, 93, 94]
thanatophoric dysplasia II								100%					lethal	[27]
hypochondroplasia						9%				1		91%	mild	[87]
Crouzon syndrome with acanthosis nigricans							100%						severe	[30]
SADDAN									100%				severe	[32, 95]
Cancer														
multiple myeloma	1								1			1	aggressive	[37, 60, 61]
bladder cancer	10%	60%	6%	1%	20%	1	1%	1%	1%	1	1		noninvasive	[75]
cervical cancer		100%												[44-46, 49]
nasopharyngeal cancer		1												[48]
seborrheic keratosis	30%	18%	8%	10%	8%		4%	10%	12%				benign	[54, 55]
epidermal nevi	96%		1			1							benign	[56, 58]

1, mutation has been found only once

However, it has also been reported that FGFR3 can not induce strong sustained Ras-dependent signals, but instead is capable of inducing the activation of Ras-independent pathways [102]. Another protein that can bind directly to FGFRs via its SH2 domain is PLCγ, which binds to the phosphorylated Y762 tyrosine residue [41, 88] (Figure 2A). Besides Ras and PLCγ, FGFR3 can also stimulate the activation of Stat pathways [103], and interacts with the IHH/PTHrPR pathway in bone growth regulation [104].

Abnormal FGFR3 signaling

FGFR3 signal transduction pathways are tissue-specific, and very different cell responses can be elicited upon its activation, e.g. cell migration, proliferation, altered survival or differentiation.

Most studies on aberrant FGFR3 signaling have been performed with the TDII mutation K652E. This mutation induces activation of Stat1, its translocation into the nucleus, and an increase in p21/CIP1 expression [105] (Figure 2B). This probably causes premature exit of proliferative chondrocytes from the cell cycle, leading to accelerated differentiation [106] and apoptosis [107]. In chondrocytes, Stat1 is also activated by the TDI mutations R248 and S249C [107]. The induced growth arrest, which is in stark contrast to the typical stimulatory response to FGF, appears to result from a unique cell-type specific downstream response of proliferating chondrocytes [108].

Constitutive Stat3 activation, which can also be induced by FGFR3-K652E [36, 103], contributes to oncogenesis in a number of human cancers such as multiple myeloma (MM), melanoma, breast-, ovarian-, lung-, pancreatic-, and prostate cancer [109]. It protects MM cells from apoptosis [103] and upregulates cyclins and downregulates p21, which leads to cell cycle transition [36], thereby contributing to disease progression.

FGFR3-K652E also activates Stat5 [110], which is predominantly expressed in hematopoietic cells. A novel interaction between FGFR3 and the nonreceptor protein-tyrosine kinase Pyk2, which regulates the control of apoptosis in MM cells, has been reported. This interaction appears to be significant in regulating the activation of Stat5. Pyk2 stimulates apoptosis which can be inhibited by Shp2-mediated dephosphorylation [111].

Next to Stats, the MAPK signaling pathway is activated in a constitutive manner by the K652E mutation [107]. This prolonged activation may be a critical event for the premature differentiation of cells, since Stat1 or 3 activation is not required to obtain a mutant phenotype [112]. Shp2 is a very important mediator

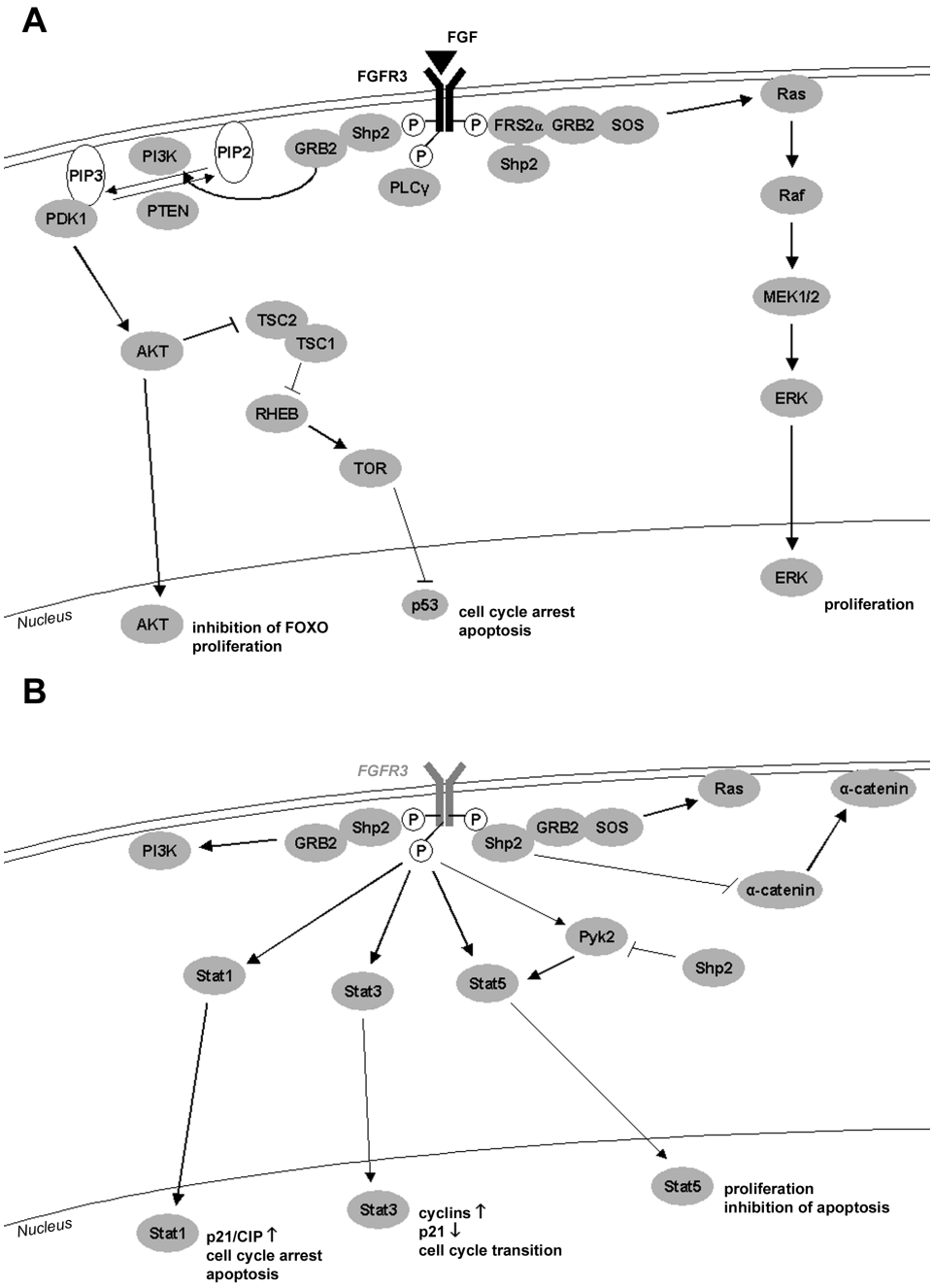

Figure 2. Signal transduction via FGFR3.
A, Ras/ERK and PI3K/Akt are two possible FGFR3 signal transduction pathways; B, pathways that were observed to be affected by aberrant FGFR3 signaling. Next to the Ras/ERK and PI3K/Akt pathways, mutated FGFR3 was also found to signal via Stat proteins.

of FGFR3-K652E transformation by activating the Ras/MAPK and PI3K signaling pathways [98], and by inhibition of α-catenin [113] (Figure 2B).

FGFR3 signaling in bladder cancer

Most observations from the extensive research that has been done on *FGFR3* mutations in skeletal dysplasias suggest that the disturbances in bone growth in human disorders result from the enhancement of normal FGFR3 signaling that can occur in the presence of ligand rather than from the activation of novel signaling pathways by the mutant receptors [112, 114], and this might also be the case for bladder cancer. A study by Jebar *et al.* showed that, similar to the findings in MM, *FGFR3* and *Ras* mutations appeared to be mutually exclusive in pTa/T1 tumors, and this is expected since they occur in the same signal transduction pathway. Therefore, the prediction is that the MAPK signal transduction pathway might be the common effector pathway for mutant *FGFR3* and Ras tumors [77] (Figure 3). Further, *PIK3CA* mutations are also present in 20% of bladder tumors of low grade and stage and occur preferentially in *FGFR3* mutant tumors, suggesting an additive role for the PI3K pathway [79]. This is also further indicated by the presence of mutations in *TSC1* [115-117], which activates downstream targets of the PI3K pathway, and loss of PTEN expression in muscle-invasive tumors [118].

Subcellular localization of FGFR3

After translation the FGFR3 receptor is transported from the endoplasmic reticulum to the plasma membrane. Accumulation of mutated FGFR3 by escape from lysosomal targeting has been found for mutations in the extracellular, transmembrane and tyrosine kinase domains [84, 85, 114]. Lievens *et al.* suggested that the K652E mutation hampers the receptor to exit the ER, and that it is the immature 120 kDa protein that mediates the abnormal signaling (Stat1 activation) from the ER [119]. This intracellular signaling seems to be different for different mutations [89]. Furthermore, they showed that FGFR3 mutants also activate MAPKs from the ER, but through an FRS2-independent pathway, probably via Src or a member of the Src family. PCLγ and Pyk2 were also recruited, but probably do not play a role in MAPK activation. Since FRS2α is a myristylated protein it is anchored on the cytoplasmic membrane, and this may be the reason that FRS2α can not be activated. This may also have implications in immature receptor turnover [120]. The full-length FGFR3-K652E receptor is able to morphologically transform NIH/3T3 cells when expressed at the plasma membrane but not when expressed in the cytoplasm or nucleus [82].

Recently, various FGFR3 mutations with different pathological severity were studied in human embryonic kidney (HEK) cells, and it was suggested that premature receptor tyrosine phosphorylation of the native receptor results in inhibition of glycosylation. This was only found for mutations in the intracellular tyrosine kinase domain, and associated with elevated receptor signaling from the Golgi apparatus [121].

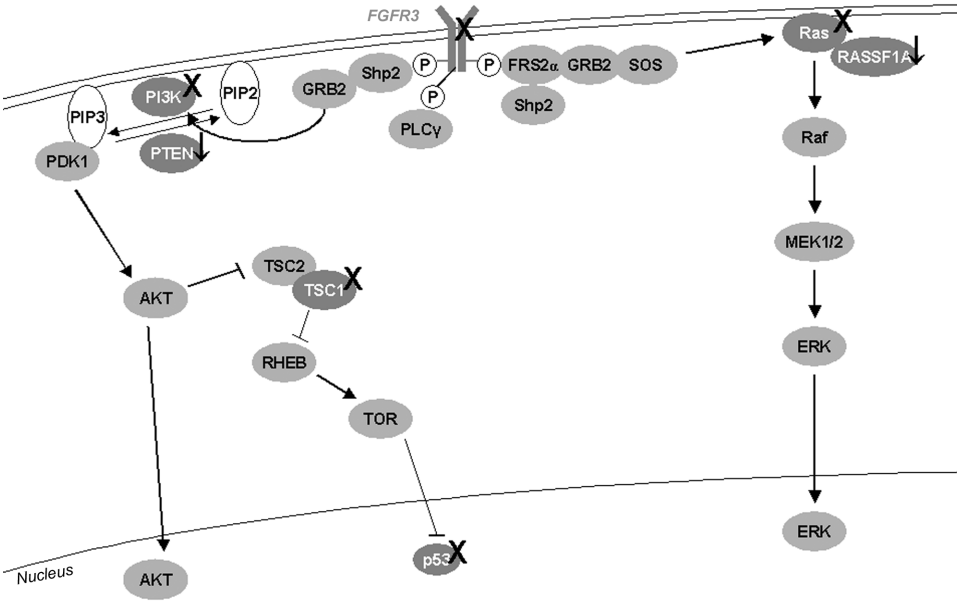

Figure 3. Mutations in potential downstream targets of FGFR3 found in bladder cancer.
The Ras and Akt pathways were associated with bladder cancer, since targets in these pathways were found to be mutated in bladder tumors. Activating mutations in *Ras* and *FGFR3* are mutually exclusive, suggesting a similar effect. Conversely, activating mutations in *PIK3CA*, which codes for the catalytic subunit of PI3K, and *FGFR3* are associated, suggesting an additive role. Inactivating mutations in TSC1 were also found, and these would lead to upregulation of TOR, and subsequently to downregulation of p53. **X**, mutation; ↓, loss.

BLADDER CANCER

Bladder cancer genetics

FGFR3 mutations

FGFR3 mutations are most frequent in tumors of low stage (pTa) and grade (G1-2), and absent in CIS. This indicates that FGFR3 activation is a key event in the development of noninvasive bladder tumors [6]. These results were confirmed by others on larger tumor series [73, 74]. Further evidence on the association between *FGFR3* mutations and benign bladder tumors was provided by a study in urothelial papilloma, which is a separate pathological entity with a benign natural behavior and no other genetic changes. These papillomas carried a mutation in 75% of tumors (9 of 12) [63].

FGFR3 mutations are correlated with mutations in a number of other genes. First, several studies showed that *FGFR3* and *TP53* mutations are almost mutually exclusive events [65, 69, 71], suggesting that these events are associated with and define two distinct tumor groups. *Ras* gene mutations (*HRAS*, *NRAS*, and *KRAS2*) and *FGFR3* mutations have been found to be absolutely mutually exclusive, and either mutation was present in 82% of low-grade tumors, indicating biological equivalence of these two mutations [77]. However, *Ras* mutations occur in tumors of all stages and grades, and are therefore not associated with either the *FGFR3* or the *TP53* pathway. Finally, *PIK3CA* mutations occur preferentially in *FGFR3* mutant tumors, suggesting an additive role for this mutation [79].

Genetic instability

Genetic instability in bladder cancer occurs at two levels. Microsatellite instability (MSI) affects the nucleotides, resulting in single-base point mutations, insertions or deletions. Tumors with MSI are therefore predominantly diploid. Chromosomal instability (CIN) is characterized by deletions or additions of fragments of chromosomes, or even entire chromosomes (aneuploidy). This results in loss or amplification (“gain”) of regions of DNA. MSI and CIN are usually mutually exclusive, since either is sufficient for carcinogenesis. This is also the case in bladder cancer: CIN is already frequent at a low level in superficial bladder tumors, and occurs at high levels in invasive tumors. In contrast, a low frequency of MSI at mono-and dinucleotides has been observed in all bladder tumors. In upper urinary tract tumors (UTT), however, the MSI rate is high, which is in accordance with the 22 times higher incidence of UTT in patients with HNPCC [122].

Loss of heterozygosity (LOH) of chromosome 9

Alterations of chomosome 9 are the most frequent genetic event in early bladder tumorigenesis, and they are even present in morphologically normal bladder tissue [123, 124]. LOH 9 is found in more than 50% of bladder tumors and is present in tumors from all stages and grades [65]. Since LOH of the q-arm of chromosome 9 is more frequent in papillary urothelial hyperplasias than loss of the p-arm, and part of these lesions showed LOH at 9q only, 9q alterations are considered the earliest event in bladder carcinogenesis [125, 126]. Many bladder tumors have LOH of the entire chromosome, which suggests loss of tumor suppressor genes on both arms. Several candidate genes have been identified in different regions on 9p and 9q, and these are currently investigated [127]. However, to date the alleged tumor suppressor gene on chromosome 9 has not been found. Considering the random development of LOH on chromosome 9q in bladder tumors, it is unlikely that the gene will be identified through LOH analysis alone [128].

Methylation

Besides alterations in the primary genetic sequence, epigenetic silencing of genes is an important mechanism in tumorigenesis. Frequent DNA methylation of CpG islands, which are often associated with promoter regions, has been reported to occur in both noninvasive and invasive bladder tumors [129]. Recently, Stransky *et al.* found that histone methylation is another mechanism that leads to the loss of gene expression in bladder tumors [130].

A molecular model for bladder carcinogenesis

Noninvasive bladder tumors have two distinct morphologies: pTa tumors are papillary, multifocal, and only occasionally progress, whereas carcinomas *in situ* (CIS) are flat and frequently progress to invasive disease. Genetic studies supported this pathological concept of a two-pathway model in bladder carcinogenesis: Spruck *et al.* observed that loss of chromosome 9 is common in pTa tumors but not in CIS. Conversely, *TP53* mutations are present in CIS lesions and invasive tumors, but not in pTa [131]. Hartmann *et al.* extended this model by the observation that LOH 9 also occurs in CIS, and that both LOH 9 and *TP53* mutations occur in adjacent dysplastic lesions but at a lower frequency, indicating that dysplasia is a precursor of CIS [132]. Conversely, frequent deletions of chromosome 9 were found in flat urothelial hyperplasias while deletions at the p53 locus at chromosome 17p13 were infrequent, suggesting that these lesions are a precursor of papillary bladder cancer [133, 134].

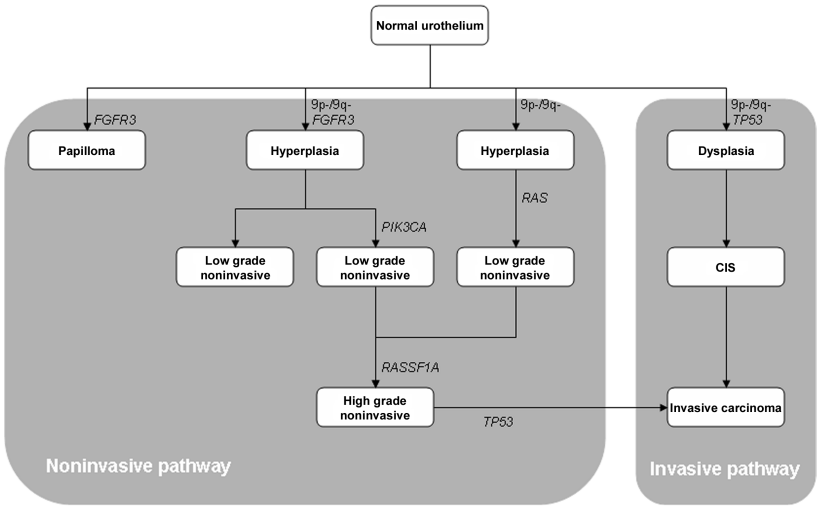

Figure 4. Model for bladder carcinogenesis.
LOH 9 occurs in all tumors except urothelial papillomas, and is the earliest known genetic event. Tumors with an *FGFR3* mutation have low malignant potential, and other genetic events (*e.g.* mutations in *PIKC3A* and/or loss of *RASSF1A*) are probably needed for tumor progression.

Thus, *TP53* mutations still delineated one arm of the model, but LOH 9 was found to be present in tumors from all stages and grades [65], and therefore can no longer serve as marker for the noninvasive pathway. When *FGFR3* mutations were found to be present in tumors of low stage and grade, and were generally absent in tumors with *TP53* mutations, this finding was considered an additional genetic confirmation of the two-pathway model of bladder carcinogenesis. It was further suggested that *FGFR3* is probably the key event for the noninvasive hyperplasia – papillary carcinoma pathway. With the current knowledge of bladder cancer genetics discussed in this paragraph, we propose an adapted model for noninvasive bladder carcinogenesis based on the two-pathway model (Figure 4). *FGFR3* mutations probably provide a growth advantage early in tumor development. In tumors without this mutation, activation of other genes (*e.g. RAS*) will cause a growth-inducing signal. For tumor progression, other genetic events, *e.g. PIKC3A* mutation of loss of *RASSF1A*, are needed.

Bladder cancer diagnosis and prognosis

After *trans*-urethral resection or cystectomy, bladder tumors are classified based on histopathological examination according to stage, grade, and a few other clinicopathologic parameters. Currently treatment of patients is based mainly on stage and grade of the tumor. It should, however, be kept in mind that interobserver variability among pathologists on especially grading has been reported to be very high [4, 68, 135]. Owing to the limitations of these traditional parameters, a frantic search is going on for identification of molecular markers which can classify bladder cancers in more detail in order to help in the selection of the optimal treatment for the patient. More specifically, the identification of molecular markers predictive of recurrence and progression is now considered of utmost importance to determine the most cost-effective surveillance protocol for a given patient.

FGFR3 mutations

Since *FGFR3* mutations in bladder cancer are related to low stage and grade, they make *FGFR3* the first marker for nonaggressive disease. *FGFR3* mutation status was related to other molecular and clinicopathologic parameters in survival analysis of 286 patients, and it appeared that pTa/T1 tumors with a mutation are less likely to progress than wild type tumors. *FGFR3* was not an indepent predictor for survival on its own, but combined with Ki-67, a marker associated with invasive tumors, it was a independent predictor of progression and disease-specific survival in pTa/T1 tumors [68].

FGFR3 mutations in pT1 tumors

pT1 bladder tumors, which penetrate the basement membrane but are not muscle-invasive, have been classified as superficial in the past, but since they have a relatively worse prognosis they are now categorized under invasive cancers (WHO 2004). They are an intermediate and heterogeneous group at the transition point in the model for bladder cancer progression, some resembling pTa and others pT2-4 tumors. This is emphasized by the observation that in T1G3 tumors *FGFR3* and *TP53* mutations are independently distributed rather than mutually exclusively [64, 67]. Especially for this group of tumors, it is difficult to determine which patients are at risk to develop muscle-invasive disease and which patients are not, and clinicians are therefore inclined to treat pT1 tumors more aggressively because of their higher risk of progression. *FGFR3* mutations fail to predict the risk of recurrence, progression, or death among patients with pT1 tumors [67,

136], suggesting that their effect is overridden by additional genetic alterations, *e.g.* activation of the PI3K pathway by *PIK3CA* mutations [79]. This has been confirmed in a large prospective study in 772 patients with pTa and pT1 tumors. After stratification according to tumor stage and grade the prognostic value of *FGFR3* mutation detection for tumor recurrence appeared to be restricted to pTaG1 tumors [66]. Unfortunately, the number of events was too low to determine the prognostic value for disease progression.

FGFR3 mutations in inverted papillomas

Inverted papilloma is a rare, benign bladder tumor with an endophytic growth pattern that has a low incidence of local recurrence and lack of invasive growth and metatstasis [137]. Conversely, papillary urothelial cell carcinomas occasionally display endophytic growth, and therefore resemble inverted papillomas, resulting in considerable difficulty in assessing invasion [138]. In a recent study on 65 inverted papillomas, five experienced pathologists have redefined 21 inverted papillomas as urothelial carcinomas. *FGFR3* mutations were present in 12 of 21 (57%) of these “carcinomas”, while only 4 of the remaining 44 inverted papillomas were mutated. *FGFR3* could therefore be a helpful marker in the differential diagnosis of inverted papilloma and urothelial cell carcinomas with an invasive growth pattern [139].

Methylation

Considering the two-pathway model, it is suggested that frequent DNA methylation is more typical of the invasive than superficial tumor pathways [140]. Moreover, methylation occurs in normal and CIS urothelial samples from patients with invasive tumors, indicating that aberrant gene hypermethylation already occurs at an early, noninvasive phase which suggests a potential prognostic role [141]. Methylation of individual genes (*APC*, *CDH1*, *CDH13, RASSF1A*) and the methylation index (MI) were related to poor prognosis and shortened survival, further presenting the methylation profile as a potential new biomarker [140, 142]. Silencing of the *RASSF1A* gene by promoter hypermethylation is associated with concomittant LOH of chromosome 3p21, which confirms the notion that *RASSF1A* acts as a tumor suppressor gene [143]. Thus, patients showing methylation of these genes in their noninvasive or superficially invasive bladder tumors might benefit of a more intense surveillance scheme in order to reduce the risk of tumor progression.

Expression profiling using cDNA microarrays

Several models to classify bladder cancer have been proposed based on expression microarray analysis of a subset of genes. These classifiers are able to identify the three major stages of bladder cancer (pTa, pT1, and pT2-4), but also several subgroups, especially within the pTa category of bladder cancer [144], and the presence of adjacent CIS [145]. Therefore, expression array analysis could become a useful tool to stratify patients with noninvasive bladder cancer for optimal follow-up. A disadvantage is that the microarray technique is very laborious and expensive, and requires frozen tissue.

Tissue biomarkers

Two widely studied immunohistochemical (IHC) markers in bladder cancer are P53 and Ki-67. Mutations in the *TP53* tumor suppressor gene are strongly associated with nuclear accumulation of P53 protein, and can therefore be determined by immunohistochemical analysis on paraffin-embedded tissue. The Ki-67 (MIB-1) labeling index is a measure for cell proliferation. Ki-67 and P53 staining increase with tumor stage and grade and are prognostic factors for disease recurrence and progression [146-148]. In pTa/T1 tumors, Ki-67 labeling index and expression of cyclins D1 and D3, which are upstream regulators of the cell cycle, might be relevant predictors of survival [149]. In spite of a plethora of publications on the prognostic impact of the above-mentioned two markers in bladder cancer, none of them is currently in routine use. In multivariate analysis these markers do not have a prognostic value in addition to the traditional clinico-pathological parameters.

Dysregulation of cytokeratin 20 (CK20) is a marker of urothelial dysplasia [150]. In normal urothelium, expression is related to differentiation and limited to superficial and occasional intermediate cells. The presence of this normal staining pattern in bladder cancer was associated with a benign disease course. An irregular expression pattern, that is throughout the full thickness of the urothelium, can already be found in a proportion of low grade tumors, which suggests that urothelial dysplasia is an early event in bladder carcinogenesis. Irregular expression was associated with progression and recurrence [151].

FGFR3 mutations are associated with increased *FGFR3* gene expression [23, 65]. Diagnosis by immunohistochemistry could therefore be possible; however, results are controversial. One study failed to demonstrate a significant relationship between FGFR3 protein expression and factors that indicate a favorable outcome [152], while another showed significant associations between FGFR3 expression and low stage and grade [153]. Furthermore, different staining

was found for different antibodies: in one study, the antibody FGFR3 C-15 showed cytoplasmic and membranous staining [152], while FGFR3 was also scored positive when cytoplasmic and nuclear staining occurred [154]. Other groups used antibody FGFR3 B-9, and here FGFR3 expression was again found in the plasma membrane and cytoplasm, but not in the nucleus [153, 155]. Moreover, the latter studies differed with regard to FGFR3 staining results in normal bladder tissue. Analysis of FGFR3 expression by IHC might therefore not be the ideal method to determine *FGFR3* mutations in bladder tumors.

In conclusion, apart from CK20, IHC markers might not be very useful in the diagnosis and prognosis of bladder cancer.

Bladder cancer surveillance by urine analysis

For follow-up of patients after TUR, the number of cystoscopies could be reduced if a reliable, noninvasive test in urine would be available. Furthermore, a major advantage is that tumors of the upper urinary tract may also be detected with some urine tests. Several markers and tests have been proposed with different sensitivity and specificity results, but so far none was considered of sufficient sensitivity to replace cystoscopy, the current gold standard for bladder cancer surveillance. Urine analysis can probably be improved by using multiple markers [156-158].

Fluorescence In Situ Hybridization

The most commonly used genetic test on urine, the UroVysion test, is based on FISH analysis and this urine test was approved by the United States Food and Drug Administration in 2001. It detects chromosomal abnormalities using three probes on the centromeres of chromosomes 3, 7, 17, and one locus-specific probe on chromosome 9p21. By including alterations that represent both stable (9p21) and instable (3, 7, 17) tumors, this test is not only helpful in diagnosis of bladder cancer, but may also be used in surveillance strategies [159].

Microsatellite analysis

Microsatellites are short, noncoding DNA sequences that are repeated many times within the genome. Because microsatellites change in length early in the development of cancers, they are useful markers for early cancer detection. Genetic changes that occur in bladder cancer, such as LOH (often displayed on chromosomes 9q, 9p, 17p (the p53 locus), 4p, 8p, and 11p) and microsatellite

instability (MSI), can therefore be detected by microsatellite analysis (MA).

A Phase II trial in patients with superficial bladder carcinoma has reported a sensitivity of 75% for MA on urine sediment. Tumors that were missed were pTaG1, and therefore the cystoscopy frequency may be reduced if MA is negative [160]. By including *FGFR3* mutation analysis in addition to MA, the sensitivity increased to 89% [76]. A major disadvantage of this technology is its very labor-intensive nature and the requirement of a substantial amount of DNA. The cost-effectiveness including quality-of-life aspects using MA on voided urine specimens in patients monitored for superficial bladder cancer is currently investigated in a randomized Phase III trial.[2]

FGFR3 mutation analysis

As mentioned above, *FGFR3* mutation analysis is also possible on urine sediments. This is especially important for the detection of low-grade Ta lesions since urine cytology has a very low sensitivity for their detection, while the sensitivity of MA is also reduced in this subset of lesions. Initially, analysis for *FGFR3* mutations was done by single strand conformation polymorphism (SSCP) analysis or sequencing, which are both labor-intensive techniques, indicating the need for a simple assay for detecting mutations [161]. An alternative assay has been set up based on allele-specific PCR, which could detect the four most common mutations in bladder cancer (R248C, S249C, G372C, Y375C). Sequencing revealed that 13% of mutations were missed because they were not included in the assay. Furthermore, the assay is still rather laborious since two simultaneous multiplex PCR reactions have to be done and an internal control is needed to calculate ratios of mutation-specific signals [78].

Methylation analysis

Methylation-specific PCR is a sensitive technique which can identify up to 1 methylated allele in 1000 unmethylated alleles, and would therefore be useful for the detection of tumor cells in urine sediments. By analyzing the hypermethylation status of the tumor suppressor genes *APC*, *RASSF1A*, and $p14^{ARF}$, which provides 100% diagnostic coverage, Dulaimi *et al.* [162] could detect tumors in 87% of urine samples, which outperformed urine cytology especially for low stage and grade tumors. Hoque *et al.* [163] reached 100% coverage using a panel of four genes (*CDKN2A*, *ARF*, *MGMT*, and *GSTP1*), which correctly identified 69% of bladder cancer patients. By subsequently analyzing methylation status of five

[2] MNM van der Aa, unpublished data

other genes for patients that were negative, sensitivity improved to 82%; however, the specificity decreased to 96%.

Bladder cancer cell lines and animal models

To date, most functional studies on the FGFR3 receptor and its mutations have been done in chondrocytes and skeletal dysplasia phenotypes. However, the function of activated FGFR3 in bladder cancer has yet to be investigated in urothelium. No animal models for FGFR3 in bladder cancer have been established yet: treatment of mice with the carcinogen BBN induced bladder tumors mimicking the CIS pathway, but these did not carry *FGFR3* mutations. BBN-induced tumors in the rat were superficial and papillary; however, *FGFR3* mutations were absent, suggesting that these tumors differ genetically from human bladder tumors [164]. Targeting the FGFR3-S249C transgene to the mouse urothelium resulted in mild hyperplasias but no tumors developed.[3]

Alternatively, bladder cancer cell lines could also be used as a model, although most bladder cancer cell lines are derived from high stage and grade tumors. Up until now two studies on the function of FGFR3 have been done in human bladder cancer cell lines: Bernard-Pierrot *et al.* [23] used MGH-U3, a bladder cancer cell line derived from a papillary grade 1 tumor with an Y375C mutation in *FGFR3* also showing overexpression of P53 [79]. This cell line was able to form colonies on soft agar and to develop tumors in nude mice but it lacks the ability for anchorage-independent growth [165]. A different bladder cancer cell line, 97-7 was used by Tomlinson *et al.* [166]. This cell line with mutations in *FGFR3* (S249C) and *TP53* was cultured from a primary T1G2-3 tumor. Both studies showed the importance of mutant *FGFR3* for these two tumor cell lines, as inhibition of FGFR3 with siRNA or FGFR inhibitors resulted in growth inhibition.

The presence of *FGFR3* mutations in a substantial group of bladder carcinomas leads to the assumption that *FGFR3* functions as an oncogene. The transforming properties of mutant FGFR3 have been shown by stable transfection of FGFR3-S249C into NIH-3T3 cells, which leads to transformation, anchorage-independent colony growth, and tumor formation in nude mice [23]. This was presented as evidence that mutated FGFR3 is oncogenic; however, transforming properties of mutant FGFR3 have yet to be investigated in urothelial cells to confirm this hypothesis.

[3] F Radvanyi, personal communication

OUTLINE OF THIS THESIS

Mutation of the *FGFR3* gene is a common finding in superficial UCC and probably a key genetic event in the development of noninvasive bladder tumors. The main goals of this thesis are 1) to explore the potential use of *FGFR3* mutations in bladder cancer diagnosis, prognosis, and in surveillance of patients with bladder cancer, and 2) to understand the functional role of mutant FGFR3 in bladder carcinogenesis.

For further clinical studies on *FGFR3* mutations and the possible use of *FGFR3* mutation analysis in bladder cancer patients, a simple and fast method for *FGFR3* mutation detection was needed. In **Chapter 2**, a new assay for the analysis of all known *FGFR3* mutations in bladder cancer is described that is fast, easy, more sensitive and less laborious than previous techniques. Furthermore, this test can easily be adjusted when additional mutations in the *FGFR3* gene are discovered. With this technique, several clinical studies were done on the role of FGFR3 in bladder carcinogenesis and patient outcome.

To provide further evidence that *FGFR3* mutation is an early event, associated with low-risk tumors, *FGFR3* mutations and LOH 9 were analyzed in urothelial hyperplasias, a precursor of low grade papillary carcinomas (described in **Chapter 3**). Furthermore, the genetic stability of *FGFR3* mutated tumors was studied by determining the number of chromosomal aberrations using CGH analysis (**Chapter 4**). CK20, a marker for terminal differentiation of urothelium, was employed to determine the degree of tumor differentiation, particularly among the cancers carrying the *FGFR3* mutation (**Chapter 5**).

In **Chapter 5** we also describe the relation of four molecular markers (*FGFR3*, CK20, Ki-67, P53) to bladder cancer patient outcome. Urothelial cell carcinomas not only occur in the bladder but also in the upper urinary tract (i.e. in the ureter and renal pelvis). However, these tumors may be genetically different from bladder tumors. **Chapter 6** describes the *FGFR3* mutation analysis and its correlation to patient outcome performed on both upper urinary tract and bladder tumors.

No studies have yet been done of the effect of mutant FGFR3 receptor on the phenotype of urothelial cells in vitro, nor is there any information on FGFR3 signaling pathways involved in urothelial or epithelial cells. In **Chapter 7**, the results are reported of an experimental study analyzing the expression of a mutant FGFR3 receptor transfected in a bladder cancer cell line and the subsequent effects on morphology and signal transduction.

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CHAPTER 2

A simple and fast method for the simultaneous detection of nine *FGFR3* mutations in bladder cancer and voided urine

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ABSTRACT

Mutations in the fibroblast growth factor receptor 3 (*FGFR3*) occur in 50% of primary bladder tumors. An *FGFR3* mutation is associated with good prognosis, illustrated by a significantly lower percentage of patients with progression and disease-specific mortality. *FGFR3* mutations are especially prevalent in low grade/stage tumors, with pTa tumors harboring mutations in 85% of the cases. These tumors recur in 70% of patients. Efficient *FGFR3* mutation detection for prognostic purposes and for detection of recurrences in urine is an important clinical issue. In this paper, we describe a simple assay for the simultaneous detection of nine different *FGFR3* mutations. The assay consists of one multiplex PCR, followed by extension of primers for each mutation with a labeled dideoxynucleotide. The extended primers are separated by capillary electrophoresis, and the identity of the incorporated nucleotide indicates the presence or absence of a mutation. The assay was found to be more sensitive than single-strand conformation polymorphism analysis. Mutations could still be detected with an input of only 1 ng of genomic DNA and in a 20-fold excess of wild type DNA. Moreover, in urine samples from patients with a mutant tumor, the sensitivity of mutation detection was 62%. In conclusion, we have developed a fast, easy to use assay for the simultaneous detection of *FGFR3* mutations, which can be of assistance in clinical decision-making and as an alternative for the follow-up of patients by invasive cystoscopy for the detection of recurrences in urine.

INTRODUCTION

Bladder cancer is the fifth most common cancer in the western world with an incidence of 20 new cases per year per 100,000 people in the U.S. [1]. Unfortunately, these statistics do not include superficial pTa bladder cancer, which represents the most common type of bladder cancer. In the Netherlands, the incidence of both superficial and invasive bladder cancer is estimated as about 30 new cases per year per 100,000 people. This is in accordance with data from global cancer statistics for the western world [2]. Superficial bladder tumors are removed by transurethral resection. However, up to 70% of these patients will develop one or more recurrences, and it has been estimated that 1 in 1,450 people is under surveillance for bladder cancer in the United Kingdom [3]. Cystoscopy is an uncomfortable, invasive, and expensive procedure, but currently remains the gold standard for detection of recurrences. Because patients have to be monitored perpetually and have a long-term survival, bladder cancer is the most expensive cancer when calculated on a per patient basis [4].

Activating mutations in the fibroblast growth factor receptor 3 (*FGFR3*) gene have been reported in >50% of primary bladder tumors [5, 6].[1] Most of the somatic mutations found in bladder cancer are identical to germ line mutations responsible for skeletal disorders such as thanatophoric dysplasia and achondroplasia [7]. It has been reported that *FGFR3* mutations are very frequent in bladder tumors of low stage and grade, indicating that they occur much more frequently in superficial bladder cancer than in invasive bladder cancer [8, 9].

Many potential molecular markers for progressive disease have been identified [10, 11], of which Ki-67 labeling (MIB-1 staining) seemed to be the most promising marker. However, no single marker was able to predict the clinical behavior of bladder tumors, and none of the markers proved to be superior to histopathological staging and grading [10]. We recently showed that the presence or absence of an *FGFR3* mutation in combination with high or low MIB-1 staining was able to characterize almost 90% of 286 primary bladder tumors [6]. In a retrospective study with a median follow-up of 5 years, multivariate analysis showed that the combination *FGFR3* and MIB-1 seemed superior to other variables for predicting progression of bladder tumors and survival of the patients. Moreover, *FGFR3* mutation analysis and MIB-1 staining were more reproducible than histochemical analysis of the tumors by expert urinary pathologists. This was the first study to show that molecular markers can be used to predict disease course more

[1] JMM van Oers, unpublished results

accurately and reproducibly than traditional clinical pathology.

Over the years, the need for an inexpensive, non-invasive, and simple procedure for the detection of bladder cancer has been expressed [11, 12]. Cytology done on voided urine is a noninvasive procedure with up to 100% specificity. Unfortunately, this method is limited by its sensitivity, which is especially poor for low-grade tumors. Because of this limited sensitivity, several other methods were developed for the detection of tumor cells in voided urine. Currently, one of the most promising approaches is microsatellite analysis, with a reported sensitivity of up to 95% [13, 14]. Recurrences that are missed are usually small pTa grade 1 tumors. We and others have previously shown that the sensitivity of molecular cytology could be enhanced by *FGFR3* gene analysis [15, 16].

Reliable and easy to perform *FGFR3* mutation detection for prognostic purposes and for detection of recurrences in urine is an important clinical issue. Therefore, a simple assay for detecting mutations is needed to replace the current labor-intensive mutation analysis by single-stranded conformation polymorphisms (SSCP) or by sequencing [16]. In this study, we report the development of a new method for *FGFR3* mutation analysis based on the detection of single nucleotide changes. With this method, the nine most common mutations can be detected in one assay simultaneously. In addition, it is fast and easy to use. The assay is more sensitive than SSCP, needs little DNA, and can reliably detect mutations in a background of up to 20-fold excess of control DNA.

MATERIALS AND METHODS

Sample collection and DNA extraction

Paraffin-embedded tumor tissue samples were collected from patients who underwent surgery at the Erasmus MC or other hospitals in the Rotterdam area. All patients signed written informed consent. Voided urine samples were collected before surgery. DNA was obtained as previously described [14, 15].

Polymerase Chain Reaction

Initially, three regions of the *FGFR3* gene, located in exons 7, 10, and 15, were amplified by PCR as described previously [8]. These regions comprise the following potential codon mutations: R248C and S249C (exon 7), G372C, Y375C, and A393E (exon 10), and K652E, K652Q, K652M, and K652T (exon 15).

After analysis of the secondary structure and folding free energy of the exon 7 and exon 10 PCR products using DNAMAN software (Lynnon Corporation, Vaudreuil,

Canada), new primer sequences were constructed. In the end, the following primer pairs were used: for exon 7, 5'-AGTGGCGGTGGTGGTGAGGGAG-3' and 5'-GCACCGCCGTCTGGTTGG-3'; for exon 10, 5'-CAACGCCCATGTCTTTGCAG-3' and 5'-AGGCGGCAGAGCGTCACAG-3'; and for exon 15, 5'-GACCGAGGACAACGTGATG-3' and 5'-GTGTGGGAAGGCGGTGTTG-3'. A multiplex reaction for all three regions was successfully set up. The multiplex PCR was done in a volume of 15 µl, containing 1x PCR buffer, 1.5 mM $MgCl_2$, 0.5 units Taq polymerase (Promega, Madison, WI), 0.17 mM deoxynucleotide triphosphates (Roche, Basel, Switzerland), 10 pmol of exon 7 and exon 15 primers, 7.5 pmol of exon 10 primers (Invitrogen, Carlsbad, CA), 5% glycerol (Fluka, Buchs SG, Switzerland), and 1 to 250 ng of genomic DNA. Cycling conditions were as follows: 5 minutes at 95°C, 35 cycles at 95°C for 45 seconds, 60°C for 45 seconds, and 72°C for 45 seconds, followed by 10 minutes at 72°C. Products were treated with Exonuclease I (*Exo*I, Amersham Biosciences, Uppsala, Sweden) and shrimp alkaline phosphatase (Amersham Biosciences) to remove excess primers and deoxynucleotide triphosphates.

FGFR3 mutation analysis

PCR products were analyzed for mutations using the ABI PRISM SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA), according to the protocol supplied by the manufacturer. The SNaPshot method is based on the dideoxy single-base extension of unlabeled oligonucleotide primers. For each of the nine mutations described above, a primer annealing adjacent to the potentially mutant nucleotide was developed. In mutations K652E and K652Q, the same nucleotide is changed, therefore one primer will detect both mutations. The same holds for mutations K652M and K652T. In total, seven SNaPshot primers were developed, with a different length of poly(dT) tail attached to the 5'-end to enable their simultaneous detection. All primers were designed with a similar melting temperature and were checked for the absence of base pairing with other SNaPshot primers.

PCR conditions were optimized in order to develop a multiplex SNaPshot reaction. Two new primers had to be developed to improve detection of the S249C and Y375C mutations (see Results). In order to enhance the detection of the most frequent mutation (S249C), two different lengths of poly(dT) tails were added. The final panel of SNaPshot primers is shown in Table 1. The multiplex SNaPshot reaction was done in a volume of 10 µl, containing 2 µl of PCR product (see above), 2.5 µl Ready Reaction Mix, 1x sequencing buffer, and SNaPshot primers

with concentrations as indicated in Table 1. Cycling conditions were: 25 cycles of rapid thermal ramp to 96°C, 96°C for 10 seconds, rapid thermal ramp to 58,5°C, and 58,5°C for 40 seconds. After treatment with shrimp alkaline phosphatase to remove excess dideoxynucleotide triphosphates, labeled products were separated in a 25-minute run on 36 cm long capillaries in an automatic sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems). GeneScan Analysis Software version 3.7 (Applied Biosystems) was used for data analysis.

Table 1. SNaPshot primers for *FGFR3* mutation detection.

Primer	Sequence	Strand	Primer extension		Conc (pmol/µl)
			wt	mut	
R248C	5'-T_{46}CGTCATCTGCCCCCACAGAG-3'	sense	C	T	2.0
S249C	5'-T_{36}TCTGCCCCCACAGAGCGCT-3'	sense	C	G	1.2
S249C	5'-T_{28}TCTGCCCCCACAGAGCGCT-3'	sense	C	G	1.2
G372C	5'-T_{29}GGTGGAGGCTGACGAGGCG-3'	sense	G	T	0.4
Y375C	5'-T_{43}ACGAGGCGGGCAGTGTGT-3'	sense	A	G	0.6
A393E	5'-T_{34}CCTGTTCATCCTGGTGGTGG-3'	sense	C	A	2.4
K652E/Q	5'-T_{50}GCACAACCTCGACTACTACAAG-3'	sense	A	G/C	1.2
K652M/T	5'-T_{20}CACAACCTCGACTACTACAAGA-3'	sense	A	T/C	0.8

wt, wild type; mut, mutant; conc, concentration

RESULTS

Development of an FGFR3 mutation detection assay

Mutation analysis was based on the ABI PRISM SNaPshot Multiplex Kit. We first designed primers adjacent to the seven nucleotides that are most frequently mutated in bladder tumors. These seven primers enabled detection of nine mutations in the *FGFR3* gene. They were modified by adding different lengths of poly(dT) tails to the 5'-end in order to allow their separation based on differences in size. These primers were annealed to a combination of *FGFR3* exon 7, 10, and 15 PCR products from control DNA samples. In a subsequent reaction (SNaPshot reaction), the primers were extended using a substrate mixture of all four fluorescently labeled dideoxynucleotide triphosphates, thus allowing the addition of only one nucleotide to each primer. The products were analyzed on an ABI automatic sequencer in a 25-minute run. With this procedure, all seven wild type nucleotides were detected as shown in Figure 1A.

When we subsequently analyzed known mutations in bladder tumor DNA samples, it seemed that the mutations in codon 248, 372, 393, and 652 could be detected reliably. However, for codon 249 the mutant peak was often too small to be distinguished from background signals, thus leading to false-negative results. Conversely, false-positive results were frequently experienced for codon 375 because of the presence of a background peak with the same color and position as expected for the mutant nucleotide. A new SNaPshot primer for codon 375 was therefore designed that annealed to the antisense strand. When using this forward primer, the background signal disappeared. A new primer on the opposite strand was also developed for codon 249. Two different lengths of poly(dT) tails were added to the new S249C forward primer, in order to enhance the possible detection of this most frequent mutation. However, the detection of the S249C signal continued to be inefficient. We next analyzed the secondary structure of the exon 7 PCR product, and observed that the single strand was able to fold into a very stable secondary structure with a free energy of –36.11 kcal/mol. When the codon 249 mutation was present, the structure was even more stable (free energy –40.05 kcal/mol). Consequently, annealing of the S249C SNaPshot primer was inefficient. A new exon 7 reverse primer was developed to create a shorter PCR product with a lower GC content, resulting in a less stable secondary structure for both wild type (free energy –20.31 kcal/mol) and codon 249 mutant (free energy –22.88 kcal/mol) DNA. As a result, we now observed that the S249C peaks were clearly visible next to the wild type peaks (detection of the codon 249 mutation is depicted in Figure 1C). Please note that the mutant peaks appear at a slightly different position than the wild type products. This is due to the differences in size of the fluorescent labels of the dideoxynucleotide triphosphates. In addition, the assay is not quantitative because the labels also have different emission efficiencies. Based on the secondary structure, a new reverse primer was also developed for exon 10, and this improved the detection of the codon 375 mutation even further (Figure 1E). In Figure 1B-J examples of the detection of all nine mutations are shown.

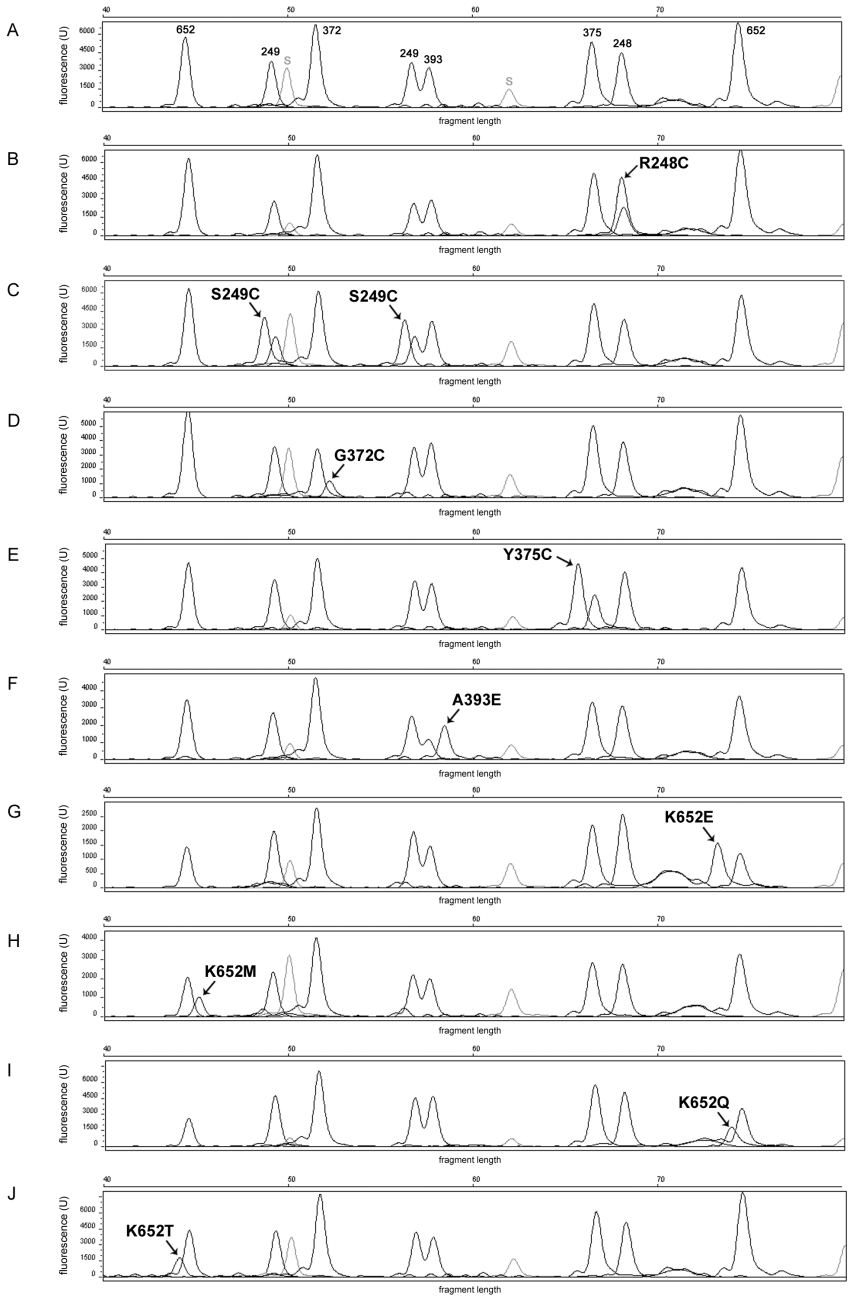

Figure 1. Detection of wild type and mutant *FGFR3* nucleotides using the *FGFR3* SNaPshot mutation assay.

A, analysis of control DNA. Peaks are labeled with the relevant *FGFR3* codon. Please note that two different primers were used for codon 249. S denotes the size standard; B-J, analysis of tumor DNA samples containing the following mutations: R248C, S249C, G372C, Y375C, A393E, K652E, K652M, K652Q, and K652T.

Sensitivity of the FGFR3 SNaPshot assay

We next determined the minimal amount of DNA required for reliable mutation detection. To this end, tumor DNA samples harboring the four most common mutations (S249C, Y375C, R248C and G372C) were diluted, and for each of these mutations we observed that 1 ng DNA was sufficient as a starting concentration for the multiplex PCR reaction (data not shown). We subsequently determined whether these mutations could also be detected in a background of normal DNA. To this end, we mixed tumor DNA heterozygous for the mutations (*FGFR3* mutations are usually heterozygous [15, 17]) with control DNA in different ratios (1:1, 1:4, 1:9, and 1:19). Figure 2 shows the result of the mutation detection using a 1:9 (G372C and Y375C) or 1:19 (R248C and S249C) ratio of mutant versus normal DNA. All four mutations were detected. This means that one mutant allele can still be detected against a background of up to at least 39 wild type alleles.

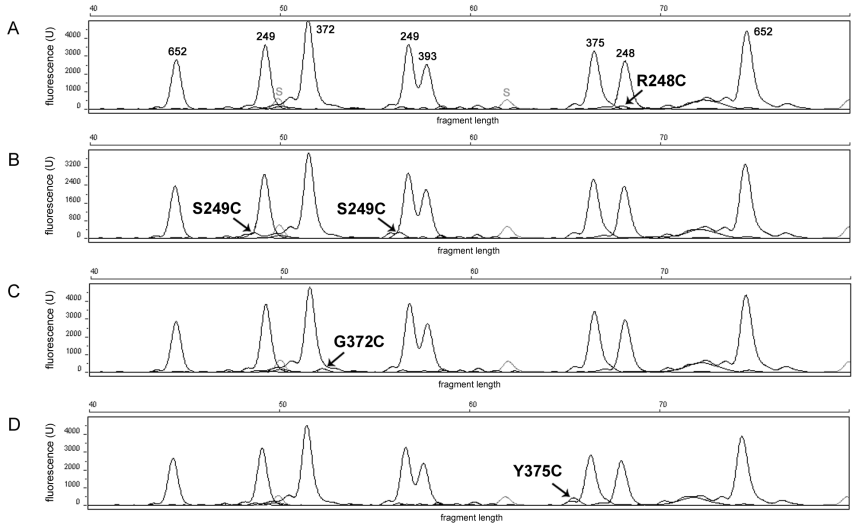

Figure 2. Dilution experiment for mutation detection.
DNA samples from tumors harboring the most common *FGFR3* mutations were diluted at a ratio of 1:9 (G372C and Y375C) or 1:19 (R248C and S249C) with control (nonmutant) DNA. A, R248C; B, S249C; C, G372C; D, Y375C.

FGFR3 SNaPshot mutation detection is superior to single-strand conformation polymorphisms

To compare the new method with analysis by SSCP, we did *FGFR3* mutation analysis on primary tumor DNA samples from 92 patients diagnosed with bladder cancer. Mutations that were found by SSCP could all be confirmed using the SNaPshot method, except for one codon 249 mutation (Table 2). Four additional codon 375 mutations were observed, thereby almost doubling the detection efficiency for this mutation. This mutation was difficult to detect by SSCP because the wild type and mutant bands migrated closely together. In summary, the SNaPshot method proved to be more sensitive than SSCP.

Table 2. Comparison of *FGFR3* SNaPshot mutation analysis with SSCP on 92 primary bladder tumor DNA samples.

FGFR3	**SSCP**	**SNaPshot**
Wild type	50	47
R248C	5	5
S249C	29	28
G372C	2	2
Y375C	5	9
A393E	0	0
K652E	0	0
K652M	1	1
K652Q	0	0
K652T	0	0
Total	92	92

FGFR3 mutation analysis in urine

We next evaluated the sensitivity of the assay with regard to the detection of recurrent bladder tumors. To this end, we selected urine samples from patients with a recurrent tumor. Urine samples from patients with primary tumors were excluded because primary tumors are often larger than recurrences and this may result in an overestimated sensitivity of the assay. *FGFR3* mutations in the tumors were detected by SSCP [14] or SNaPshot analysis.[2] A total of 64 urine samples were analyzed. Of these, 29 were derived from patients with a mutant tumor, and in

[2] MNM van der Aa, unpublished results

35 cases the corresponding tumor was wild type for *FGFR3*. Table 3 illustrates the results obtained with the SNaPshot assay on DNA from urinary cells. The results show that the sensitivity of the assay was 62% (18 of 29) and the specificity 89% (31 of 35). The positive predictive value of the test was 82% (18 of 22) and the negative predictive value was 74% (31 of 42). We conclude that *FGFR3* mutations are readily observed in a considerable proportion of urinary DNA samples.

Table 3. Sensitivity of *FGFR3* mutation detection by SNaPshot analysis in urine samples.

	Urine (mut)	Urine (wt)	Total
Tumor (mt)	18*	11	29
Tumor (wt)	4	31	35
Total	22	42	64

mut, *FGFR3* mutation present; wt, wild type *FGFR3* gene
*In three cases the mutation found in urine differed from the mutation in the tumor

DISCUSSION

FGFR3 mutation detection is of great clinical importance in bladder cancer. Here we describe a simple assay to detect *FGFR3* mutations in bladder tumors and urine samples. Previous mutation detection was by SSCP and sequencing, both very laborious techniques as the mutation hotspots on exons 7, 10, and 15 have to be screened separately. With the SNaPshot technique the analysis of the three exons can be combined into one assay, thus allowing a sample to be screened for all *FGFR3* mutations simultaneously. Furthermore, there is no need for additional sequencing because both techniques are identical.

The frequency of *FGFR3* mutations in primary bladder tumors is ~50%. Because the presence of an *FGFR3* mutation is associated with a favorable disease course [6], a first analysis of tumors for mutations can select this 50% of patients with a low chance of progressive disease, which could reduce the number of cystoscopies. *FGFR3* also has potential as a molecular marker in urine. A previous study by Rieger-Christ *et al.* [16] showed an overall frequency of *FGFR3* mutations in urine DNA samples of 43%. In this work, the mutation status of the corresponding tumors was not analyzed, therefore it is not possible to calculate the sensitivity of *FGFR3* mutation detection. We have shown that

FGFR3 mutation detection on urine by the SNaPshot assay is an efficient method to detect recurrent bladder tumors (i.e., the sensitivity of the assay was 62%). That the mutations in the urine DNA samples were not observed in all cases might in part be due to the fact that *FGFR3* mutant tumors are often pTaG1, and it is possible that these tumors shed fewer cells than tumors containing a wild type *FGFR3* gene, as these are more often of higher grade and stage. In three cases, the mutation in the urinary DNA sample did not match the mutation in the tumor and in four tumors that were wild type for *FGFR3*, the corresponding urine contained an *FGFR3* mutation. A possible explanation for these findings is that we have observed in another study that there is a small group of patients in whom wild type and mutant bladder tumors seem to occur simultaneously, and that some tumors also appear heterogeneous for the *FGFR3* mutation.[3] For follow-up of patients with superficial bladder cancer (pTa, pT1), we suggest the following scheme: cystoscopy is done at 3 months after transurethral resection and subsequently once per year. *FGFR3* mutation analysis on urine DNA is done every 3 months. When positive, cystoscopy follows. When negative, further screening is done by loss of heterozygosity analysis. Thus, *FGFR3* mutation analysis on voided urine could replace a large percentage of cystoscopies without the risk of negatively affecting the disease course in these patients. In addition, a decrease in the number of invasive cystoscopies would considerably improve the quality of life of the patients.

In conclusion, we developed a high-throughput method for *FGFR3* mutation analysis that is more sensitive than SSCP, and that can be used on both tumor and urine DNA samples. In theory, it is possible to add additional primers to the SNaPshot reaction; for instance, for the detection of other less common mutations such as G382C and S373C [7, 16, 18].

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[3] MNM van der Aa, unpublished results

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CHAPTER 3

Chromosome 9 deletions are more frequent than *FGFR3* mutations in flat urothelial hyperplasias of the bladder

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ABSTRACT

Flat urothelial hyperplasias (FUHs) in patients with papillary bladder tumors frequently show deletions of chromosome 9, suggesting that FUH could be the first neoplastic step in the development of papillary bladder cancer. *FGFR3* mutations are frequent in noninvasive papillary tumors with low risk of progression. Our aim was to investigate the frequency of *FGFR3* mutations and deletions of chromosomes 9p/q and 8p/q in FUH. Thirty FUH and nine simultaneous or consecutive tumors were detected by 5-ALA-based photodynamic cystoscopy. DNA was isolated from frozen sections and whole genome amplification was done by I-PEP-PCR, followed by LOH analysis on chromosomes 8p/q and 9p/q. *FGFR3* mutations were detected by SNaPshot analysis. LOH analysis on FUH revealed deletions at 9p/q (11/30, 37%) and 8p/q (3/30, 10%). *FGFR3* mutations were found in 7/30 FUH (23%). Only 2 FUH showed an *FGFR3* mutation without deletions of chromosome 9. In contrast, 6 FUH revealed chromosome 9 deletions but wild type *FGFR3* ($p = .03$). These results suggest that chromosome 9 deletions are the earliest genetic alterations in bladder cancer. The detection of *FGFR3* mutations in FUH further supports the role of this lesion as precursor of papillary bladder cancer.

INTRODUCTION

Flat urothelial hyperplasia (FUH), which is defined as markedly thickened mucosa without cytological atypia, is frequently found adjacent to papillary urothelial tumors. For many years, this lesion has been regarded non-neoplastic without malignant potential, although clinicopathological studies documented an association between hyperplasia and papillary neoplasia [1]. Genetic analysis on FUH and concomitant papillary tumors in bladder cancer patients revealed that many hyperplasias showed the same genetic aberrations as the papillary tumor, suggesting that FUH could be the first neoplastic step in the development of urothelial carcinoma [2, 3]. Chromosome 9 alterations, especially loss on 9q, are the most frequent genetic events in bladder tumors, and can be found in histologically normal urothelium [2, 4, 5, 6, 7]. It has therefore been suggested that loss of 9q may be the earliest event in the initiation of bladder cancer [8, 9]. Deletions at chromosome arm 8p are also frequent but predominantly associated with advanced papillary bladder tumors [10].

Mutations in the fibroblast growth factor receptor 3 (*FGFR3*) gene, which occur in the germ line of patients with skeletal dysplasias, are present in about 50% of bladder tumors. The majority of these tumours are of low stage and grade, and the presence of an *FGFR3* mutation correlates with a favorable clinical outcome; that is, significantly fewer patients show progression and disease-specific mortality [11]. *FGFR3* mutations were also detected in 75% of urothelial papillomas and are the only genetic events reported to date in these benign lesions [12, 13]. Since mostly tumors of low stage and grade present with *FGFR3* mutations, *FGFR3* mutations are considered early genetic events in bladder tumorigenesis [14, 15].

The aim of the present study was to analyze FUH for *FGFR3* mutations and loss of heterozygosity (LOH) of chromosomes 9 and 8 to investigate these early events in preneoplastic lesions. Since hyperplasia might be the first morphologically identifiable lesion in the development of papillary bladder tumors, we hypothesized that molecular analyses of these lesions could identify early genetic alterations in bladder cancer development.

MATERIALS AND METHODS

Patient material was obtained from a clinical trial assessing the photodynamic diagnosis of neoplastic urothelial lesions [16]. All patients gave written informed consent for this study. Biopsies were taken during cystoscopy after instillation

of 5-ALA into the bladder from both fluorescence-positive, but otherwise inconspicuous areas, and fluorescence-negative papillary tumours. The biopsies were immediately snap-frozen in the operating room. Serial frozen sections stained with haematoxylin and eosin were used for histological diagnosis. Grading was performed according to the WHO classification [17] and staging according to the Union Internationale Contre le Cancer (UICC) [18]. FUH was diagnosed if there was a markedly thickened mucosa (at least 10 layers of cells) without any cytological atypia in serial sections. Surface umbrella cells were preserved. Cases with significant inflammatory infiltrate and edema in the adjacent stroma were excluded as well as lesions with urothelial atypia [2, 3].

Table 1. Histopathological and clinical characteristics of 24 patients with flat urothelial hyperplasia and simultaneous or consecutive papillary bladder tumors.

Case no	Gender/ age	Hyperplasia		Tumor		
		Time point	Localization	Stage/ grade	Time point	Localization
1	M67	-	LW right	-	-	-
2	M37	S	LW right	pTaHG	S	LW left
3	M68	S	LW right	pTaLG	S	LW right
4	M60	-	PW	-	-	-
5	F81	-	LW left	-	-	-
6	M62	-	LW left	-	-	-
7	M64	S	D	pTaLG	S	PW
8	M57	S	PW	pTaLG	S	B
9	M73	-	LW left	-	-	-
10a	F49	S	D	-	-	-
10b		S	D	-	-	-
11	M58	-	LW left	-	-	-
12	F73	C4	PW	pT1HG	C4	LW left
13	M83	-	B	-	-	-
14	M73	S	PW	pT1HG	S	PW
15	F78	-	D	-	-	-
16a	M55	S	PW	-	-	-
16b		S	LW left	-	-	-
17	M56	C2	B	pTaLG	C2	B
18	M60	-	B/LW left	-	-	-
19a	M89	S	PW	-	-	-
19b		S	B	-	-	-
19c		S	B	-	-	-
20	M60	S	LW left	pT2HG	S	LW left
21	M62	S	LW left	pT1HG	S	LW left
22a	M68	S	B	-	-	-
22b		S	PW	-	-	-
23	M81	-	LW right	-	-	-
24a	M73	S	PW	-	-	-
24b		S	B	-	-	-

M, male; F, female; S, simultaneous; C, consecutive/months; LW, lateral wall; PW, posterior wall; D, bladder dome; B, bladder base; LG, low grade; HG, high grade

Microdissection and DNA isolation of the urothelial lesions was performed as described previously [3]. The microdissected samples contained at least 90% urothelial cells. Normal DNA as a reference for LOH analyses was isolated from EDTA blood, or from microdissected stromal or muscle tissue from the urinary bladder.

Whole genome amplification using I-PEP-PCR was performed for all samples to obtain sufficient amounts of DNA [3, 19]. For all lesions, at least 500 cells were microdissected to avoid preferential amplification of one allele in LOH analysis because of insufficient amounts of DNA.

After preamplification, exons 5-9 of the *TP53* gene were directly sequenced using single exon amplification and subsequent cycle sequencing on an ABI 373 sequencer as described previously [20].

Analysis of the *FGFR3* gene for mutations was based on the ABI PRISM SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA), and performed as described before [21]. In short, three regions of interest comprising nine mutations were amplified in one multiplex polymerase chain reaction, followed by extension of primers for each mutation with a labelled dideoxynucleotide. Extended primers were separated by capillary electrophoresis, and the presence or absence of a mutation was indicated by the incorporated nucleotide.

LOH analysis was performed using two microsatellite markers at chromosome arm 8p (D8S1817, D8S1145), two markers at chromosome arm 8q (D8S587, D8S591), two markers at chromosome arm 9p (D9S304, pky11), and three at chromosome arm 9q (D9S303, D9S747, D9S1113) as described previously [22]. Informative cases were scored as allelic losses when the intensity of a signal for a tumor allele had decreased to at least 50% relative to the matched normal allele. All cases of allelic loss (LOH) were confirmed at least once.

RESULTS AND DISCUSSION

Thirty lesions diagnosed as FUH and 9 simultaneous or consecutive papillary carcinomas from 24 patients were investigated in this study (additional clinicopathological data are detailed in Table 1). Good results were obtained for all lesions, however repeated LOH analyses failed in some hyperplasias (8% of the samples could not be analysed (NA), see Figure 1).

Microsatellite analysis showed LOH in 12 of 30 hyperplasias (40%) and in 6 of 9 tumours (67%, Figure 1). Most of the loss was observed at chromosome arm 9q (6/30 hyperplasias and 4/9 tumors), but LOH was also found on 9p (5/30

hyperplasias and 1/9 tumors) and 8p (3/30 hyperplasias and 2/9 tumors). Loss on 8q was not observed.

FGFR3 mutation analysis showed that 23% (7/30) of hyperplasias had an *FGFR3* mutation (Figure 1). Of the 9 papillary carcinomas, two had an *FGFR3* mutation (22%, cases 3 and 7), of which one corresponded with the mutation in the concurrent hyperplasia. In the other case, the tumor was mutant and the concomitant hyperplasia wild type for *FGFR3*. Both tumors were of low stage and grade (Table 1). None of the high grade or invasive tumors carried an *FGFR3* mutation. On the other hand, two high-grade tumours (cases 14 and 20) showed a mutation in *TP53* after analysis of all tumors (Figure 1). This is in accordance to previous studies that demonstrated the mutual exclusiveness of *FGFR3* and p53 alterations, and their role in the different molecular pathways of disease pathogenesis in bladder cancer [23, 24].

Hyperplasia

Case	LOH									FGFR3
	D9S303	D9S747	D9S1113	D9S304	pky11	D8S1817	D8S1145	D8S587	D8S591	
1	I	I	I	I	I	NI	I	I	I	wt
2	NI	I	I	I	I	NI	LOH	I	NI	wt
3	NI	NI	I	I	LOH	I	NA	I	NA	wt
4	NI	I	I	I	I	I	NA	I	NI	wt
5	I	NI	I	I	I	NI	I	I	I	wt
6	LOH	NI	NI	I	I	I	LOH	I	NA	Y375C
7	LOH	I	NI	I	I	NI	I	I	I	Y375C
8	I	I	I	I	I	NI	I	I	I	wt
9	I	NI	I	I	I	I	I	NI	NA	S249C
10a	I	I	I	I	I	NI	I	I	NA	wt
10b	I	I	I	I	I	NI	I	I	I	wt
11	I	I	I	I	I	NI	I	I	I	wt
12	I	LOH	NI	I	I	LOH	I	I	NA	wt
13	NI	I	NI	NI	I	NI	NI	I	NA	wt
14	LOH	NI	I	NI	NI	I	NI	I	NI	wt
15	NI	I	NI	NI	NI	NI	I	I	I	wt
16a	NI	I	NI	NI	I	NI	NI	I	I	wt
16b	NI	I	NI	NI	I	NI	NI	I	I	S249C
17	NI	LOH	NI	I	I	I	NI	I	I	wt
18	I	I	NA	I	NI	I	I	I	NA	wt
19a	I	NI	NA	I	LOH	NI	I	I	I	wt
19b	I	NI	NA	I	LOH	NI	I	I	NA	wt
19c	I	NI	NA	I	I	NI	I	I	I	wt
20	I	I	I	I	I	NI	NA	NI	NA	wt
21	NA	I	NI	I	I	I	I	NI	NA	wt
22a	I	I	NI	LOH	I	I	NI	I	I	R248C
22b	I	I	NA	LOH	I	I	NI	I	NA	R248C
23	LOH	NI	NA	I	I	I	NI	I	NI	R248C
24a	I	NI	I	I	NI	NI	NI	I	I	wt
24b	I	NI	I	I	NI	NI	NI	I	I	wt

Tumor

Case	LOH									FGFR3	P53
	D9S303	D9S747	D9S1113	D9S304	pky11	D8S1817	D8S1145	D8S587	D8S591		
2	NI	I	I	I	I	NI	LOH	I	NI	wt	wt
3	NI	NI	I	I	LOH	I	I	I	I	S249C	wt
7	LOH	I	NI	I	I	NI	I	I	I	Y375C	wt
8	I	I	I	I	I	NI	I	I	I	wt	wt
12	I	LOH	NI	I	I	LOH	I	I	I	wt	wt
14	LOH	NI	I	NI	NI	I	NI	I	NI	wt	mut
17	NI	LOH	NI	I	I	I	NI	I	I	wt	wt
20	I	I	I	I	I	NI	I	NI	I	wt	mut
21	I	I	NI	I	I	I	I	NI	I	wt	wt

Figure 1. Summary of *FGFR3* and *TP53* mutation analysis and LOH data in hyperplasias and tumors.

LOH, loss of heterozygosity; NA, not available; NI, not informative; I, informative; wt, wild type; mut, mutation.

When the results of LOH and *FGFR3* mutation analysis for the hyperplasias were combined, two hyperplasia samples of two patients (cases 9 and 16a) displayed an *FGFR3* mutation, without having deletions at chromosomes 9 or 8. These deletions, however, may be missed because of the limited set of markers we used. Alternatively, samples could be contaminated with normal tissue in spite of microdissection, and this may influence the results of LOH analysis, whereas *FGFR3* mutation analysis is very robust in a large background of nontumor DNA. Conversely, 7 hyperplasia samples of 6 patients with deletions at chromosomes 9 or 8 (cases 2, 3, 12, 14, 17, 19a, and 19b) were *FGFR3* wild type. Six of the 7 samples had deletions at chromosome 9. This means that 55% (6/11) of hyperplasias with LOH at chromosome 9 lacked *FGFR3* mutations, while 71% (5/7) of mutated hyperplasias already had LOH (Table 2, $p = 0.03$). This is in concordance with a previous study by Van Tilborg *et al.* in which we showed that *FGFR3* mutations occur after loss of chromosome 9 in evolutionary genetic trees of patients with multiple tumor recurrences [25]. Urothelial papillomas may also be regarded as precursors of low-grade urothelial cancer [12], and the percentage of *FGFR3* mutations in these lesions is high. Interestingly, so far no LOH in papillomas has been observed, although only a limited number of samples have been investigated [13]. This suggests that mutations in *FGFR3* may play a leading role in the formation of papillomas, whereas in this study we show that chromosome 9 deletions are more frequent in FUH. An explanation for this observation could be that *FGFR3* mutations are associated with neoplasias with a papillary growth pattern, while chromosome 9 deletions might be important for all urothelial neoplasias. We previously showed that chromosome 9 deletions occur both in hyperplasias [3] and in dysplasias and CIS [20].

Table 2. LOH at chromosome 9 and *FGFR3* mutations in flat urothelial hyperplasias.

	FGFR3		
	Mutant	Wild type	Total
LOH 9 (yes)	5	6	11
LOH 9 (no)	2	17	19
Total	7	23	30

In summary, *FGFR3* mutations were detected in flat urothelial hyperplasias of patients with bladder tumors, further supporting the role of this lesion as a precursor of papillary bladder carcinoma. Chromosome 9 deletions may occur earlier than *FGFR3* mutations in most cases and still remain the earliest known genetic alterations in bladder cancer.

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CHAPTER 4

FGFR3 mutations in bladder tumors correlate with low frequency of chromosome alterations

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Submitted

ABSTRACT

It has been suggested that mutation of *FGFR3* is associated with noninvasive tumors of low malignant potential and low risk of recurrence and progression. We hypothesized that *FGFR3* mutated tumors are more genetically stable than bladder tumors without a mutation. The aim of this study was to analyze the distribution of *FGFR3* mutations in bladder tumors of different grade and stage, and determine the relation of *FGFR3* mutations to chromosomal alterations detected by CGH. Frozen sections of 100 bladder cancer samples served as templates for manual microdissection. DNA was isolated from dissected samples containing at least 80% tumor cells. Mutations in *FGFR3* were analyzed by SNaPshot analysis. CGH was carried out according to standard protocols. *FGFR3* mutations were detected in 45 out of 92 samples (49%). Concerning T-category, the following mutation frequencies occurred: pTa 69%, pT1 38%, pT2/3 0%. The mutation frequency was significantly associated with tumor grade: G1 72%, G2 56%, G3 4%. In pTaG1 tumors, mutations were found in 74%. A significant lower number of genetic alterations per tumor detected by CGH was associated with *FGFR3* mutations (2 vs. 8). This association was also seen in pTaG1 tumors: 2.5 (with mutation) vs. 7.5 (without mutation). Our results confirm that *FGFR3* mutations characterize noninvasive low-risk tumors of low malignancy. The low malignant potential of these tumors is underlined by a low number of genetic alterations per tumor. Therefore, *FGFR3* represents a valuable prognostic marker of tumors with low malignant potential and could be used as surrogate marker for detection of genetically stable bladder tumors with good clinical outcome.

INTRODUCTION

Urothelial carcinomas of the urinary bladder represent the fifth most common cancer, and 357,000 new cases are diagnosed every year worldwide [1]. Most of these tumors are noninvasive, well-differentiated papillary tumors (pTa, low grade), and can be treated by endoscopical *trans*-urethral resection. However, up to 70% of these tumors recur, and of these 15-30% are characterized by tumor progression. An early detection of invasive tumors is necessary for an effective therapy. At the moment, no prognostic parameters are available to predict the risk of recurrence or progression for the individual patient. Therefore, new prognostic markers are required for an individual prognosis of patients with bladder cancer. The knowledge of tumor biology and especially the identification of genes and proteins involved in tumor development and progression are essential for new diagnostic and prognostic tools.

The fibroblast growth factor receptor 3 (*FGFR3*) gene could represent a promising biomarker for bladder cancer. FGFR3 is a glycoprotein and belongs to the tyrosine kinase receptor family. Constitutive activation of FGFR3 by germline point mutations in the *FGFR3* gene leads to congenital anomalies such as achondroplasia and thanatophoric dysplasia [2, 3]. Recently, it has been shown that somatic mutations of the *FGFR3* gene occur frequently in urothelial tumors of the bladder and less frequently in carcinomas of the cervix uteri, suggesting that *FGFR3* plays an oncogenic role [4]. Further studies demonstrated that mutations in *FGFR3* occur frequently in noninvasive urothelial tumors of the bladder but not in invasive tumors and might correlate with favorable clinical outcome [5-7].

In the last 10 years it was clearly demonstrated that two different pathways exist in the development of urothelial carcinomas [8]. Noninvasive papillary tumors are genetically stable with few chromosomal alterations, and have a low malignant potential with frequent recurrences but very infrequent progression to invasive disease. In contrast, flat urothelial lesions like dysplasia and carcinoma *in situ* are genetically unstable, with mutiple chromosomal alterations and a rapid progression to invasive highly malignant tumors with unfavorable outcome [9, 10].

The aim of this study was to analyze both *FGFR3* mutations and chromosomal alterations of urothelial tumors of the bladder in order to investigate whether tumors with *FGFR3* mutations are genetically more stable than tumors without mutations. To our knowledge, this is the first study investigating chromosome alterations over the whole genome and *FGFR3* mutation status in correlation with

histopathological data in a large consecutive series.

MATERIALS AND METHODS

Sample collection and DNA extraction

One hundred primary consecutive urothelial carcinomas with different T-categories and grade were included in this study (Table 1). Tumor samples were obtained immediately after *trans*-urethral resection or radical cystectomy and were snap-frozen in liquid nitrogen. Frozen sections were done for all specimens and stained by haematoxylin and eosin to define areas with a high amount of tumor cells. Areas with at least 80% tumor cells were manually microdissected and DNA was isolated with a commercial kit (Qiagen, Germany).

Informed consent was obtained from all patients. Histopathology was assesed on the paraffin-embedded material of the patients by one surgical pathologist. Grading was done according to the 1973 WHO classification.

Table 1. Contribution of histopathological categories.

Classification	Number
pTa	49
pT1	27
pT2-3	14
G1	36
G2	30
G3	22

FGFR3 mutation analysis

Analysis of the *FGFR3* gene for mutations was based on the ABI PRISM SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA), and performed as described before [11]. In short, three regions of interest comprising nine *FGFR3* mutations were amplified in one multiplex polymerase chain reaction, followed by extension of primers for each mutation with a labeled dideoxinucleotide. Extended primers were separated by capillary electrophoresis, and the presence or absence of a mutation was indicated by the incorporated nucleotide.

Comparative genomic hybridization

CGH was performed in cases where mutation analysis was successfully performed. In order to obtain sufficient amounts of DNA for CGH analysis, tumor DNA was amplified according to a modified protocol for DOP-PCR [12, 13]. This protocol employs Sequenase during the first eight cycles of nonspecific PCR, followed by 30 additional cycles under specific conditions using Taq polymerase (Stoffel fragment). Labeling of tumor DNA and normal DNA was achieved by 20 PCR cycles using Biotin-16dUTP and Digoxigenin-11dUTP, respectively.

One µg of both tumor DNA and normal DNA were hybridized with 50 µg Cot-1 DNA on normal metaphases at 37°C for 48h. Detection of fluorescent signals was carried out with Avidin-FITC (tumor DNA) and Anti-Digoxigenin-Rhodoamine (normal DNA). DAPI-Antifade was used for chromosome counterstaining. Fifteen metaphases were analyzed in each case using an Axioplan-Microscope (Zeiss, Germany), and a computer system from "Metasystems" (Germany). Chromosomal alterations could be detected as shifts of the profile to the red borderline (loss of chromosomal region in the tumor DNA) or to the green borderline (gain of chromosomal region in the tumor DNA).

Statistical analysis

Statistical analysis was performed using SPSS software. The χ^2 test and the Mann-Whitney-U test were used.

RESULTS

FGFR3 mutation analysis

Mutation analysis was possible in 92 out of 100 cases. *FGFR3* mutations were detected in 45 out of 92 cases (49%). Most frequently, mutations in codon 249 (S249C) occurred (25 cases). Codon 375 (Y375C), 372 (G372C), 248 (R248C), and 652 (K652E and K652T) mutations were found in 10, 4, 3 and 3 cases, respectively (see Table 2).

A strong correlation between mutations and stage was found. Mutations occurred only in pTa (69%) and pT1 tumors (38%), but never in higher stage tumors (pT2-3 0%, see Figure 1A). These results were statistically significant ($p < 0.001$). Comparison of mutation frequency between pTa and pT1 tumors was also significant ($p = 0.01$).

Mutations were detected mostly in G1 and G2 tumors, but only in one G3 tumor (see Figure 1B). These results were statistically significant ($p < 0.001$).

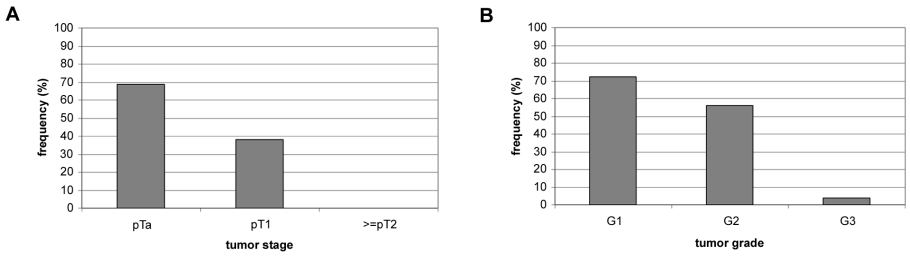

Figure 1. *FGFR3* mutation frequency.
The frequency of *FGFR3* mutations correlated to T-category (A), and tumor grade (B; p<0.001).

Mutations occurred in 72% of G1 and in 56% of G2 tumors (p = 0.23). The mutation frequency was 74% in pTaG1 tumors and 54% in pTaG2 tumors, respectively.

Comparative Genomic Hybridization

CGH was performed on the 92 cases where mutation analysis was possible. Results were obtained in 90 cases. Alterations were detected in 62 cases (69%) (see Table 2). The mean number of genetic alterations per tumor was 6.4. The following genetic alterations were found frequently: losses of chromosomes 9 (55%), 8p (29%), 6 (23%), 11p (23%), 5q (20%), and gains of chromosomes 8q (44%) and 1 (21%).

The mean number of aberrations per tumor was 4.73, 7.86 and 9.11 in pTa, pT1 and pT2-3 tumors, respectively (p = 0.03). The frequency of specific alterations was higher with higher T-category for many chromosomes (see Figure 2). However, statistical significance was reached only for loss of chromosome 5q and loss of chromosome 9 (more frequent in lower T).

Table 2. Results after mutation analysis and CGH analysis.

Case No	pT	G	*FGFR3*	CGH results
102	pTis	G C	o	o
95	pTa	G1	o	dim(8p), enh(3p21qter,6p,7q31qter,8q,10p12pter,12q23 qter)
149	pTa	G1	o	o
155	pTa	G1	o	o
156	pTa	G1	o	o
168	pTa	G1	o	o
194	pTa	G1	o	dim(2q,3p14p23,6,8p,9,14,17p), enh(8q)
196	pTa	G1	o	dim(9q), enh(8q)
430	pTa	G1	o	o
582	pTa	G1	o	dim(5q,6q,9p,14), enh(5,16q,20)

367	pTa	G1	G372C	dim(Y)
583	pTa	G1	G372C	dim(9q13q31)
172	pTa	G1	K652E	dim(8p,9p,17p,18q12q21)
373	pTa	G1	K652T	dim(6q13qter,9q,17p), enh(1q,3q26qter,6p12q13,7p15pter,8q23)
190	pTa	G1	R248C	dim(9)
366	pTa	G1	R248C	dim(3p13p24,5q12q21,6q16qter,8p12pter,9,10q21qter,17p), enh(1q24,2q14q34,17q)
590	pTa	G1	R248C	o
15	pTa	G1	S249C	enh(1p,15q), dim(9,11p)
23	pTa	G1	S249C	o
27	pTa	G1	S249C	dim(9q,Y), enh(8q)
70	pTa	G1	S249C	o
144	pTa	G1	S249C	dim(9,16q21qter,17p13pter), enh(1q24q41)
153	pTa	G1	S249C	dim(9q22q33)
157	pTa	G1	S249C	o
179	pTa	G1	S249C	o
204	pTa	G1	S249C	o
207	pTa	G1	S249C	dim(9q21qter)
214	pTa	G1	S249C	o
365	pTa	G1	S249C	dim(9q), enh(10q24q25)
379	pTa	G1	S249C	o
140	pTa	G1	Y375C	dim(9)
333	pTa	G1	Y375C	dim(Y)
371	pTa	G1	Y375C	o
378	pTa	G1	Y375C	dim(3p12p24,2q,4p15pter,6p?,8p,9q,14q22qter,17p), enh(5q14q31,8q,13q21qter)
440	pTa	G1	Y375C	dim(9q)
588	pTa	G1	Y375C	dim(9), enh(11q14q22)
71	pTa	G2	o	o
110	pTa	G2	o	
137	pTa	G2	o	o
441	pTa	G2	o	enh(3p21pter,3q,5q,8q,10q,16q,17q), dim(4,5q,6q,8p,9,11,12,18q), amp(11q13)
571	pTa	G2	o	enh(6p,7p,8,20),amp(11q13)
573	pTa	G2	o	enh(1q32qter,2q15q22,5q,7q,8q,11q,13q21qter,18p,20), dim(2q32qter,5q,6p21.2q22,7p11.2p21,9,11p,14,18q,Y), amp(8p11.1p22)
580	pTa	G2	G372C	dim(Y)
13	pTa	G2	S249C	dim(9q,Xp), enh(14q23qter,17,20q)
372	pTa	G2	S249C	o
577	pTa	G2	S249C	enh(1p,8q,15), dim(8p,9,11p)
584	pTa	G2	S249C	enh(1q,8,13)
574	pTa	G2	Y375C	o
579	pTa	G2	Y375C	dim(9q,11), enh(7), amp(12q21q22)
587	pTa	G3	S249C	dim(9,10q24q25,11p)
195	pT1	G1	o	o
62	pT1	G2	o	dim(5q?,5q33qter,6q22,8p12pter,9,11p,17p12pter), enh(6p?,17q?,18q?,20p), amp(8q23)
109	pT1	G2	o	dim(4q,5q23qter,8p,9q,11p,Y), enh(3q,6,7p,8q,10p,12p,18p,21)
138	pT1	G2	o	o
161	pT1	G2	o	dim(8p), enh(8q)
183	pT1	G2	o	dim(2q31qter,4q,5p,5q23qter,6p11.1p21.3,8q23qter,9,10q,13q12q14,Y), enh((3q25qter,6q12q21,10p,11p11.2q13,15q,16), amp(5p13q11), amp(7p14pter), amp(8)(q11.1q12), amp(12)(q14q21)
429	pT1	G2	o	o
591	pT1	G2	o	enh(5p,8p11.1p22,10p,18p)
96	pT1	G2	K652E	enh(3,8)
4	pT1	G2	S249C	dim(9q), enh(8q)
61	pT1	G2	S249C	dim(9), enh(1q)

89	pT1	G2	S249C	dim(5q32q34,7p,9), enh(7q,15q)
201	pT1	G2	S249C	o
215	pT1	G2	S249C	dim(8p,9p,10q23qter?,11p,18q21q22), enh(2p,3q?,12q14q21)
382	pT1	G2	S249C	dim(8p12q11.2)
581	pT1	G2	S249C	dim(3p14pter,(9q21qter)
300	pT1	G2	Y375C	enh(7), dim(9q,Y)
576	pT1	G2	Y375C	dim(2q33qter,11)
22	pT1	G3	o	dim(4q32.1qter,12q21qter), enh(1q21q31,2q,3p25pter, 5p,18p)
106	pT1	G3	o	dim(8p,18q), enh(5p,8q21q23,17p,18p)
185	pT1	G3	o	enh(8q22qter,16,17q,20)
210	pT1	G3	o	enh(5p,6p,10p,11q13q23,13q,16p,17q), dim(5q,Y), amp(8q22)
225	pT1	G3	o	dim(5q?,9q?), enh(1p13q31)
575	pT1	G3	o	dim(5q,7q32qter,8p,Y), enh(2p,3q,4p,4q31q33,5p,6p, 8q21q23,10p,11q14q23,13q21qter,18p)
585	pT1	G3	o	enh(2p,3,5p,7,8,9,10p,20), dim(1p,5q11.2q15,6q,9,10q, 14,17p)
586	pT1	G3	o	dim(2q32qter,4q13q31.1,5q,8p,10q,11p,13,14q11.2q24, 16q), enh(1q21q32,2p,5p,-5q,6p,7q,8q,10p,11q,16p,17, 20)
589	pT1	G3	o	dim(4q26qter,6q,8p,10q23qter,11p,14,17p,18q), enh(1q24qter,2p11.2p22,2q24q25,3p22pter,3q,5p,6p,7p, 7q35qter,8p11.2q11.2,8q23q24,9p,11q23qter,20)
60	pT2	G3	o	dim(2q32qter,5q,11p12pter,12p,Y), enh(3,5p,8q21.3q22.3 ,13q21.3qter,14q,20q)
135	pT2	G3	o	dim(2q14,2,4p,6q,8p12pter,11p,13q13q31.3,14q21qter,1 6p,18q), enh(1p31p1q32.2,3q,4q?,5p,7,16q22.1qter,18p, 20q), amp(8q23)
178	pT2	G3	o	dim(1p,2q36qter,5q,6q,8p,10q,18q), enh(1q,5p,6p22p24, 9p,10p12pter,13q31qter,17q23.2qter,18p,20q)
223	pT2	G3	o	dim(8p,11q23qter,18q21qter?), enh(3q24qter,6p22pter, 8q,9,11p?,18q,14q)
224	pT2	G3	o	dim(2q36qter,10q,11q22qter), enh(6p23pter)
228	pT2	G3	o	o
578	pT2	G3	o	o
311	pT2b	G3	o	o
66	pT3a	G3	o	dim(6q), enh(8q21.1qter,11q14.3qter)
101	pT3a	G3	o	o
108	pT3a	G3	o	
143	pT3a	G3	o	dim(6p22pter,17p), enh(3q25q26,8q,9p23pter)
139	pT3b	G2	o	dim(4q31qter,9,15q22qter,17p,Y), enh(7), amp(10q22q23)
381	pT3b	G3	o	dim(4p,5q11.2q23,8p,18q), enh(5p,8q,10q25qter,20)
181	pTx	G2	G372C	o

Mutations are specified for each codon. o, no alterations were detected; dim, loss of chromosome region; enh, gain of chromosome region.

For all tumors, the number of aberrations per tumor is significantly higher in tumors without mutations (median 8.0) compared to that with *FGFR3* mutations (median 2.0, $p < 0.001$). Correspondingly, the number of alterations per tumor differs significantly between tumors with (median 2.5) and without (median 7.5) mutations ($p = 0.01$) in the subgroup of pTa tumors.

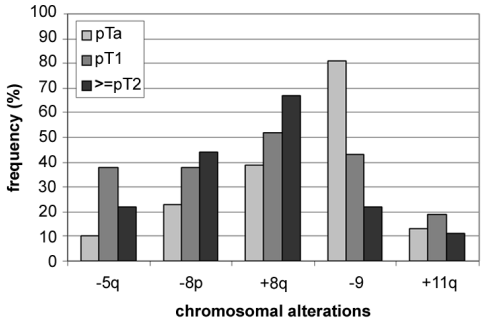

Figure 2. Contribution of chromosomal alterations detected by CGH concerning T-category.
Statistical significance was reached for loss of chromosome 5q between Ta and T1 and for loss of chromosome 9 between Ta, T1 and T2-3.

Analysis of specific chromosomes revealed significantly more gains of chromosomes 8q ($p < 0.001$), 5p ($p = 0.003$), 10p ($p = 0.01$), 11q ($p = 0.02$) as well as losses of chromosomes 8p ($p = 0.04$) and 5q ($p = 0.002$) in tumors lacking mutations (see Figure 3A). Interestingly, losses of chromosome 9 were on the other hand significantly more frequent in tumors with mutations ($p = 0.01$).

In pTa tumors, the above described alterations were also different in tumors with or without mutations (see Figure 3B). However, statistical significance was reached for chromosomes 5q ($p = 0.04$), 6 ($p = 0.02$), 8q ($p = 0.004$) and 11q ($p = 0.01$). Losses of chromosome 9 were similarly distributed in both groups.

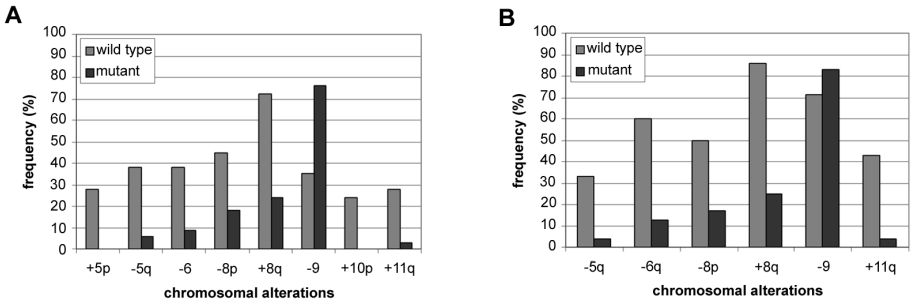

Figure 3. Frequency of chromosomal alterations detected by CGH in tumors with and without *FGFR3* mutations.
A, all tumors. Statistical significance was reached for all presented chromosomes. B, pTa tumors only. Statistical significance was reached for chromosomes 5q, 6, 8q and 11q.

DISCUSSION

Despite of good therapy options and favorable outcome, a high recurrence rate is a serious problem in noninvasive bladder tumors of low malignancy. In addition, urothelial carcinomas with stromal invasion (pT1) show a considerable risk of progression to muscle invasive disease. Until now, no prognostic parameters are available to predict the individual outcome of these patients. During the last years, molecular investigations were performed to understand the tumor biology of bladder cancer, and thereby to identify genes and proteins which are involved in tumor development and progression. Several genes were identified which could be of prognostic significance in bladder cancer. It was shown that accumulation of p53 is an independent prognostic predictor of recurrence-free and overall survival [14, 15]. However, some studies failed to show the relationship with outcome, possibly because of different protocols for immunohistochemistry or patient selections [16]. Because of the rare occurrence of *TP53* mutations in pTaG1 tumors other prognostic factors are necessary to predict the outcome of these patients.

FGFR3 mutations were recently detected in bladder cancer by several groups and described to be associated with low recurrence and progression rate [4, 6]. Fibroblast growth factor receptors belong to the tyrosine kinase receptor family. They regulate cellular processes like cell growth, differentiation, and angiogenesis.

In our study, we found *FGFR3* mutations in about 50% of all tumors. There was a strong correlation with stage and grade. Mutations were restricted to

pTa and pT1 tumors with high or moderate differentiation, and never occurred in muscle-invasive tumors. Therefore, *FGFR3* mutations were associated with noninvasive low malignant tumors or tumors with limited invasive potential. These results confirmed findings of other studies. While the frequency in pTa tumors was similar compared to these studies, the percentage of pT1 tumors with mutations differs between the studies [5, 6, 17]. One reason might be the different number of cases which were investigated and a different distribution of tumor grade in these cases. If there were many pT1G3 tumors included, the percentage of cases with mutations was very low. This strong correlation with grade was striking in all studies. The majority of mutations were found in low malignant tumors, whereas mutations in G3 tumors were very rare. These results from different studies underline that *FGFR3* mutations characterize tumors with favorable histological features. Furthermore, in a study from van Rhijn *et al.* it was shown that the presence of an *FGFR3* mutation is a strong indicator of superficial bladder tumors with a favorable clinical outcome [5]. Recently, Hernandez *et al.* found *FGFR3* mutations to be associated with a higher rate of recurrence but again with good clinical outcome [7]. Previous studies clearly showed that bladder cancer cases can be separated in two distinct tumor entities: 1) genetically stable low malignant tumors with few genetic alterations which are mainly deletions of chromosome 9, and 2) genetically unstable highly malignant tumors with multiple genomic aberrations.

Based on these findings, we hypothesized that tumors with and without *FGFR3* mutations were also characterized by different chromosomal patterns. For that reason, we performed comparative genomic hybridization (CGH), which allows the detection of chromosomal losses and gains over the whole genome. Mutation analysis and CGH were carried out on the same material and were correlated. To our knowledge, this is the first study which combines *FGFR3* mutation analysis with a whole genome chromosome analysis. The number of genetic alterations was significantly higher in tumors without *FGFR3* mutations. This clearly indicates that tumors with mutations are genetically more stable than tumors without mutations. In order to exclude that this correlation was due to the higher percentage of high grade tumors in the group of invasive tumors, we analyzed the pTa tumors separately. Even in this group a strong correlation of *FGFR3* mutations with low number of chromosomal alterations was found. Therefore, mutations in *FGFR3* characterize noninvasive low malignant tumors which are genetically stable.

Looking at specific chromosomes, we found that losses of 5q and 6 as well

as gains of chromosomes 8q, 10p and 11q were significantly more frequent in tumors without mutations. A similar picture was seen in pTa tumors; however, statistical significance was reached only for chromosomes 6, 8q and 11q. Losses on chromosome 5 in bladder cancer are known from other studies [18, 19], in which losses of the chromosome regions 5q22-q23 and 5q33-q34 were found to be associated with tumor progression [19]. Gain of 8q and loss of 8p are known genetic alterations which are associated with progression in many solid tumors as well as in bladder cancer [20-22]. Interestingly, deletions of chromosome region 8p12-22 were found to be associated with invasive papillary bladder cancers [23]. To exclude that the statistical correlation between specific genetic alterations and *FGFR3* mutation is based on a correlation between stage and genetic alterations, we performed statistical analysis between T-category and each chromosomal alteration. As a result, we could not find statistical significance even if there was an accumulation of the above alterations in invasive tumors. Because the correlation between *FGFR3* wild type status and gains of 8q was shown, we can conclude that gain of 8q is a specific feature of more aggressive tumors without *FGFR3* mutations.

Another significant alteration was a gain on 11q. Most often, loss on 11q was described as associated with bladder cancer, but we found it only in 3 tumors. On the other hand, several groups detected an amplification in the region 11q13q23 [22, 24]. In our cases, gains on 11q occurred mostly as amplifications or gains of a restricted region between 11q13 and q23. This region contains oncogenes like *CCND1*, *FGF3*, and *FGF4*, which could be activated by amplification [25]. Amplification of these genes is associated with stage and survival in pT1 tumors [26].

Loss on chromosome 9 correlated with *FGFR3* mutations in the whole tumor group. However, this correlation is based on distribution of chromosome 9 losses in different stages. There is a strong correlation between loss of chromosome 9 and stage (see Figure 2). A higher number of chromosome 9 alterations in pTa tumors compared to pT1-pT4 tumors was also described by Richter *et al.* [27]. Losses on chromosome 9 are apparently typical features of noninvasive low malignant papillary tumors (see Figure 2). In pTa tumors, loss of chromosome 9 is similar distributed in tumors with and without mutations. Therefore, a correlation of *FGFR3* mutations and chromosome 9 alterations does not exist in pTa tumors. Obviously, alterations of chromosome 9 precede *FGFR3* mutations in pTa tumors. This is underlined by the fact that losses on chromosome 9 occur earlier than *FGFR3* mutations in hyperplasias [28].

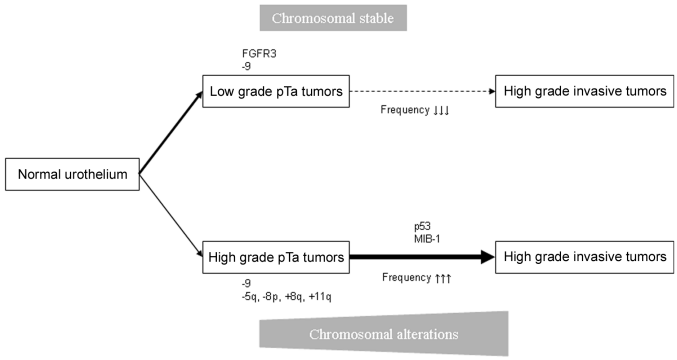

Figure 4. Scheme of different pathways in bladder cancer development

The results of our study underline that different pathways of bladder cancer pathobiology exist with different genetic alterations. This hypothesis was described by van Rhijn *et al.* and Bakkar *et al.* [17, 29, 30]. The authors investigated *FGFR3* mutations and expression of several markers correlated with invasive tumors like p53, MIB-1, or p27. They found that overexpression of p53 and MIB-1 is very rare in tumors with *FGFR3* mutations. The presence of p53 and *FGFR3* mutations is mutually exclusive in bladder tumors. Furthermore, the combination of *FGFR3* mutation and proliferation index (MIB-1) gives the base of a molecular grading which correlates with clinical course and outcome. From these studies, and the results of the present investigation, two distinct pathways of bladder cancer development can be hypothesized (Figure 4). The first more frequent group of tumors has *FGFR3* mutations, no alterations in p53, and low proliferation. These papillary tumors are low malignant and possess a low recurrence rate and, if at all, only a minimal progression risk. The second group consists of highly malignant solid and papillary tumors without *FGFR3* mutations, but frequent p53 alterations and high proliferation index. These tumors frequently recur and have a considerable progression risk. Our results clearly support this hypothesis. In addition to the described features, the second pathway is characterized by genetic instability on the chromosome level and by specific chromosomal alterations like gain of 8q, amplification on 11q and loss of 5q. Furthermore, specific molecular expression signatures for progressive and non-progressive pTa/pT1 tumors were identified by Dyrskjøt *et al.* [31]. These authors suggested that it will be possible to identify patients with high risk of disease progression at an early stage of disease.

The identification of molecular markers and the combined use for a molecular grading will be helpful in the future to predict the prognosis of bladder cancer patients at time of first diagnosis, and to select a specific therapy at an early time point. Furthermore, the reproducibility of molecular markers is superior to that of the classical parameters stage and grade. Because most studies on molecular markers such as *FGFR3*, p53 or specific chromosomal markers were performed on retrospective material, prospective studies are required to evaluate the clinical relevance.

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CHAPTER 5

FGFR3 mutations and a normal CK20 staining pattern define low-grade noninvasive urothelial bladder tumors

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ABSTRACT

Molecular markers superior to conventional clinicopathologic parameters are needed to predict disease courses in bladder cancer patients. In this study, we investigated four markers (Ki-67, TP53, CK20, *FGFR3*) in primary urothelial bladder tumors and compared them with traditional pathologic features. Tissue microarrays were used to analyse CK20, TP53, and Ki-67 expression immunohistochemically in 255 unselected patients. *FGFR3* mutations were detected by SNaPshot analysis. Abnormal CK20 expression was strongly associated with higher tumor grades and stages ($p < 0.001$); however, 65% of pTa tumours revealed an abnormal CK20 pattern. In the group of pTaG1 tumors, 59% presented with an abnormal CK20 pattern, whereas 82% carried the *FGFR3* mutation. In the group of bladder tumors with normal CK20 pattern, the *FGFR3* gene was mutated in 89%, whereas a mutated *FGFR3* gene was found only in 37% of cases with abnormal CK20 expression ($p < 0.001$). All markers proved to be strong predictors of disease-specific survival in univariate studies. However, in multivariate analyses they were not independent from classical pathologic parameters. None of the molecular markers was significantly associated with tumor recurrence. We conclude that dysregulation of CK20 expression is an early event in the carcinogenesis of papillary noninvasive bladder cancer, but occurs later than *FGFR3* mutations. The group of low-grade noninvasive papillary tumors is defined by the presence of an *FGFR3* mutation and a normal CK20 expression pattern.

INTRODUCTION

Most bladder tumor patients (75-80%) present with papillary noninvasive (pTa) or superficially invasive (pT1) urothelial tumours at first presentation, whereas the remaining 20-25% of primary tumors are already muscle-invasive (≥pT2). pTa and pT1 tumors can be removed by transurethral resection (TUR); however 70% of patients will have one or more recurrent tumors, and up to 25% will eventually develop muscle-invasive disease. Bladder cancer patients therefore have to be monitored thoroughly for disease recurrence and progression. Recent attempts in predicting prognosis include image analysis [1] and uPAR expression analysis [2]; still there are no established markers, molecular or classical, that are able to predict which tumors will progress and which will not.

Mutations in the fibroblast growth factor receptor 3 (*FGFR3*) gene are very frequent in pTa bladder tumors (about 75%) [3, 4, 5], less frequent in pT1G3 tumors [6, 7], and rare in carcinoma *in situ* (pTis) [3, 7]. Furthermore, patients with primary bladder cancers with an *FGFR3* mutation were shown to have a significantly better prognosis than patients without a mutation [5].

Increased proliferative activity using Ki-67 immunohistochemistry (IHC), and *TP53* mutations are both markers that indicate an unfavorable disease course [8, 9]. However, bladder tumors with an increased immunoreactivity for Ki-67 and TP53 progress less often when an *FGFR3* mutation is present [5]. *FGFR3* and *TP53* are almost mutually exclusive, and represent different pathways in bladder cancer development [5, 10, 11]. The combination of *FGFR3* mutation analysis and Ki-67 IHC, defined as molecular grading, proved to be superior to other parameters in the prediction of progression and survival of bladder cancer patients [5].

Cytokeratin 20 (CK20) is another marker for tumors of low stage and grade, and is expressed in the umbrella cells of normal urothelium [12] and reactive atypia. When CK20 expression in bladder tumors is limited to the umbrella cells, it is associated with a mild disease course, while expression in the entire urothelium in more than 10% of the tumor cells is associated with higher tumor grade [13] and an increased risk of progression and recurrence [14, 15]. In urothelial carcinoma *in situ*, intense CK20 expression is found in the majority of malignant cells [16, 17].

To study the possible prognostic value of these four molecular markers and their relation to each other in the pathogenesis of bladder cancer, we analyzed a large series of unselected primary urothelial bladder tumors for *FGFR3* mutations, and for expression of CK20, TP53, and Ki-67 in relation to tumor stage, grade,

multifocality, adjacent carcinoma *in situ*, and prognosis in these patients.

MATERIALS AND METHODS

Bladder cancer tissue microarray

A tissue microarray was constructed [18] from 255 consecutive, formalin-fixed, paraffin-embedded, primary urothelial bladder cancer tissues (Institute of Pathology, University of Regensburg, Regensburg, Germany). Clinical data were obtained from the Central Tumor Registry, Regensburg, Germany, and by telephone interviews (MB, SD) in case of missing data. The Institutional Review Board of the University of Regensburg approved analysis of tissues from human subjects.

Haematoxylin-eosin-stained slides of all tumors were evaluated by a single surgical pathologist (AH). Tumor stage and grade were assigned according to Union Internationale Contre le Cancer (UICC) and World Health Organization (WHO) criteria. Growth pattern was determined for all invasive tumors (≥pT1). Papillary growth was defined by the presence of a papillary tumor component (≥20%) with a histologic grade identical to the invasive tumor. All other tumors were considered to have a solid growth pattern. Clinicopathologic data are summarized in Table 1. Retrospective clinical follow-up data were available regarding the end points recurrence-free survival and overall survival for all patients with a median follow-up period of 75 months (range: 0-147 months). The median follow-up for censored patients was 81 months. Recurrences were defined as cystoscopically visible tumors with histologic verification. Data on progression-free survival were not available.

Immunohistochemistry

Immunohistochemical studies utilized an avidin-biotin peroxidase method with a diaminobenzidine (DAB) chromatogen. After antigen retrieval (microwave oven for 30 min at 250 W), immunohistochemistry was carried out in a NEXES immunostainer (Ventana, Tucson, AZ) following manufacturer's instructions. The following primary antibodies were used: anti-TP53 (mouse monoclonal IgG, clone Bp53-12 (sc-263); Santa Cruz Biotechnology, Inc, Santa Cruz, CA; dilution 1:1000), anti-CK20 (mouse monoclonal IgG2a, clone IT-Ks20.8 (61026); Progen Biotechnik GmbH, Heidelberg, Germany; dilution 1:10), and anti-Ki-67 (mouse monoclonal IgG1, clone MIB-1 (M7240); Dako, Glostrup, Denmark; dilution 1:50). One surgical pathologist (A.H.) performed a blinded evaluation of the slides.

Table 1. Patient and tumor characteristics and results of molecular and immunohistochemical analyses.

Variable	Categorization	Patients	
		n	%
Age at diagnosis			
	<70 years	141	55
	≥70 years	114	45
Gender			
	female	64	25
	male	191	75
Tumor stage[a]			
	pTa	146	57
	pT1	48	19
	pT2	56	22
	pT3	2	1
	pT4	3	1
Histologic grade[a]			
	G1	81	32
	G2	69	27
	G3	105	41
Adjacent carcinoma *in situ*			
	no	222	87
	yes	33	13
Multifocality			
	unifocal tumor	53	21
	multifocal tumor	202	79
Growth pattern[b]			
	papillary	61	56
	solid	47	44
FGFR3			
	wild type	110	53
	mutant	98	47
Ki-67 IHC			
	≤ 25%	168	71
	> 25%	68	29
TP53 IHC			
	≤ 10%	179	73
	> 10%	66	27
CK20 IHC[c]			
	normal	49	20
	abnormal	192	80

[a]staging and grading according to the 1973 WHO classification system
[b]pT1-4 only
[c]CK20 IHC staining pattern according to Harnden *et al*. Lancet 1999;353:974-7

TP53 positivity was defined as strong nuclear staining in more than 10% of the tumor cells. The percentage of Ki-67 positive cells of each specimen was determined as described previously [19]. High Ki-67 labelling index was defined if more than 25% of the tumor cells were positive [5]. CK20 staining was defined as normal (superficial staining pattern) or abnormal (negative or more than 10% stained) according to Harnden *et al.* [14].

DNA isolation

Genomic DNA of tumors was isolated from 1.5 mm punch biopsies of the paraffin blocks (one tissue core per case). Tumor areas were marked by a surgical pathologist (A.H.) to ensure a tumor cell content of at least 80%. DNA isolation was performed with the use of the Magna Pure DNA isolation kit (Roche, Penzberg, Germany) according to manufacturer's instructions.

FGFR3 mutation analysis

FGFR3 mutation analysis was performed with the use of the SNaPshot method [20]. In short, three regions of the *FGFR3* gene, comprising all *FGFR3* mutations found in bladder cancer [21], were amplified simultaneously in a multiplex polymerase chain reaction (PCR). After removal of excess primers and dNTPs, eight SNaPshot primers detecting nine *FGFR3* mutations were annealed to the PCR products and extended with a labelled dideoxynucleotide. These extended primers were analyzed on an automatic sequencer, with the label on the incorporated nucleotide indicating the presence or absence of a mutation. All mutations were verified by a second and independent SNaPshot analysis.

Statistical analysis

Statistical analyses were completed with the Statistical Package for the Social Sciences, version 11.0 (SPSS Inc, Chicago, IL). Differences were considered significant if $p < 0.05$. Associations between measured parameters were obtained by applying chi-square and two-sided Fisher's exact tests. The Kaplan-Meier method was used to compare curves for the different variables with regard to recurrence-free and disease-specific survival, with significance evaluated by two-sided log rank statistics. For the analysis of recurrence-free survival, patients were censored at the date when cystectomy was performed or at the time of their last tumor-free clinical follow-up appointment. For survival analysis, patients were censored at the time of their last tumor-free clinical follow-up appointment or at their date of death if it was not related to the tumor. The proportionality assumption for all variables was assessed with log-negative-log survival distribution functions. Cox proportional hazard ratios were estimated to obtain risks of recurrence and death and to find independent prognostic factors in a multivariate model.

RESULTS

Molecular markers and histopathology

Activating mutations in the *FGFR3* gene were detected in 98 (47%) of 208 bladder tumors, of which 61% (94 of 154) occurred in pTa and pT1 tumors. Table 2 shows the association of *FGFR3* mutation status with pathologic parameters. *FGFR3* mutations were predominantly present in tumors with stage pTa (73%) and grade 1 (82%). Interestingly, almost all *FGFR3* mutations occurred in tumors without adjacent carcinoma *in situ* ($p < 0.001$). With only 1% (3 of 208) concurrence, *FGFR3* mutation and adjacent carcinoma *in situ* must be considered exclusive events. These data confirm that *FGFR3* mutations are associated with favorable histopathological characteristics.

Table 2. Comparison of the four molecular markers with traditional pathologic parameters.

Variable	Category	*FGFR3* mutations			Ki67 expression			TP53 expression			CK20 staining pattern		
		wt	mut	p^a	≤25%	>25%	p^a	≤10%	>10%	p^a	norm	abn	p^a
Tumor stage													
	pTa	31	85		127	9		130	10		48	90	
	pT1	29	9		24	19		20	26		1	44	
	pT2	46	3	**<0.001**	16	36	**<0.001**	26	28	**<0.001**	0	53	**<0.001**
	pT3	2	0		1	1		0	2		0	2	
	pT4	2	1		0	3		3	0		0	3	
Histologic grade													
	G1	11	49		71	1		73	2		30	44	
	G2	20	38	**<0.001**	61	7	**<0.001**	58	10	**<0.001**	18	50	**<0.001**
	G3	79	11		36	60		48	54		1	98	
Adjacent carcinoma *in situ*													
	no	81	95	**<0.001**	155	51	**0.001**	165	48	**<0.001**	48	164	**0.01**
	yes	29	3		13	17		14	18		1	28	
Multifocality													
	unifocal	25	14	0.20	30	18	1.0	33	19	0.10	10	40	1.0
	multifocal	85	84		138	50		146	47		39	152	

[a]Fisher's exact test (2-sided); bold face representing *p*-values < 0.05.
wt, wild type; mut, mutant; norm, normal; abn, abnormal.

In addition to *FGFR3* mutation status, we studied the CK20 expression pattern in relation to histopathologic parameters. Examples of CK20 expression are shown in Figure 1. A normal CK20 pattern occurred only in low-stage tumors (pTa and pT1, Table 2), and most tumors with a normal CK20 pattern were grade 1 (61%). Moreover, tumors with a normal CK20 staining pattern almost never revealed adjacent carcinoma *in situ* (48 of 49, 98%). Abnormal CK20 expression was

strongly associated with higher tumor stages and grades ($p < 0.001$), even though 65% (90 of 138) of pTa bladder tumors revealed an abnormal CK20 pattern.

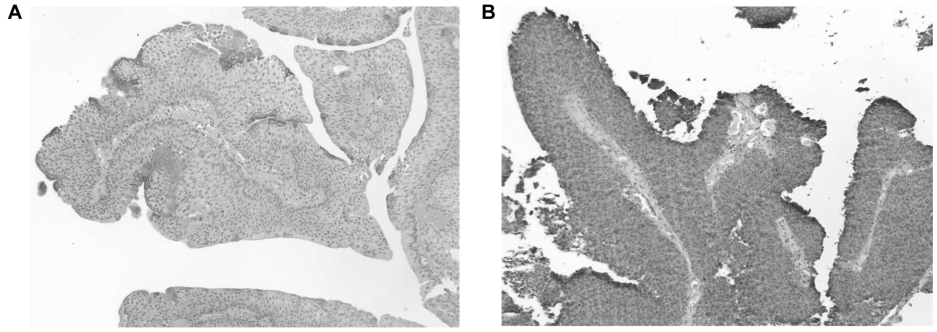

Figure 1. Immunohistochemical staining with CK20.
A, normal CK20 expression pattern in which only the superficial urothelial cells are positive; B, abnormal CK20 staining pattern, represented by CK20 expression in all cell layers.

In contrast, TP53 overexpression (>10%) and high proliferation (% Ki-67 positive cells >25) were seen in 3% and 1% of grade 1 tumors, and in 53% and 63% of grade 3 tumors, respectively (Table 2). Furthermore, overexpression of TP53 and high Ki-67 labeling index was frequently present in tumors with adjacent carcinoma *in situ* (65% and 75%, respectively). Mutations of the *FGFR3* gene and positive TP53 immunoreactivity (>10%) were exclusive events, with a coincidence of only 2% (5 of 203, $p < 0.001$, Table 3).

Table 3. *FGFR3* mutation status related to other molecular markers.

Variable	Categorization	*FGFR3*		
		wild type	mutant	*p*[a]
Ki-67 IHC				
	≤25%	54	84	**<0.001**
	>25%	48	10	
TP53 IHC				
	≤10%	59	91	**<0.001**
	>10%	48	5	
CK20 IHC				
	normal	4	32	**<0.001**
	abnormal	102	61	

[a]Fisher's exact test, 2-sided

Molecular markers and disease course

The end points in this study were recurrence-free and disease-specific survival. Unfortunately, data on progression-free survival were not available. Kaplan-Meier analyses for disease-specific survival are depicted in Figures 2A–D, and show that bladder cancer patients with an *FGFR3* mutation have a significantly better survival than patients without a mutation. In contrast, patients with aberrant expression of Ki-67, TP53 or CK20 have a significantly worse survival. Table 4 shows *p*-values and 5-year survival rates for these molecular markers. None of the parameters showed correlation with tumor recurrence (data not shown).

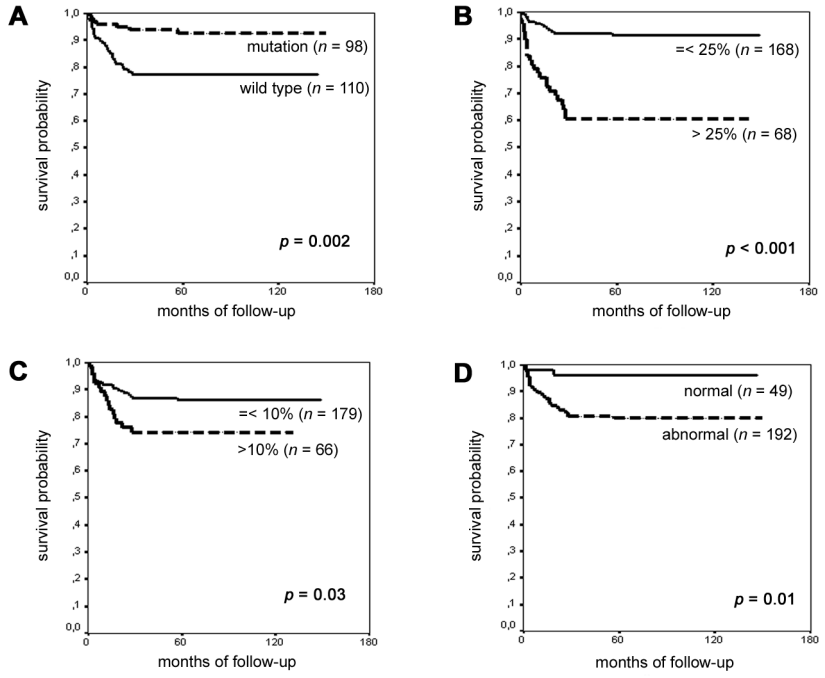

Figure 2. Kaplan-Meier analyses for disease-specific survival.
A, *FGFR3*; B, Ki-67; C, TP53; D, CK20.

We investigated the association between the molecular markers and disease-specific survival more closely by adjusting a Cox regression model. Univariate Cox regression analysis showed that patients with wild type *FGFR3* status, abnormal CK20 staining pattern, increased proliferation fraction, and positive

TP53 immunoreactivity, have a significantly higher risk of tumor-related death, with hazard ratios ranging from 2.2 to 5.2 (Table 4). However, in the global model (including tumor stage, grade, adjacent carcinoma *in situ*, multifocality, *FGFR3* status, CK20 IHC, TP53 IHC, and Ki-67 IHC), none of the molecular markers proved to be an independent predictor of tumor-related death.

We also investigated the combination *FGFR3* status/Ki-67 IHC, together with all other possible combinations of molecular markers (*FGFR3*/CK20, *FGFR3*/TP53, CK20/Ki-67, CK20/TP53, Ki-67/TP53) in various Cox regression models. None of these combinations of molecular markers was an independent predictor of recurrence-free survival and disease-specific survival.

Table 4. Univariate Cox regression analysis for disease-specific survival

Variable	Categorization	*n*	events	5-year survival rate	p^a	HR	95% CI	p^b
FGFR3								
	mutation	98	7	93%	**0.002**	1		**0.008**
	wild type	110	24	78%		3.2	[1.4-7.4]	
TP53 IHC								
	≤10%	179	24	87%	**0.03**	1		**0.02**
	>10%	66	16	76%		2.2	[1.1-4.1]	
Ki-67 IHC								
	≤25%	168	14	92%	**<0.001**	1		**<0.001**
	>25%	68	25	63%		5.2	[2.7-10.1]	
CK20 IHC								
	normal	49	2	96%	**0.01**	1		**0.03**
	abnormal	192	37	81%		5.0	[1.2-20.6]	

[a]Log rank test, 2-sided; bold face representing *p*-values < 0.05.
[b]Cox regression; bold face representing *p*-values < 0.05.

FGFR3 status and CK20 staining pattern

Since *FGFR3* mutations and a normal CK20 staining pattern both occur in tumors of low stage and low grade, we investigated those two markers more closely. *FGFR3* was mutated in 89% (32 of 36) of bladder tumors with normal CK20 pattern, but in only 37% (61 of 163) of cases with abnormal CK20 expression ($p < 0.001$, Table 3). Furthermore, in the group of pTaG1 tumours 82% (49 of 60) carried an *FGFR3* mutation, and only 59% (44 of 74) revealed an abnormal CK20 pattern, whereas in the group of pTaG2 tumours 69% carried an *FGFR3* mutation and 68% an abnormal CK20 staining pattern. Only 4 of 199 tumors presented with a normal CK20 pattern and *FGFR3* wild type status.

Tumors with a normal CK20 pattern and *FGFR3* mutation were all pTa G1/2 (Figure 3), whereas *FGFR3* mutant tumors with an abnormal CK20 pattern were of higher stage and grade ($p < 0.001$). In detail, 18% (11 of 61) were stage pT1 or higher, and 15% (9 of 61) presented as grade 3 tumors. This finding suggests that *FGFR3* status in combination with CK20 IHC can be used to further define the benign (low-grade) lesions in the group of *FGFR3* mutant tumors.

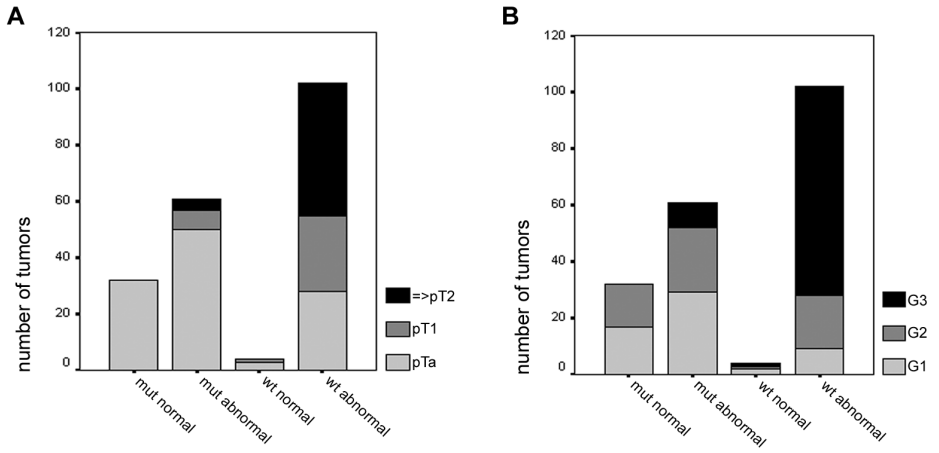

Figure 3. Combination of *FGFR3* status and CK20 IHC.
Distribution of these two markers compared to stage (A) and grade (B). mut, mutant *FGFR3*; wt, wild type *FGFR3*; normal, normal CK20 staining pattern; abnormal, abnormal CK20 staining pattern.

DISCUSSION

The aim of this study was to investigate *FGFR3*, CK20, Ki-67, and TP53, four established molecular markers with prognostic potential for bladder cancer, in a large patient group with clinical follow-up data. The mutation status of the *FGFR3* gene has previously proven to be a robust marker for the prognosis of bladder cancer patients, as is described by van Rhijn *et al.* [5, 11]. In this study, we confirmed the correlation of *FGFR3* mutations with tumors of low stage and grade [3, 5], and the mutual exclusiveness of *FGFR3* mutations and TP53 overexpression [10, 11]. However, in contrast to the results of van Rhijn, the combination *FGFR3*/Ki-67 was not an independent predictor of disease-specific survival in our study. Contradictory results from different studies regarding the

markers CK20, Ki-67 and TP53 suggests that this could be due to differences in efficiency and interpretation of immunohistochemical staining. For example, CK20 is an excellent marker for urothelial differentiation in superficial bladder tumors; however, the results for prediction of disease course differ. Harnden *et al.* [14, 22] showed that noninvasive pTa tumors with normal CK20 expression were less likely to recur, while others [23] did not find a significant correlation between CK20 expression and recurrence in low-grade tumors. In our study, CK20 expression was not significantly correlated to recurrence-free survival for tumors of all stages and grades or for pTa tumours separately ($p = 0.90$). Several other studies have investigated the use of Ki-67 expression, which is a strong marker for cell proliferation, and nuclear TP53 expression, which is thought to be a marker of progression, as prognostic indicators. Most studies did not manage to demonstrate independent prognostic significance for both markers, although recently Quintero *et al.* [24] showed Ki-67 as an independent predictor for progression-free and overall survival in low-stage bladder tumors. In our study, Ki-67 and TP53 were strongly associated with disease-specific survival; however, they were not independent predictors.

We confirmed in this study that *FGFR3* mutation status is not an independent marker, but is influenced by the pathologic parameters stage and grade for the prediction of disease course, which means that stage and grade are still better predictors than *FGFR3*. Nevertheless, *FGFR3* is a useful marker to identify superficial tumours with low malignant potential, since mutation analysis is more robust (100% reproducible) than pathologic staging and grading (high interobserver variability) [5]. An important limitation of this study is that a major end point for bladder cancer, progression-free survival, could not be assessed in this patient cohort.

The observation that *FGFR3* mutations are not frequent in tumors from patients with adjacent carcinoma *in situ* has been described previously [5]. This association between *FGFR3* and carcinoma *in situ* has also been considered by others: Dyrskjøt *et al.* [25] studied two groups of superficial bladder tumors with and without surrounding carcinoma *in situ* for gene expression patterns, and found that the *FGFR3* gene was upregulated in tumors without surrounding carcinoma *in situ*. In a second study [7], they found a highly significant correlation between the tumors with *FGFR3* mutations and tumors that did not display the molecular signature associated with adjacent carcinoma *in situ* in gene expression analysis. We verified these results in our study by demonstrating that the presence of an *FGFR3* mutation predicts that there is no adjacent carcinoma *in situ* ($p < 0.001$),

which confirms that *FGFR3* can be a useful marker in the follow-up of bladder cancer patients.

In the past few years, it has become clear that the *FGFR3* mutation is a prognostically favourable molecular alteration in bladder tumors; this concept is consolidated by the strong correlation of *FGFR3* mutations with a normal CK20 pattern, which we demonstrate here for the first time. Harnden *et al.* [14, 17] showed that retention of a normal CK20 pattern is a powerful indicator of normal differentiation. The observation that 65% of pTa tumors revealed an abnormal CK20 pattern implies that dysregulation of CK20 is an early event in the development of papillary bladder cancer; however, the correlation of *FGFR3* mutations with a normal CK20 pattern indicates that the mutation probably occurs earlier. In pTaG1 tumors, *FGFR3* mutations are more frequent (82%) than an abnormal CK20 pattern (59%), suggesting that *FGFR3* mutations precede dysregulation of CK20 in the development of papillary noninvasive bladder cancer.

The presence of a normal CK20 pattern and of a mutation in the *FGFR3* gene correlates with the group of pTa G1-2 tumors, while *FGFR3* mutant tumors with an abnormal CK20 pattern are less differentiated, and some are of higher stage (18%) and grade (15%), and are presumably one step closer to an invasive tumor. We think that the combination of *FGFR3* and CK20 is an excellent prognostic marker for pTa bladder tumors, since it can identify the well-differentiated tumors within the already favorable group of *FGFR3* mutated tumors. *FGFR3*/CK20 was not an independent predictor of recurrence-free survival and disease-specific survival in our study; however, prospective studies regarding progression-free survival have to be performed to examine the prognostic value of this combination of markers more closely.

In conclusion, we found that the four molecular markers *FGFR3*, TP53, Ki-67, and CK20 are significant markers for disease-specific survival of bladder cancer patients; however, they were not independent markers. *FGFR3* mutations occur earlier than dysregulation of CK20 expression, and the correlation of *FGFR3* mutations with a normal CK20 staining pattern further indicates the association of these mutations with well-differentiated tumors. The two alternative genetic pathways in bladder cancer development are characterized by *FGFR3* mutations and a normal CK20 pattern on one side, and TP53 alterations, high proliferation fraction, and the presence of carcinoma *in situ* on the other side.

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CHAPTER 6

FGFR3 mutations and methylation are prognostic factors in tumors from the bladder, ureter, and renal pelvis

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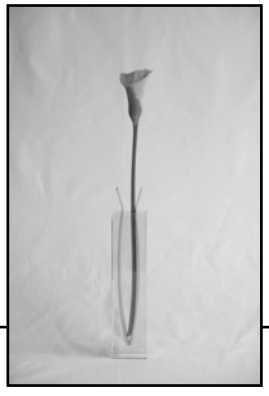

Submitted

ABSTRACT

Methylation and microsatellite instability are frequent in tumors of the upper urinary tract (UTT) and infrequent in bladder tumors. *FGFR3* mutations and loss-of-heterozygosity (LOH) are common findings in bladder tumors and *FGFR3* mutations are associated with a good prognosis. We investigated the occurrence of *FGFR3* mutations and LOH in UTT and determined the prognostic effect of these genomic changes. Tissue was obtained from 280 patients (117 bladder tumors and 163 UTT). *FGFR3* mutations were detected by SNaPshot analysis. LOH of chromosome 9 and 17p was established using microsatellite analysis. Analysis for hypermethylation at 11 CpG islands was performed previously on these tumors. *FGFR3* mutations occurred with the same frequency in bladder and upper tract tumors, and were associated with a milder disease course in bladder, ureter, and renal pelvis tumors. LOH 9 and 17p was equally distributed between upper and lower tract tumors, however, LOH 9 was inversely correlated with progression in bladder tumors. Only one of 25 patients with an *FGFR3* mutant bladder tumor in which *RASSF1A* was not methylated progressed. We conclude that *FGFR3* mutations and LOH 9 and 17p occur at the same frequency in bladder tumors and UTT. In the bladder, ureter and the renal pelvis the presence of an *FGFR3* mutation seems to protect a tumor from progressing. Moreover, the combination of *FGFR3* mutation and lack of *RASSF1A* methylation identifies a group of bladder tumors that have a very low risk of progression.

INTRODUCTION

The majority of urothelial cell carcinomas (UCC) occur in the bladder, with only 5% in the ureter or renal pelvis (upper tract tumors (UTT)). Urothelial tumors from the upper and lower tract show histological and morphological similarities and were thought to develop similarly. However, several studies have shown that upper and lower tract tumors are genetically and epigenetically different: microsatellite instability (MSI) was found to be more frequent in upper tract tumors [1] while bladder tumors displayed more frequent nucleotide instability at selected tetranucleotides [2]. Analysis for hypermethylation at 11 CpG islands subsequently showed that promoter hypermethylation is both more frequent and more extensively present in UTT compared to bladder tumors [3]. These results indicate that urothelial tumors differ with location and may have different pathogenesis pathways.

Fibroblast growth factor receptor 3 (*FGFR3)* and *TP53* mutations are the most frequent somatic mutations found in bladder UCC. *FGFR3* mutations occur in 50% of primary bladder tumors, and are associated with low stage and grade tumors [4] and a good prognosis [5]. Conversely, mutations in the *TP53* gene, which are present in about 25% of tumors, occur primarily in invasive bladder tumors. Based on the mutual exclusivity of *FGFR3* and *TP53* mutations in bladder tumors, a two-pathway model has been proposed for urothelial carcinogenesis [6, 7]. In colon cancer, a difference in *TP53* mutation frequency according to location has recently been reported: distal colon tumors were found to have significantly more mutations than proximal colon tumors [8]. In addition, methylation and MSI also differ in frequency according to tumor site in this type of cancer [9, 10].

Chromosome 9 loss-of-heterozygosity (LOH) is the most frequent genetic alteration in bladder UCC, regardless of stage and grade. Loss of the q-arm of chromosome 9 is most frequent, and probably represents the earliest genetic alteration in bladder cancer [11]. LOH 9 has not been investigated with relation to disease course. LOH of chromosome 17 indicates loss of the *TP53* gene and is therefore predominantly present in invasive tumors.

The aim of this study was to compare the frequency of *FGFR3* mutations, LOH of chromosome 9 and 17, and promoter hypermethylation of specific CpG islands in upper and lower tract urothelial tumors and correlate these results to etiology and disease course of UCCs of different locations.

MATERIALS AND METHODS

Sample collection and DNA extraction

We studied 280 patients with primary UCC (Table 1) who underwent surgery at the Royal Hallamshire Hospital Sheffield, UK, or at the Institut Mutualiste de Montsouris, Hôpital Tenon, and Hôpital de St. Louis, Paris, France [3]. Superficial bladder tumors were removed by transurethral resection and radical cystectomy was used for muscle-invasive disease. Nephroureterectomy was performed for all UTT in this series. These samples have previously been analyzed for microsatellite instability [2], mismatch repair protein expression [12], and promoter hypermethylation [3].

Table 1. Patient and tumor characteristics according to tumor location.

Variable	Categorization	Bladder		Ureter		Renal pelvis	
		n	%	n	%	n	%
Age at diagnosis							
	<70 years	57	49	32	48	40	48
	≥70 years	60	51	35	52	43	52
Gender							
	male	79	68	48	71	59	70
	female	38	32	20	29	25	30
Tumor stage							
	pTa	43	37	22	32	15	18
	pT1	27	23	10	15	14	17
	≥pT2	47	40	36	53	55	65
Histologic grade							
	G1	21	18	11	16	9	11
	G2	36	31	33	49	40	47
	G3	60	51	24	35	35	42
Family history							
	no	9	82	39	91	65	96
	yes	2	18	4	9	3	4
Other cancer							
	no	93	85	32	65	52	74
	yes	17	15	17	35	18	26
FGFR3							
	wild type	57	54	26	41	49	61
	mutant	48	46	37	59	31	39
LOH 9							
	conserved	48	58	44	65	49	58
	loss	35	42	24	35	35	42
LOH 17							
	conserved	65	80	56	82	62	75
	loss	16	20	12	18	21	25
methylation index (MI)							
	≤20%	78	67	35	51	41	49
	>20%	39	33	33	49	43	51
RASSF1A							
	unmethylated	49	46	24	37	28	34
	methylated	57	54	41	63	55	66

From each patient we obtained normal and tumor DNA using microdissection (>80% pure) of 6 x 10 µm thick formalin-fixed, paraffin-embedded tissue sections. DNA was extracted using the phenol chloroform method.

Methylation analysis

Promoter hypermethylation analysis was performed using bisulphite conversion, methyl specific PCR and sequencing, as described elsewhere [3]. Eleven CpG islands were analyzed and were selected to include the promoters of two MMR genes (hMLH1 and hMSH2), genes involved in urothelial carcinogenesis (p14, p16, and E-cadherin), other frequently methylated genes in human cancer (RARB, DAPK, MGMT, RASSF1A, and GSTP1) and one CpG island (MINT31) specific to the hypermethylator phenotype. Methylation index (MI) was defined as the number of methylated islands/total number of successfully analyzed islands, as a percentage.

FGFR3 mutation analysis

FGFR3 mutation analysis was performed on 252 of the 280 primary tumors using the SNaPshot method [13]. In short, three regions of the *FGFR3* gene, comprising all *FGFR3* mutations found in bladder cancer [14], were amplified simultaneously in a multiplex PCR reaction. After removal of excess primers and dNTPs, eight SNaPshot primers detecting nine *FGFR3* mutations were annealed to the PCR products and extended with a labeled dideoxynucleotide. These extended primers were analyzed on an automatic sequencer, with the label on the incorporated nucleotide indicating the presence or absence of a mutation.

LOH analysis

Chromosome 9 and 17 LOH analysis was performed for each tumor using paired PCR reactions with normal and tumor DNA. Four microsatellite markers were studied on chromosome 9 (D9S304, D9S156, and D9S1751 on 9p and D9S747 on 9q) and two on chromosome 17 (D17S796 and TP53). PCR was performed in a 12 µl volume reaction, composed of 1 pmol fluorescence labeled forward and unlabelled reverse primers, 50 ng of DNA template and manufactured PCR mastermix solution of Taq DNA polymerase, dNTPs, 1.5 mM $MgCl_2$ and buffers (Abgene, Surrey, UK). For each locus a standard reaction was performed with 35 cycles of amplification using a 'Primus 96' thermal cycler (MWG Biotech, UK). Annealing temperatures varied for each microsatellite locus and with primer details are described elsewhere [15]. Analysis of the PCR products was performed on an

automated LICOR sequencer (MWG Biotech, UK). LOH was determined when the tumor allelic strength was less than 60% that of corresponding normal DNA. All reactions were duplicated to confirm the results.

Statistical methods

Two-tailed statistical analyses were performed using SPSS version 11 (SPSS Inc, Chicago, IL). Categorical variables were compared using the χ^2 and Fisher's exact tests. Recurrence, progression and survival probabilities were analyzed using the Kaplan-Meier method and log rank test. Cox proportional hazard ratios were estimated to obtain risks of recurrence, progression, and death, and to find independent prognostic factors in a multivariate model. Since there were no progression and death events in categories pTa and G1 of the renal pelvis tumor group, Cox regression analysis was not possible. Also, a multivariate Cox regression model on bladder tumors could not be fitted when LOH 9 was included. Results were considered significant if $p < 0.05$.

RESULTS

Mutations in FGFR3 and LOH 9 and 17 occurred at the same frequency in bladder and upper tract tumors

FGFR3 mutations occurred in 46% (48 of 105) of bladder tumors and in 48% (71 of 147) of UTT (Table 1). Mutations were found in six different codons, and their frequencies were consistent for bladder tumors and UTT, respectively: the mutation in codon 249 (S249C) was the most frequent (62/58%), followed by Y375C (18/20%), R248C (10/14%), G372C (8/4%), K652M (2/3%), and A393E (0/1%). In one bladder tumor sample and one UTT sample, the double mutation S249C/Y375C was found.

The group of UTT consisted of 63 ureter and 80 renal pelvis tumors, and when these groups were studied separately we found that *FGFR3* mutations occurred in 59% (37 of 63) of ureter tumors, and in only 39% (31 of 80) of renal pelvis tumors ($p = 0.02$). Thus, *FGFR3* mutations are less frequent in renal pelvis tumors.

We also analyzed chromosome 17p LOH. Frequencies did not differ significantly between the locations: 20% (16 of 81) of bladder tumors and 21% (34 of 162) of UTT showed LOH for one of the two markers of chromosome 17. In the group of renal pelvis tumors, we found that LOH of chromosome 17 predominantly occurred in *FGFR3* wild type tumors ($p = 0.02$). Tumors were also analyzed for loss of chromosome 9, which is a common event in bladder cancer. LOH on

Table 2. Comparison of *FGFR3*, LOH, and methylation with tumor stage and grade

Organ	Variable	Categorization	Stage				Grade				Total
			pTa	pT1	≥ pT2	*p*[a]	G1	G2	G3	*p*[a]	
bladder	*FGFR3*										
		wild type	11	13	33	**<0.001**	4	13	40	**<0.001**	57
		mutant	27	13	8		15	19	14		48
	LOH 9										
		conserved	13	14	21	**0.02**	6	10	32	0.08	48
		loss	19	7	9		5	16	14		35
	LOH 17										
		conserved	24	19	22	0.51	8	19	38	0.17	65
		loss	8	3	5		3	7	6		16
	methylation index (MI)										
		≤ 20%	37	13	28	**0.009**	16	25	37	0.20	78
		> 20%	6	14	19		5	11	23		39
	RASSF1A										
		unmethylated	29	6	14	**<0.001**	16	18	15	**<0.001**	49
		methylated	10	18	29		4	14	39		57
ureter	*FGFR3*										
		wild type	3	2	21	**0.002**	2	11	13	0.06	26
		mutant	15	7	15		8	18	11		37
	LOH 9										
		conserved	15	5	24	0.99	8	19	17	0.83	44
		loss	7	5	12		3	14	7		24
	LOH 17										
		conserved	19	8	29	0.59	11	26	19	0.22	56
		loss	3	2	7		0	7	5		12
	methylation index (MI)										
		≤ 20%	18	7	10	**<0.001**	4	23	8	0.35	35
		> 20%	4	3	26		7	10	16		33
	RASSF1A										
		unmethylated	12	4	8	**0.005**	5	10	9	0.88	24
		methylated	8	5	28		6	21	14		41
renal pelvis	*FGFR3*										
		wild type	5	6	38	**0.009**	5	20	24	0.21	49
		mutant	10	5	16		4	17	10		31
	LOH 9										
		conserved	8	7	34	0.45	5	26	18	0.47	49
		loss	7	7	21		4	14	17		35
	LOH 17										
		conserved	12	12	38	0.31	9	27	26	0.36	62
		loss	3	2	16		0	12	9		21
	methylation index (MI)										
		≤ 20%	12	6	23	**0.02**	5	23	13	0.12	41
		> 20%	3	8	32		4	17	22		43
	RASSF1A										
		unmethylated	9	3	16	0.07	3	13	12	0.84	28
		methylated	6	11	38		6	27	22		55

[a]Fisher's exact test (2-sided)

chromosome 9 occurred at the same frequency in bladder tumors (35 of 83, 42%) and UTT (65 of 163, 40%) ($p = 0.78$).

FGFR3 mutations are associated with a milder disease course

The frequency of *FGFR3* wild type and mutant tumors did not differ significantly regarding age, sex, family history or other cancer. *FGFR3* mutations were more frequent in low-stage bladder, ureter, and renal pelvis tumors, and in low-grade bladder and ureter tumors (Table 2). However, *FGFR3* wild type and mutant tumors were equally distributed in low-grade renal pelvis tumors (p = 0.21). *FGFR3* mutations are therefore not associated with low tumor grade in renal pelvis tumors.

To investigate whether *FGFR3* mutations have the same effect on survival in upper and lower tract tumors, we studied the behavior of tumors after treatment with regard to *FGFR3* mutation status. Figure 1 shows the Kaplan-Meier analyses for progression-free survival in all tumor groups, and with the log rank test we demonstrated that tumors with an *FGFR3* mutation progressed significantly less often than wild type tumors in all three groups (see also Table 3). However, this association was less clear in the group of renal pelvis tumors. Similar results were found for overall survival (Table 4). *FGFR3* was not correlated to tumor recurrence.

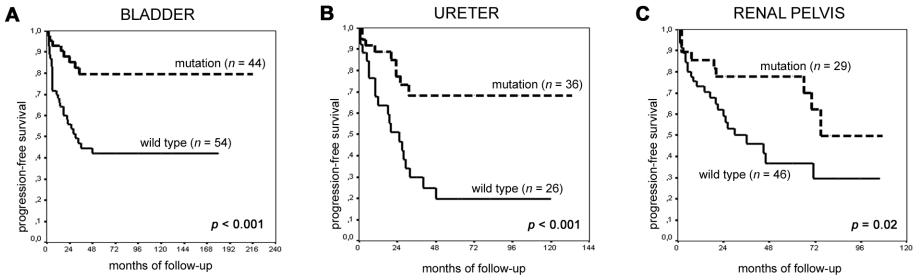

Figure 1. Tumor behavior according to *FGFR3* mutation status for different tumor locations.

FGFR3 mutated tumors have lower progression rates in the bladder (A), ureter (B), and renal pelvis (C), than those without a mutation. Dashed lines, survival curves of patients with an *FGFR3* mutation.

Univariate analysis using Cox regression analysis (Table 3) also showed that patients had a significantly higher risk of tumor progression (HR varying from 2.4 to 3.8) when *FGFR3* was wild type in all three tumor groups. In tumors from the bladder multivariate Cox regression analysis showed that none of the investigated

parameters was significantly independent in predicting progression- or disease-free survival (Tables 3 and 4). The investigated parameters included stage, grade, *FGFR3* status and *RASSF1A* methylation (see further below). However, *FGFR3* status was the only independent variable predicting progression-free survival (Table 3, $p = 0.02$) and disease-free survival (Table 4, $p = 0.03$) in tumors from the ureter. Cox regression analyses were not possible in renal pelvis tumors for stage and grade (no events in the pTa and G1 tumors).

LOH of chromosome 9 is associated with better survival in bladder tumors

When loss of chromosome 9 was compared to clinicopathologic parameters, we found that significantly more LOH 9 was present in pTa bladder tumors than in tumors of higher stage (≥pT1, $p = 0.02$). This difference was not observed in ureter or renal pelvis tumors. LOH 9 was not correlated to *FGFR3* mutation status.

We subsequently looked at disease course, and found that bladder tumors with loss of chromosome 9 progressed less often than tumors with a conserved chromosome 9 ($p = 0.01$, Figure 2A). This was not the case for ureter (Figure 2B) or renal pelvis (Figure 2C) tumors. Similar results were obtained for overall survival (Table 4). In a Cox regression model, univariate analysis showed that patients had a significantly higher risk of bladder tumor progression (HR 2.9, Table 3) and death (HR 2.7, Table 4) when chromosome 9 was conserved. LOH 9 was not correlated to tumor recurrence.

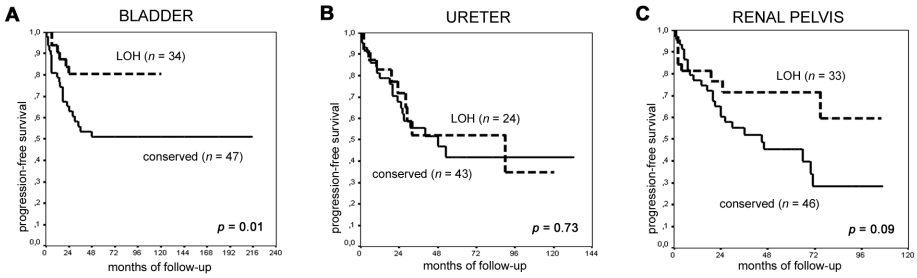

Figure 2. Tumor behavior according to loss of chromosome 9.
In the bladder, tumors with LOH 9 have a higher progression-free survival rate than tumors with a conserved chromosome 9 (A). This is not the case for ureter (B) and renal pelvis (C) tumors. Dashed lines, survival curves of patients with LOH of chromosome 9.

Table 3. Progression-free survival analysis according to tumor location

							univariate			multivariate		
Organ	**Variable**	**Categorization**	**n**	**events**	**5-year survival rate**	***p*[a]**	**HR**	**95% CI**	***p*[b]**	**HR**	**95% CI**	***p*[b]**
bladder	stage											
		pTa	43	4	91%		1			1		
		pT1	27	13	52%	**<0.001**	6.2	[2.0-19.0]	**<0.001**	4.0	[0.7-24.5]	0.07
		pT2-4	40	24	40%		9.7	[3.3-28.0]		6.1	[1.2-31.5]	
	grade											
		G1	19	1	95%		1			1		
		G2	32	6	81%	**<0.001**	3.2	[0.4-26.7]	**0.001**	1.9	[0.2-17.2]	0.75
		G3	59	34	42%		12.9	[1.8-94.0]		2.5	[0.2-27.4]	
	FGFR3											
		mutant	44	8	82%	**<0.001**	1		**0.001**	1		0.12
		Wild type	54	29	46%		3.8	[1.7-8.3]		2.1	[0.8-5.5]	
	LOH 9											
		Loss	34	6	82%	**0.01**	1		**0.02**		NA[c]	
		conserved	47	22	53%		2.9	[1.2-7.2]				
	RASSF1A											
		unmethylated	48	13	73%	**0.04**	1		**0.04**	1		0.80
		methylated	51	25	51%		2.0	[1.0-3.9]		0.9	[0.4-2.0]	
ureter	stage											
		pTa	21	5	76%		1			1		
		pT1	10	4	70%	**0.04**	1.4	[0.4-5.3]	0.06	0.9	[0.2-4.2]	0.26
		pT2-4	36	21	42%		3.0	[1.1-8.1]		2.1	[0.7-6.2]	
	grade											
		G1	11	1	91%		1			1		
		G2	32	16	53%	**0.03**	5.1	[0.7-38.3]	0.06	3.9	[0.5-30.5]	0.22
		G3	24	13	46%		8.9	[1.2-68.1]		5.6	[0.7-43.6]	
	FGFR3											
		mutant	36	9	75%	**<0.001**	1		**0.002**	1		**0.02**
		Wild type	26	19	27%		3.5	[1.6-7.8]		2.6	[1.2-5.9]	
	LOH 9											
		Loss	24	10	62%	0.73	1		0.73			
		conserved	43	20	53%		1.1	[0.5-2.5]				
	RASSF1A											
		unmethylated	23	9	61%	0.56	1		0.56			
		methylated	41	20	54%		1.3	[0.6-2.8]				

renal pelvis	stage								
		pTa	12	0	100%				
		pT1	13	4	77%	**0.002**		NA[c]	
		pT2-4	54	30	50%				
	grade								
		G1	8	0	100%				
		G2	37	15	70%	**0.008**		NA[c]	
		G3	34	19	44%				
	FGFR3								
		mutant	29	9	79%	**0.02**	1		**0.03**
		Wild type	46	24	50%		2.4	[1.1-5.1]	
	LOH 9								
		Loss	33	9	76%	0.09	1		0.10
		conserved	46	25	52%		1.9	[0.9-4.1]	
	RASSF1A								
		unmethylated	27	7	74%	0.11	1		0.12
		methylated	51	26	57%		1.9	[0.8-4.5]	

[a] log rank test, 2-sided; bold face representing *p*-values < 0.05.
[b] Cox regression; bold face representing *p*-values < 0.05.
[c] Cox regression analysis was not applicable.

Table 4. Disease-specific survival analysis according to tumor location

							univariate			multivariate		
Organ	**Variable**	**Categorization**	**n**	**events**	**5-year survival rate**	***p*[a]**	**HR**	**95% CI**	***p*[b]**	**HR**	**95% CI**	***p*[b]**
bladder	stage											
		pTa	43	4	91%		1			1		
		pT1	27	11	59%	**<0.001**	4.9	[1.6-15.4]	**<0.001**	2.4	[0.3-17.5]	0.08
		pT2-4	40	23	42%		9.5	[3.3-27.6]		4.8	[0.8-30.4]	
	grade											
		G1	19	1	95%		1			1		
		G2	32	5	84%	**<0.001**	2.6	[0.3-22.4]	**0.001**	1.5	[0.2-14.4]	0.54
		G3	59	32	46%		11.5	[1.6-84.5]		3.3	[0.3-41.9]	
	FGFR3											
		mutant	44	8	82%	**0.002**	1		**0.003**	1		0.53
		Wild type	54	26	52%		3.3	[1.5-7.3]		1.4	[0.5-3.5]	
	LOH 9											
		Loss	34	6	82%	**0.03**	1		**0.04**		NA[c]	
		conserved	47	20	57%		2.7	[1.1-6.6]				
	RASSF1A											
		unmethylated	48	12	75%	0.07	1		0.08			
		methylated	51	23	55%		1.9	[0.9-3.8]				
ureter	stage											
		pTa	21	5	81%		1			1		
		pT1	10	3	70%	**0.04**	1.1	[0.3-4.7]	0.05	1	[0.2-4.4]	0.35
		pT2-4	36	20	44%		2.9	[1.1-7.8]		1.9	[0.6-5.8]	
	grade											
		G1	11	1	91%		1			1		
		G2	32	14	59%	**0.02**	4.3	[0.6-33.1]	**0.048**	3.4	[0.4-26.5]	0.19
		G3	24	13	46%		8.7	[1.1-66.5]		5.4	[0.7-42.4]	
	FGFR3											
		mutant	36	9	75%	**0.003**	1		**0.005**	1		**0.047**
		Wild type	26	18	35%		3.1	[1.4-6.9]		2.3	[1.0-5.3]	
	LOH 9											
		Loss	24	9	62%	0.67	1		0.67			
		conserved	43	1	58%		1.2	[0.5-2.6]				
	RASSF1A											
		unmethylated	23	9	65%	0.72	1		0.72			
		methylated	41	18	56%		1.2	[0.5-2.6]				

renal pelvis	stage								
		pTa	12	0	100%				
		pT1	13	4	77%	**0.002**		NA[c]	
		pT2-4	54	30	50%				
	grade								
		G1	8	0	100%				
		G2	37	15	70%	**0.006**		NA[c]	
		G3	34	19	44%				
	FGFR3								
		mutant	29	9	79%	**0.02**	1		**0.03**
		Wild type	46	24	50%		2.4	[1.1-5.2]	
	LOH 9								
		Loss	33	9	76%	0.10	1		0.11
		conserved	46	25	52%		1.9	[0.9-4.0]	
	RASSF1A								
		unmethylated	27	7	74%	0.14	1		0.15
		methylated	51	26	57%		1.9	[0.8-4.3]	

[a] log rank test, 2-sided; bold face representing *p*-values < 0.05.
[b] Cox regression; bold face representing *p*-values < 0.05.
[c] Cox regression analysis was not applicable.

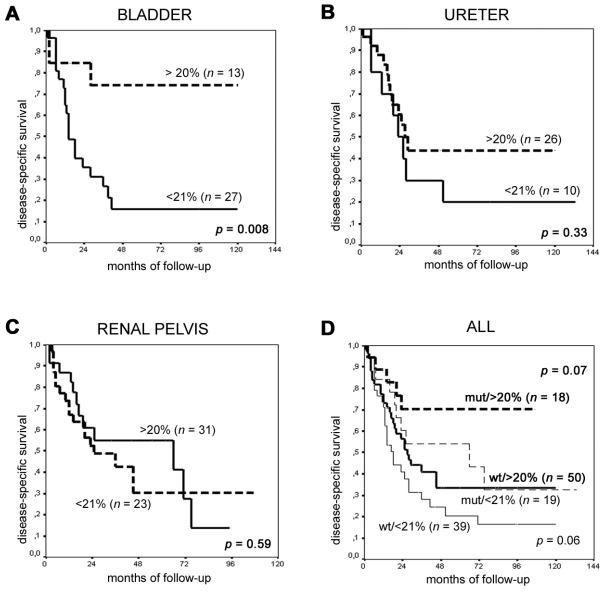

Figure 3. Behavior of muscle-invasive tumors according to methylation status.
A, muscle-invasive (pT2-4) bladder tumors with higher MI have lower mortality rates. In ureter (B) and renal pelvis (C) tumors MI status is not significant. D, muscle-invasive tumors with an *FGFR3* mutation have a higher overall survival rate for both high (>20%) and low (≤20%) MI. Dashed lines A-C, survival curves of patients with MI >20%; dashed lines D, survival curves of patients with an *FGFR3* mutation; bold D, survival curves of patients with MI >20%.

Invasive bladder tumors with a high methylation index have lower mortality rates

We previously investigated methylation of CpG islands in the promoters of 11 genes. MI was significantly correlated to stage in all three tumor groups (Table 2), and pT2-4 tumors with a higher methylation index (MI) had lower mortality rates [3]. Upon reexamination of these data we observed that this effect could be ascribed to the group of bladder tumors (compare Figures 3A, B, and C). When we combined methylation index with *FGFR3* status, we observed that for both tumor groups (high and low MI) the *FGFR3* mutant tumors had a better survival, although this difference was not significant ($p = 0.07$ and 0.06 for high and low MI respectively, Figure 3D). A further evaluation of the combination of MI and *FGFR3* status and disease course in the different tumor groups was unfortunately not possible because the number of tumors in each group was too low.

Bladder tumors with an FGFR3 mutation that lack methylation of the RASSF1A gene rarely progress

We observed that of the 11 promoters, methylation of the *RASSF1A* promoter occurred preferentially in *FGFR3* wild type tumors (p = 0.002), and studied the combination of these two markers for survival. For the group of wild type tumors progression-free survival was the same for tumors with methylated and unmethylated *RASSF1A* in all three tumor groups (Figure 4). In the group of bladder tumors with an *FGFR3* mutation, however, we found that only 1 of 25 tumors with unmethylated *RASSF1A* promoter progressed, whereas *FGFR3* mutant/*RASSF1A* methylated tumors were indistinguishable from the *FGFR3* wt tumors (p = 0.002, Figure 4A). This association is apparently restricted to bladder tumors since it was not observed in ureter tumors (p = 0.99) and renal pelvis tumors (p = 0.32, see Figure 4B and 4C). Similar results were found for overall survival (data not shown).

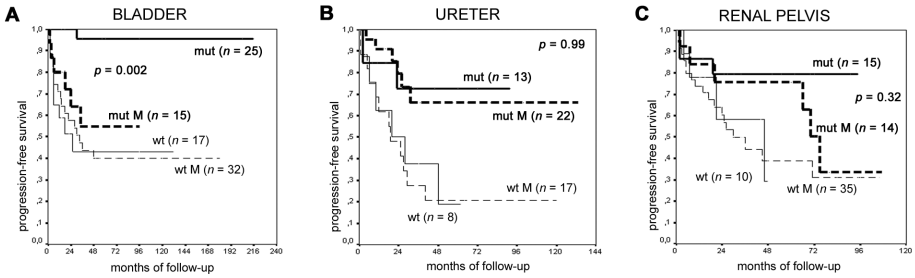

Figure 4. Tumor progression according to a combination of *FGFR3* mutation status and *RASSF1A* methylation.

In the bladder (A), *FGFR3* mutated tumors with unmethylated *RASSF1A* (mut) have a lower progression rate than those with methylated *RASSF1A* (mut M). *RASSF1A* methylation does not distinguish mutant tumors with low progression in ureter (B) and renal pelvis (C). Dashed lines, survival curves of patients with methylation of *RASSF1A*; bold, survival curves of patients with an *FGFR3* mutation.

DISCUSSION

This study aimed to investigate the pathogenesis of bladder tumors versus UTT and the effect of various molecular aberrations on disease course. In invasive bladder tumors genetic instability is frequent, as has been observed by numerous LOH and CGH analyses, and mutations in the *TP53* gene are often present [16].

In non-invasive bladder tumors LOH of chromosome 9 predominates as well as activating point mutations in the *FGFR3* gene [17]. We previously showed that in UTT microsatellite instability and CpG island methylation are far more common than in UCCs of the bladder [2, 3]. However, no data on *FGFR3* mutations and few data on LOH of chromosome 9 [18] were available for these tumors. Because these findings suggested that the pathogenesis pathways in UCCs may differ according to location, here we investigated the occurrence of *FGFR3* mutations as well as LOH for chromosomes 9 and 17p (the location of the *TP53* gene) in UCCs of the bladder, ureter and renal pelvis. To our surprise we detected that the percentage of *FGFR3* mutant tumors was similar: 46% in the bladder and 48% in UTT. Likewise, the frequency of LOH of chromosome 9 and 17p was independent of tumor location. These results suggest that there are no great differences in the genetic aberrations that accumulate in the development of UCCs of different locations. However, it will be interesting to investigate whether large scale LOH as observed in invasive bladder tumors is perhaps less frequent in upper tract tumors where methylation of tumor suppressor gene promoters may play an equivalent role.

FGFR3 mutations were significantly more frequent in pTa tumors of all locations. However, the correlation of mutations with low grade in tumors of the bladder and ureter was not apparent in renal pelvis tumors. Using log rank and Cox regression tests we found that *FGFR3* mutant tumors have a significantly lower progression and mortality rate in all three tumor groups, which confirms and extends previous studies performed for bladder tumors only [5, 19, 20]. In the multivariate Cox regression analysis the presence of an *FGFR3* mutation appeared the only independent predictor of progression and death in ureter tumors. We conclude that *FGFR3* mutation analysis, which is very easy to perform, offers an additional practical tool to stratify UCCs into a low or high progression risk. Moreover, mutation analysis is more reproducible than pathology, even when performed by expert pathologists as we have shown before [5]. It is for this reason that we think it is advisable to evaluate molecular markers by their own merit in predicting disease course instead of first stratifying tumors by stage.

Chromosome 9 alterations are the most frequent and also the earliest known genetic events in bladder cancer. Here we have shown that this is probably the same for UCCs of the upper tract. In general, previous studies indicated that LOH 9 in bladder tumors is independent of tumor grade and stage [16] as was recently very elegantly demonstrated by a whole genome SNP analysis by Koed *et al.* [21]. In this study, however, we found that LOH 9 is somewhat more pronounced in

low-stage bladder tumors. To our knowledge the possible relation between LOH9 and disease course in bladder tumors has not been studied before. Here we show that bladder cancer patients progress less often when the tumor displays LOH for chromosome 9. These results have recently been confirmed in an independent patient group.[1] To our knowledge, this is the first report on the correlation of LOH 9 with event-free survival.

The 11 CpG islands that were studied for methylation were chosen based on their involvement in urothelial carcinogenesis or mismatch repair. *RASSF1A*, a splice variant of the *RASSF1* gene, is frequently inactivated by promoter hypermethylation in many cancers, and functions as a tumor suppressor gene through RAS-mediated pathways. A study by Chan *et al.* [22] demonstrated high frequency of *RASSF1A* methylation in bladder cancer, and suggested that inactivation of this gene may be an early event in bladder carcinogenesis. Our results for promoter hypermethylation are described elsewhere [3], and showed that tumors with *RASSF1A* methylation had higher progression and mortality rates, and that bladder tumors were more affected than UTT. We combined methylation of *RASSF1A* with the *FGFR3* mutation, which is also an early event [19, 23] and a marker for progression [5] in bladder cancer, and uses RAS-mediated pathways [24]. We observed that *FGFR3* mutant bladder tumors, by themselves a group with lower progression than wt tumors, can be further divided into a prognostically benign group with no methylation of *RASSF1A* and a group with an adverse prognosis when *RASSF1A* is methylated. The combination of these two markers identifies the truly benign tumors (*FGFR3* mutant and *RASSF1A* unmethylated), and could be an excellent tool in prognosis prediction in patients with bladder cancer. Furthermore, we observed that *FGFR3* status and methylation index (MI) for muscle-invasive tumors (pT2-4), can identify patients with a better disease specific survival, although unfortunately this analysis could not be done in the bladder group only where the MI index by itself had the strongest effect. This interesting finding warrants a further study in a larger group of bladder tumors. Selection of invasive tumors with a high or low mortality risk could be used to optimize cystectomy timing and the use of neodajuvant therapy. An important advantage is also that both methylation (additional band) and mutation analysis (additional peak) assays produce positive signals when compared to LOH analysis (partial loss of one allele), and are therefore suitable for diagnostic application in the clinic.

[1] A Hartmann, personal communication

In conclusion, we have shown that mutations in *FGFR3* and LOH of chromosomes 9 and 17p occur at the same frequency in bladder tumors and UTT. The *FGFR3* mutation seems to protect bladder, ureter, and renal pelvis tumors from progressing, and in ureter tumors *FGFR3* is an independent predictor of progression. Promoter hypermethylation of *RASSF1A* further distinguishes between mutant bladder tumors that show more (methylated) or less (unmethylated) progression, and the combination of *FGFR3* and *RASSF1A* can therefore be used as a prognostic and diagnostic marker in bladder cancer. Furthermore, *FGFR3* mutation status and methylation index can be used to select the invasive tumors with a lower risk in both low and high MI groups. Finally, the presence of LOH 9 is another marker that is associated with lower progression in bladder tumors.

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CHAPTER 7

Expression of mutant FGFR3 in a human bladder cancer cell line results in loss of integrin expression and inability to grow in three-dimensional colonies

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Submitted

ABSTRACT

Mutations in the *FGFR3* gene occur mostly in noninvasive UCC, and they are associated with a favorable prognosis. To elucidate the mechanism behind this "protective" effect of *FGFR3* mutations, we created human bladder tumor cell lines stably expressing wild type and mutant FGFR3. Cells expressing mutant and wild type FGFR3 grew in multiple layers on plastic substrate, in contrast with the monolayer growth pattern of untransfected and mock transfected cell lines. Compared to wild type cells, cells expressing mutant receptor showed phosphorylation of FGFR3 and enhanced Ras/MAPK signaling. Mutant FGFR3 cells had much lower integrin expression compared to the other cell lines, and during 3-D culture their capacity to produce multicellular spheres was much reduced. Strikingly, mutant FGFR3 expressing cells that retained the potential to form multicellular spheres in Matrigel had lost their FGFR3 protein expression. The results therefore suggest that mutant FGFR3 somehow inhibits integrin expression, thereby reducing the contacts between cells and extracellular matrix. This results in multiple cell layers when the cells are cultured on plastic, and in an inability to grow out when surrounded by Matrigel. The findings may explain why UCCs with an *FGFR3* mutation have reduced invasive properties.

INTRODUCTION

Activating mutations in the fibroblast growth factor receptor 3 (*FGFR3*) gene occur in 50% of primary bladder tumors, and the presence of a mutant *FGFR3* gene is significantly associated with noninvasive, well-differentiated tumors with few genetic changes, and with better patient survival [1, 2].[1] Bladder tumors of low stage and grade represent the majority (80%) of tumors at first presentation. In contrast, high-grade invasive tumors that arise from carcinoma *in situ* have multiple genetic changes such as *TP53* mutations, they largely lack *FGFR3* mutations, and their prognosis is much worse. Since *FGFR3* and *TP53* mutations have been shown to be almost mutually exclusive [3, 4], a two-pathway model for bladder carcinogenesis was suggested indicating these two mutations as the key genetic events for the development of noninvasive and invasive tumors, respectively.

FGFR3 is a member of the FGF receptor tyrosine kinase family that encompasses five receptors and 23 ligands [5]. The receptor consists of an extracellular domain that includes an NH_2-terminal hydrophobic signal peptide followed by three immunoglobulin (Ig)-like domains, a hydrophobic transmembrane domain, and an intracellular split tyrosine kinase domain. Through alternative RNA splicing two isoforms exist with distinct tissue expression patterns [6, 7]: FGFR3 IIIc, lacking exon 8, is found mainly in mesenchymal structures, whereas the expression of FGFR3 IIIb, lacking exon 9, was shown to be restricted to epithelial cells and tissues [8]. This lineage-specific expression of the IIIb and IIIc isoforms of FGFRs is thought to play a role in the interaction between the epithelial and mesenchymal layers [9].

Activating germline mutations in the *FGFR3* gene cause several forms of skeletal dysplasias by inducing growth inhibition and differentiation in chondrocytes. In contrast, somatic mutations have been found in multiple myeloma with a t(4,14) translocation [10], in epithelial cancer of the cervix [11, 12, 13] and bladder [1, 2, 14, 15], and in the brown or senile warts of the skin called seborrheic keratosis [16, 17, 18].

Functional studies on the FGFR3 receptor and its mutations have been done in chondrocytes and skeletal dysplasia phenotypes. Unfortunately, mouse models for bladder carcinogenesis are difficult to establish. Therefore, common cell lines such as NIH-3T3 or bladder cell lines that already carry an *FGFR3* mutation were used to study the function of FGFR3 [19, 20]. In this study, we set out to analyze the effect of FGFR3 mutations in bladder cancer compared to the wild type

[1] JMM van Oers, unpublished data

receptor by introducing both wild type and mutant receptors into urothelial cells. We created stably transfected FGFR3 wild type and mutant cell lines using the noninvasive bladder cancer cell line JO'N, and compared their expression levels, morphology, and downstream signaling.

MATERIALS AND METHODS

Cell culture

The human bladder carcinoma cell line JO'N was kindly provided by Prof.dr. J.A. Schalken (Urologic Research Laboratory, University Medical Center Nijmegen, the Netherlands). This cell line was cultured from a recurrent bladder carcinoma from a Caucasian, 69-year old male by O'Toole *et al.* at the MSKCC in 1979. Cells did not invade mouse urothelium in vitro, and although the tumor take in vivo was high, muscle invasion was completely lacking [21]. Furthermore, this cell line displays a homogeneous expression pattern of E-cadherin, and it forms tight colonies of epithelial cells lacking invasion in Matrigel [22].

JO'N was analyzed for *FGFR3* mutations by the SNaPshot assay [23]. Analysis for H-*ras* mutations was done by amplifying exons 1 and 2 of the H-*ras* gene using primers 5'-ACTGGGTCATTAAGAGCAAGTGG-3' and 5'-CTAGAGGAAGCAGGAGACAG-3' for exon 1, and primers 5'-GGAGAGGCTGGCTGTGTGAACT-3' and 5'-AGGTGGAAAGCGAGAGCTGG-3' for exon 2. Products were sequenced for mutations in codons 12, 13, and 61.

Cells were maintained in standard DMEM culture medium supplemented with 10% heat-inactivated FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37˚C in 5% CO_2.

FGFR3 constructs

pcDNA3-FGFR3 IIIb cDNA was a kind gift from Dr. D.K. Podolsky (Harvard Medical School, Boston, MA) [8]. The R248C mutation was introduced as follows: cDNA from U937, H3B, and MG63 cell lines was combined and an 1841 bp region of FGFR3 cDNA containing the potential mutation site was amplified using the primers 5'-GCATTGGAGGCATCAAGCTG-3' and 5'-ACCAGTGGCCCTTCACG-3'. The R248C mutation was introduced into this fragment with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using the mutagenic oligonucleotide primers 5'-GGACGTGCTGGAGTGCTCCCCGCACC-3' and 5'-GGTGCGGGGAGCACTCCAGCACGTCC-3'. The *Rsr*II-*Bbr*PI fragment containing the mutation was excised from the PCR product and inserted in place

of the corresponding wild type sequence in the pcDNA3-FGFR3 IIIb construct.

Generation of stable cell lines

For stable transfection, pcDNA3, pcDNA3-FGFR3 and pcDNA3-FGFR3-R248C were linearized with *Pvu*I. JO'N cells were seeded in T75 flasks and transfected with 6 µg of linearized plasmid in the presence of FuGENE 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN). After 24h, cells were seeded in 6-well plates and allowed to attach first, before adding G418 (800 µg/ml; Invitrogen, Carlsbad, CA). Drug selection was continued for two weeks, and single colonies were expanded.

RNA extraction and RT-PCR analysis

Total RNA was extracted with TRIzol® Reagent (Invitrogen) and used as a template for cDNA synthesis by random priming: 3 µg RNA was treated with Dnase (Promega, Madison, WI), and subsequently 0,5 µg random primers were added in the presence of 200 U M-MLV RT, 4 µl RT-buffer, 2 µl DTT (all from Invitrogen) , 1 µl dNTPs, 9 µl mQ, and 1 µl RNAguard Ribonuclease Inhibitor (Amersham Biosciences/GE Healthcare, Little Chalfont, Buckinghamshire, UK), and incubated for 1h at 37°C, and 15 min at 99°C. The amount of FGFR3 cDNA was determined on agarose gel after PCR with specific primers, using POLR2A (polymerase II, RNA, subunit A) as an internal control. The primers used were 5'-GCATTGGAGGCATCAAGCTG-3' and 5'-TACACCTTGCAGTGGAACTC-3' for FGFR3, and 5'-GCTGAGAGAGCCAAGGATAT-3' and 5'-CACCACCTCTTCCTCCTCTT-3' for POLR2A. Primers were designed to bind two different exons.

The amount of FGFR3 mRNA was determined by TaqMan® real-time PCR. To 12,5 ng cDNA, 7,5 pmol of each of the FGFR3 or POLR2A primers was added and subsequently incubated in the ABI PRISM® 7700 Sequence Detection System with 12,5 µl of SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA).

Immunoprecipitation and Western blotting

Cells were lysed in buffer containing 25 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA pH 8.0, 10% glycerol, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM glycerolphosphate, 1 mM DTT, 1 mM PMSF, 15 µg/ml aprotinin, 10 mM sodium fluoride, and 2 µl/ml sodium pervanadate. Lysates were cleared by centrifugation, and protein concentrations were determined using

the Bradford protein assay (Bio-Rad, München, Germany). For immunoprecipitation of FGFR3, lysates were incubated overnight with an antibody that recognizes the C-terminus of FGFR3 (clone C-15, Santa Cruz, Heidelberg, Germany). Lysates were mixed by rotation for 2h with protein G PLUS-agarose beads (Santa Cruz). After washing the beads twice in lysis buffer, they were resuspended in Laemmli sample buffer and boiled for 3 min. Proteins were separated in an 8% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore, Billerica, MA). Blots were incubated in blocking buffer (5% BSA in TBS 0.2% Tween) followed by incubation with anti-phosphotyrosine antibody (clone 4G10, Upstate/Millipore). Bound antibody was detected using chemoluminescence (ECL Western Blotting Substrate, Pierce, Rockford, IL). As a loading control, blots were stripped in 50 mM Tris pH 7.5 and 10 mM urea at 55°C for 1h and subsequently incubated with FGFR3 antibody (clone B-9, Santa Cruz). In addition, phosphorylation of FGFR3 was determined in whole cell lysate using an antibody directed against phosphorylated FGFR (Cell Signaling Technology, Danvers, MA).

Antibodies for downstream targets that were analyzed on Western blot included p44/42, P-p44/42 (Thr202/Tyr204), Akt, P-Akt (Ser473), PLCγ1, P-PLCγ1 (Tyr783), P-FRS2α (Tyr436), Stat1, P-Stat1 (Tyr701 and Ser727, all from Cell Signaling Technology), Stat3, P-Stat3 (Tyr705), Stat5, P-Stat5 (Tyr694), FRS2, SH-PTP2, p53, and p16 (Santa Cruz). Anti-β-actin (Sigma, Saint Louis, MI) was used as a loading control. All primary antibodies were incubated overnight at 4°C.

Phenotypic assays

For growth curves, 2x 10^5 cells per well were seeded in 6-well dishes. Cells were counted on a Coulter Counter® (Beckman Coulter, Fullerton, CA) in triplicate wells on days 1-4.

To study cells in a three-dimensional matrix, 1x 10^4 cells were mixed with 200 µl of Growth Factor Reduced Matrigel™ Matrix and seeded on semipermeable PET membranes in 12-well dishes (BD Biosciences, Bedford, MA). Duplicate cultures were harvested on day 3, 6, 9, or 12. Cells could be recovered from Matrigel for further analysis using BD Cell Recovery Solution. One hour before termination, Matrigel cultures were incubated with 40 µg/ml bromodeoxyuridine (BrdU) to study proliferation.

To study apoptotic cells in culture, cells were seeded in 6-well dishes. Subconfluent cells were incubated with 0.5 µM YO-PRO®-1 iodide (Molecular Probes™, Invitrogen) for 15 min, and subsequently analyzed under a fluorescent

microscope. As a control, Human Umbilical Vein Endothelial Cells (HUVECs) that were deprived of culture medium for 15 min were used.

Immunohistochemistry

For immunohistochemical analysis, Matrigel cultures were fixed for 48h in buffered 10% formalin at 4°C. Membranes were cut in two and embedded in 2% agar, which was subsequently embedded in paraffin. Five µm thick sections were cut 4 times for each culture with intervals of 100 µm, mounted on Starfrost® slides (Knittel, Braunschweig, Germany), dried overnight at 37°C, and deparaffinized.

For antigen retrieval, sections were boiled for 20 min in a microwave oven in Tris/EDTA buffer (pH 9.0) for RCK108 (Euro-Diagnostica, Arnhem, The Netherlands), BrdU (Dako, Glostrup, Denmark), and FGFR3 (clone B-9, Santa Cruz) staining, and in citrate buffer (pH 6.0) for caspase 3 (R&D Systems, Minneapolis, MN) staining. For BrdU staining, slides were subsequently incubated in 2N HCl for 30 min at 37°C, followed by a two times 5 min wash with Borax buffer [24]. Non-specific binding was blocked with 0.5% nonfat dry milk (Protifar plus, Nutricia, Zoetermeer, The Netherlands) diluted in PBS 1% Tween. Incubation with primary antibodies overnight at 4°C was followed by horseradish peroxide labeling and 3,3-diaminobenzidine (DAB) staining using the EnVision™ Detection System (Dako). Slides were counterstained with Mayer's hematoxylin, dehydrated and mounted.

E-cadherin staining was done on cells cultured on uncoated glass slides using clone 5H9 antibody (Euro-Diagnostica).

Flow cytometry

Analysis of cell adhesion molecule expression was done by incubating cells with fluorochrome-conjugated monoclonal antibodies for α_6 (CD49f-FITC) and β_4 (CD104-PE) integrins, or for β_1 integrin (CD29-PE, all from BD Biosciences). Cells were studied for apoptosis by an annexin V-FITC/propidium iodide double staining using Vybrant® Apoptosis Assay Kit #3 (Molecular Probes™, Invitrogen). Cells were analyzed on a FACScan using CellQuest software (BD Biosciences). Data analysis was done using Windows Multiple Document Interface for Flow Cytometry (WinMDI, http://facs.scripps.edu/software.html).

RESULTS

Characterization of the stably transfected JO'N bladder carcinoma cell lines

To study the effect of the FGFR3 mutant receptor in urothelial cells, we searched for a bladder cancer cell line lacking *FGFR3* or *Ras* mutations, since FGFR3 and Ras are in the same signal transduction pathway. Using the SNaPshot assay for *FGFR3* and sequencing of codons 12, 13 and 61 for H-*ras*, we did not find mutations in these two genes in the JO'N bladder cancer cell line. We stably transfected JO'N cells with the wild type and mutant FGFR3 cDNA, and with the empty pcDNA3 vector as a control (mock transfection). For each transfection, one clone was selected for further studies based on qualitative RNA expression. When FGFR3 mRNA levels were analyzed using quantitative RT-PCR, we found that expression of FGFR3 was low in the untransfected JO'N cell line (as described previously by Bernard-Pierrot *et al.*) [19] and in the mock transfected cells. To compare wild type and mutant FGFR3 signaling, we intended to select two clones with similar FGFR3 expression levels. In Q-RT-PCR analysis we found that all eight mutant clones had an expression of at least 20 times higher than the untransfected cell line, and the majority (5/8 clones) had a relative expression between 1000 and 10,000. However, the clone with a relative expression of 55,000 was the only one with a detectable level of FGFR3 protein in Western blot analysis (data not shown). This clone was therefore selected for further studies together with the highest FGFR3 wild type mRNA expressing clone, which was 5 times higher than in the original cell line (Figure 1A).

We subsequently studied the morphologies of the three stable cell lines in culture, and compared them to untransfected cells. The original JO'N cell line and the mock-transfected JO'N cells showed a characteristic epithelial growth pattern. Cells were variable in size, and large cells with 2 or 3 nuclei were occasionally present. In contrast, cells transfected with the wild type or mutant receptor did not grow in sheets, but formed clusters of cells that were rounded and they grew in heaps (Figure 1B). These cells did not attain confluency. More large, multinucleated cells were present in the mutant cell line, and these included cells with up to 10 nuclei. Untransfected JO'N cells were able to attach to the plastic substrate within approximately 2h after seeding, but even after 24h a significant proportion of the cells expressing the mutant receptor were not yet attached. Nevertheless, the proliferation rate did not significantly differ between cell lines. Staining with an antibody against cytokeratin 19 (RCK108) was positive for all cell lines, which showed that the transfected cells remained epithelial. Furthermore, they also

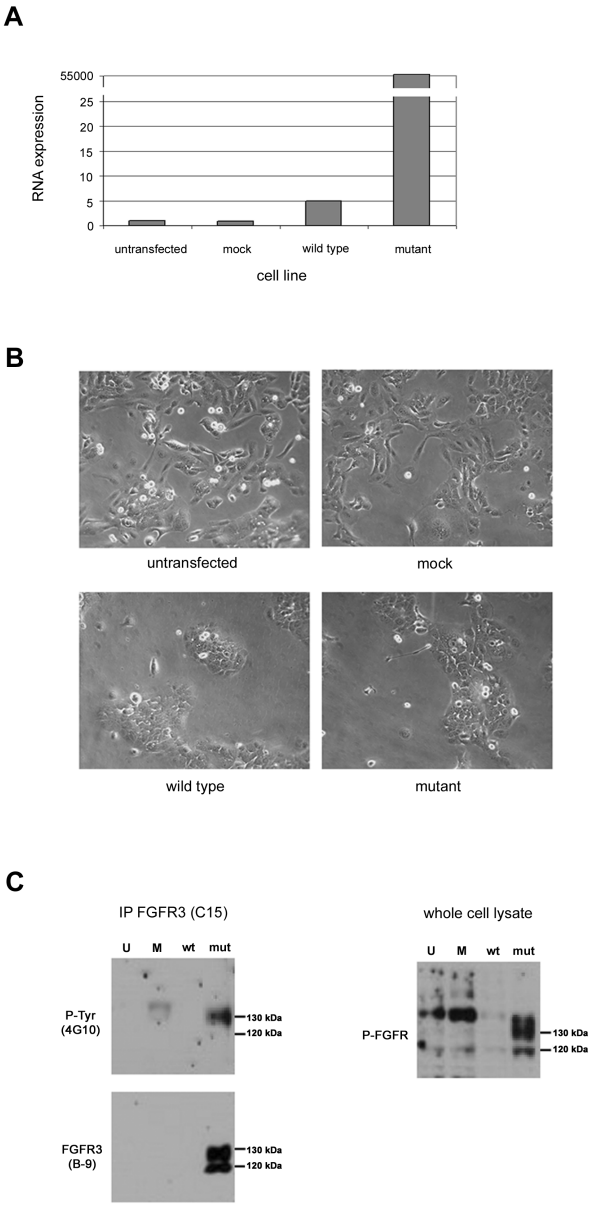

Figure 1. Characterization of the stably transfected JO'N cell lines used in this study.

A, quantitative FGFR3 mRNA expression. B, morphology of stably transfected cell lines in adherent plastic culture. C, FGFR3 protein expression and phosphorylation was determined by Western blot analysis of immunoprecipitated cell lysates using anti-FGFR3 (clone B9) and anti-phosphotyrosine (clone 4G10) antibodies, respectively. Tyrosine phosphorylation for all FGFRs was analyzed in whole cell lysate with anti-P-FGFR antibody.

U, untransfected; M, mock; wt, wild type; mut, mutant.

maintained their homogeneous E-cadherin expression pattern (data not shown).

FGFR3 protein expression was analyzed on Western blot with a specific monoclonal antibody. We found that both glycosylated forms of the FGFR3 protein were strongly expressed in the mutant cells, and that FGFR3 protein expression was not detectable in the untransfected, mock transfected and wild type transfected cells (Figure 1C), which is in accordance with the mRNA levels. To see whether the mutant FGFR3 receptor is activated, we analyzed the phosphorylation of FGFR3 after immunoprecipitation with an FGFR3 antibody directed to the intracellular part of the receptor. Immunoblots against phosphorylated tyrosine demonstrated a strong signal for the 130 kDa isoform in the mutant FGFR3 transfected JO'N cell line, while no signal was observed in the other three cell lines. We confirmed these results by determination of phosphorylated FGFR in whole cell lysate, which showed an additional phosphorylated band of the 120 kDa form (Figure 1C). In summary, we have shown that the mutant FGFR3 receptor was activated in stably mutant FGFR3 transfected cells.

Downstream targets of FGFR3

We subsequently analyzed the expression and activation of possible downstream targets of FGFR3. The first pathway we studied was the Ras/MAPK pathway, and we found that activation of Erk1 and 2 was stronger in the mutant JO'N cell line (Figure 2A). Targets directly downstream of FGFR3 in this pathway, the docking protein FRS2 and the phosphatase SH-PTP2, were also higher expressed in the mutant cell line; however, activation of FRS2 could not be demonstrated. For the other major pathways we did not find differences between the cell lines: Akt was both expressed and activated equally in all four cell lines (Figure 2B), and PLCγ, Stat1, Stat3, and Stat5 were expressed equally but not activated (data not shown). These results suggest that the Ras/MAPK and Akt pathways were already activated in JO'N, and that expression of mutant FGFR3 in JO'N increased MAPK signaling.

Mutant FGFR3 transfected cells undergo p53-independent apoptosis in Matrigel

To study the three-dimensional growth of the wild type and mutant cell lines, cells were cultured in growth factor reduced Matrigel matrix. All cell lines showed an epithelial morphology with formation of densely packed colonies or spheres. However, more small colonies were present in mutant cultures than in wild type or control cultures (Figure 3A), and in cross-sections it was visible that about 50% of these small mutant colonies consisted of only one cell, compared to 10% of

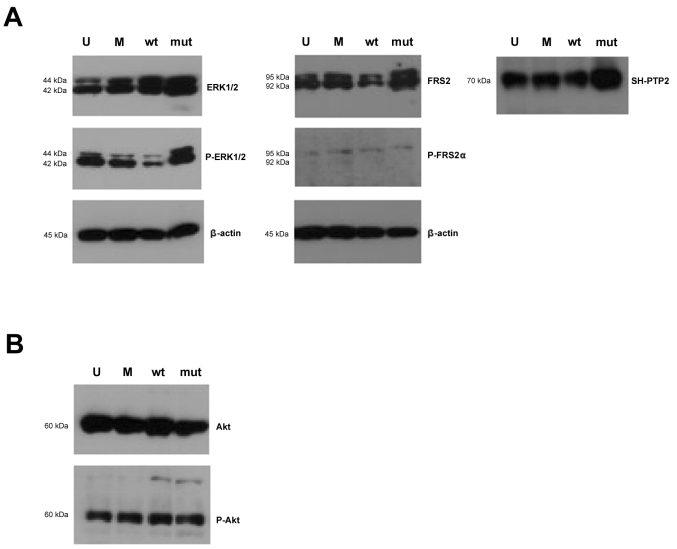

Figure 2. Expression and activation of downstream FGFR3 targets in stably transfected cell lines by Western blot analysis.
A, expression and activation of proteins in the Ras/MAPK pathway was analyzed in whole cell lysates using antibodies against p44/42, phosphorylated p44/42, FRS2, phosphorylated FRS2α, and SHP2. p44, ERK1; p42, ERK2. B, analysis of Akt expression and activation.
U, untransfected; M, mock; wt, wild type; mut, mutant.

single-cell spheres in the other cultures (data not shown). For control and wild type colonies, BrdU staining showed an increase in cell proliferation up to day 9, with subsequent decrease on day 12. In contrast, for mutant cells proliferation was low on day 9 and increased afterwards (Figure 3B).

Since the BrdU labeling index increased in mutant cells between day 9 and 12, we wondered if the small spheres represented colonies with delayed growth. We therefore cultured these cells until day 16, and by daily monitoring the spheres while in culture, we observed that colonies that were already larger grew out to the same size as colonies from untransfected JO'N cells; however, the small spheres did not grow any further (data not shown). The small spheres also did not represent senescent colonies, which was demonstrated by the absence of staining for senescence-associated acidic beta-galactosidase (data not shown). Furthermore, the number of apoptotic figures was high in mutant colonies, especially on day 9, and decreased afterwards, while the apoptotic index was lower in colonies from the other cell lines (Figure 3B). We therefore concluded that these colonies were not retarded in growth but failed to grow out and went into apoptosis.

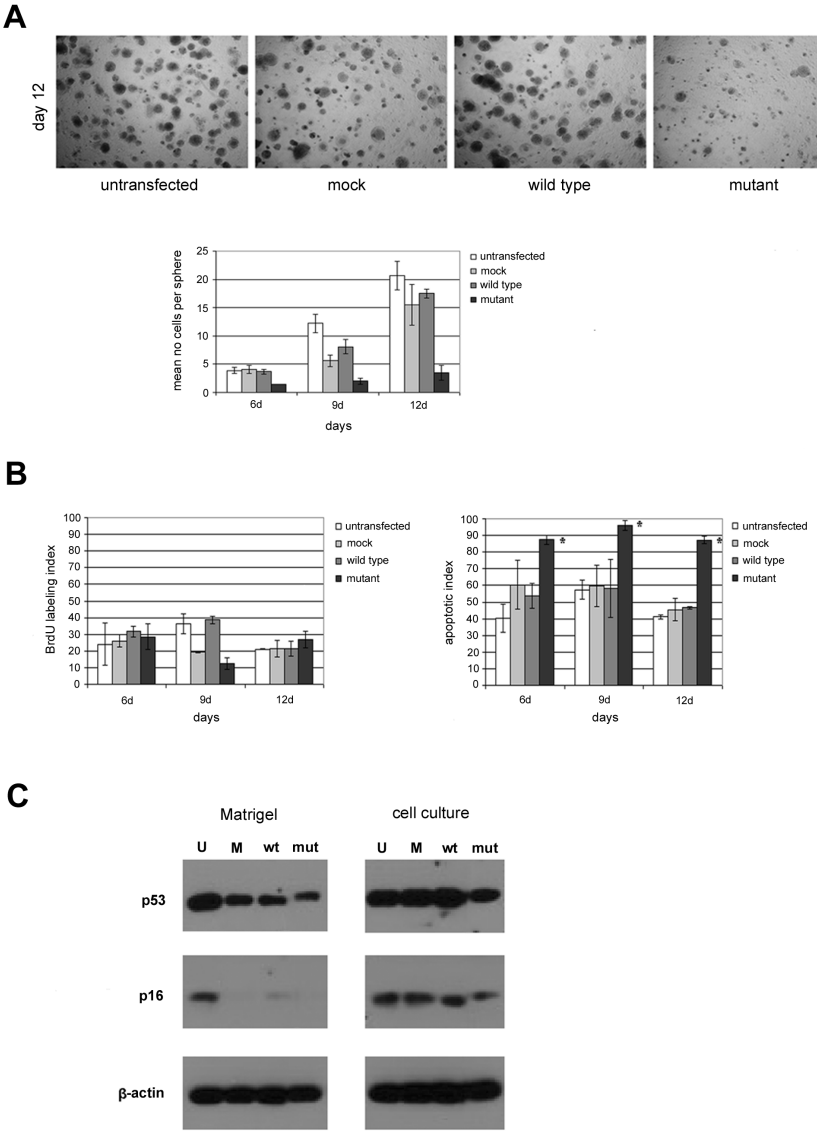

Figure 3. Analysis of stable cell lines cultured in Matrigel.
A, phase contrast micrograph of stable cell lines in Matrigel 12 days after seeding. The average size of the colonies was quantified by the number of cells per sphere. B, proliferation was measured using the BrdU labeling index, defined as the percentage of BrdU positive cells. Apoptosis was measured using the apoptotic index, defined as the number of caspase 3 positive cells plus the cells that were negative for caspase 3 but showed distinct apoptotic figures. C, expression of p53 and p16 in whole cell lysates from stably transfected cells cultured in Matrigel, and in cells from adherent plastic culture.
U, untransfected; M, mock; wt, wild type; mut, mutant.

We hypothesized that mutant cells would have a higher expression of apoptosis-related proteins related to their higher apoptotic index. During Matrigel cell culture, cell lysates were made from colonies on day 9. Analysis of the p53 and p16 proteins on Western blot revealed that these proteins were equally expressed in all four cell lines in normal cell culture, and even lower expressed in transfected cells from Matrigel culture (Figure 3C). The considerable overexpression of p53 in JO'N cells observed on Western blot indicated the presence of a mutation, and subsequent sequence analysis on our JO'N DNA for *TP53* exon 8 revealed the same mutation in codon 285 that was recently reported by Lopez-Knowles *et al.* [25] (data not shown). This suggests that apoptosis in the mutant JO'N cells was induced in another way than through the p53 pathway.

Mutant FGFR3 transfected cells do not undergo apoptosis in cell culture

To verify whether mutant cells were already apoptotic in cell culture on plastic substrate, adherent cells were stained with a green fluorescent iodide that is able to identify apoptotic cells. However, we found that none of the cell lines had increased levels of apoptosis, and the mutant FGFR3 line did not differ in apoptotic level from the other lines (Figure 4A). Similar results were observed when both adherent and floating cells were harvested and analyzed for Annexin-V expression by flow cytometry (Figure 4B). In summary, mutant FGFR3 expressing JO'N cells were able to survive in cell culture on a plastic substrate, but became apoptotic when cultured in 3-D.

Loss of $\alpha_6\beta_4$ complexes and β_1 expression in mutant FGFR3 transfected cells

Since mutant FGFR3 transfected cells went into apoptosis when cultured in Matrigel, we suggested that this might be due to loss of contact with the extracellular matrix (ECM). We therefore studied the expression of $\alpha_6\beta_4$ integrin complexes in our cell lines. In untransfected, mock transfected, and wild type transfected cell lines, more than 90% of the cells was $\alpha_6\beta_4$ double positive (Figure 5A), although in FGFR3 wild type expressing cells the population of negative cells was somewhat increased (15% vs. 9% in untransfected and mock transfected cells). In cells transfected with the mutant receptor, however, a subpopulation of cells negative for β_4 and largely negative for α_6 was found that comprised 30% of the total living cell population. To see if the loss of $\alpha_6\beta_4$ complexes was specific or that it was due to a more common loss of integrin expression, we subsequently analyzed cells for expression of the ubiquitously expressed β_1 integrin. Again, about 30%

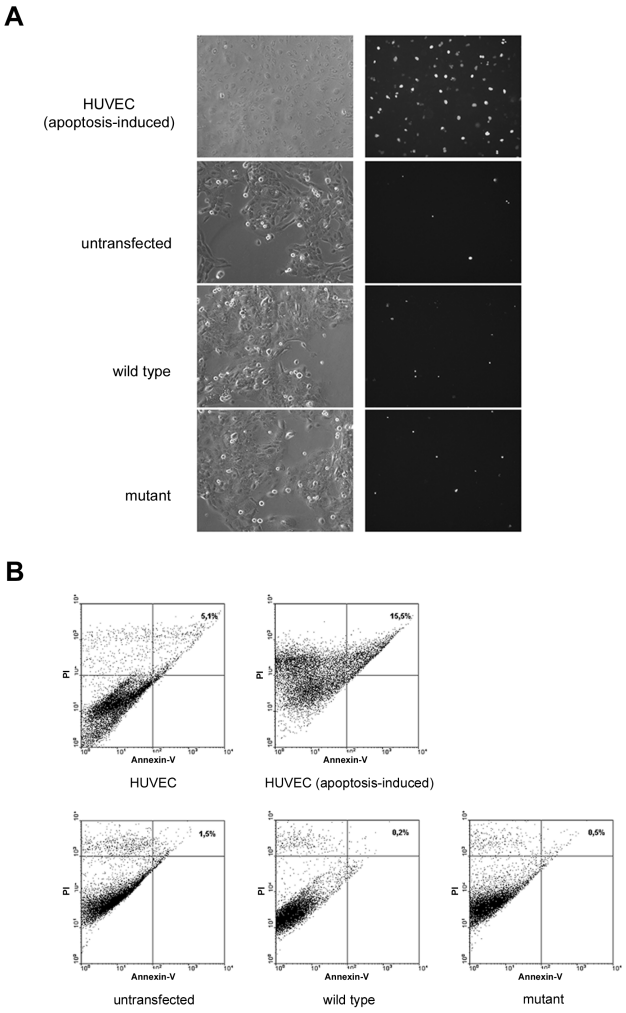

Figure 4. Apoptosis in cultured cells.
Apoptotic cells were identified by A, YO-PRO staining of adherent cells, and B, Annexin-V/PI labeling of the total cell population. Cells were stained with anti-annexin V-FITC antibody (x-axis) and propidium iodide (y-axis). Human umbilical vein endothelial cells (HUVECs) were used as a control.

of the mutant transfected cells were negative for β_1 integrin, whereas in the other cell lines almost all cells were positive for this integrin (Figure 5B). Strikingly, in contrast to the other cell lines, this subset of mutant cells was completely negative for β_1 integrin expression.

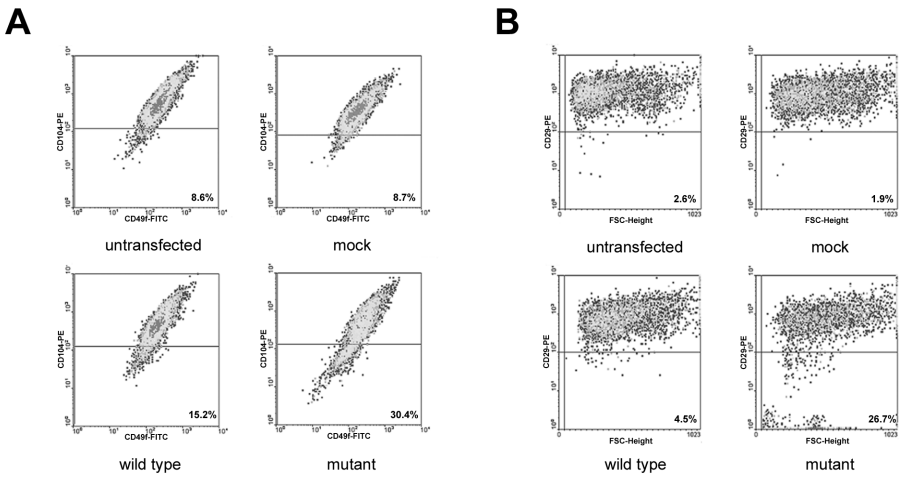

Figure 5. Integrin expression on adherent cells.
A, Expression analysis of the $\alpha_6\beta_4$ complex was done using anti-CD49f-FITC antibody for α_6 (x-axis), and anti-CD104-PE antibody for β_4 (y-axis). B, expression of β_1 was analyzed using anti-CD29-PE antibody (y-axis). Please note the large subset of mutant cells that is completely negative for β_1.

Mutant FGFR3 transfected cells that are able to grow out in Matrigel have lost FGFR3 protein expression

Untransfected, mock transfected, and wild type transfected cells, which lacked FGFR3 protein expression (Figure 1C), all formed large colonies in Matrigel. Although the majority of mutant FGFR3 transfected cells went into apoptosis, we found that some of these cells were indeed able to grow out and form colonies comparable in size to those of the other cell lines. We subsequently analyzed the colonies for FGFR3 protein expression by immunohistochemistry, and we observed that FGFR3 protein was not expressed in spheres from control and wild type cell lines. In the mutant Matrigel culture, however, the small colonies show a strong FGFR3 expression, whereas the large spheres were FGFR3 negative (Figure 6). These results indicate that only cells that lost FGFR3 expression were capable of 3-D growth.

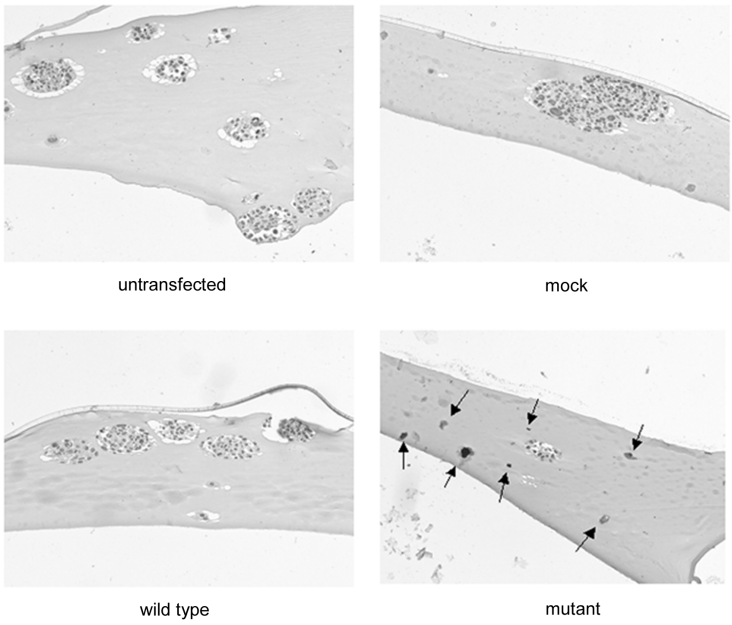

Figure 6. FGFR3 protein expression in Matrigel cultures.
Immunohistochemical staining of colonies in Matrigel was done with anti-FGFR3 (clone B-9) antibody. Arrows indicate positive cells/colonies.

DISCUSSION

Since the discovery of *FGFR3* mutations in bladder cancer, the function of this mutation in urothelial cells has remained elusive. Our aim was to study the tissue-specific effects of *FGFR3* mutations in bladder cancer compared to the wild type receptor by introducing both wild type and mutant receptors into urothelial cells. The lack of wild type clones with high FGFR3 mRNA expression, and the overexpression of FGFR3 mRNA in almost all (6/8) mutant clones suggests that mutant mRNA is more stable than wild type mRNA. This association of *FGFR3* mutations with high FGFR3 RNA expression has previously been found in gene expression profiling by Lindgren *et al.* [26]. The extensive research that has been done on the mutant FGFR3 receptor in skeletal dysplasias suggests that the corresponding phenotype results from enhancement of normal ligand-induced FGFR3 signaling, which is consistent with the findings in our transfection model.

During our studies on the stably transfected cell lines, we observed that

cells expressing mutant FGFR3 show very different behavior from cells that do not. This suggests that different mechanisms are activated in mutant FGFR3 transfected cells, and that alternative signaling pathways are involved. Analysis of the expression and activation of downstream targets of FGFR3 revealed that the Ras/MAPK and PI3K/Akt pathways are both activated in the original JO'N cell line. These two signaling pathways have been implicated in bladder carcinogenesis previously, since most mutations that were found in, especially superficial, bladder tumors occur in genes that code for proteins in these pathways [25, 27, 28]. In our study, the absence of activated proteins from other pathways, such as PLCγ and Stats, confirmed that Ras/MAPK and PI3K/Akt are the major pathways involved in bladder cancer, although mutations in *FGFR3*, H-*ras*, and *PIK3CA* [25] have not been found in JO'N. In mutant cells, which expressed activated FGFR3, an increase in Erk1/2 activation was observed, suggesting that this pathway could be enhanced when *FGFR3* is mutated. This corresponds with the results for FGFR3 splice variant IIIc, where mutations causing thanatophoric dysplasia, including the R248C mutation, lead to constitutive activation of ERK1/2 [29, 30]. We did not find activated proteins that were not expressed or activated in wild type or control cells; nevertheless, the perspective of differentially activated signaling pathways in mutant FGFR3 expressing cells merits further study by kinomic and proteomic approaches.

Although *FGFR3* is considered an oncogene, we found induction of apoptosis in bladder cancer cells with activated FGFR3 receptors when they were cultured in Matrigel. The first protein we studied that could be related to the observed apoptosis was p53; however its expression was not different in mutant colonies compared to wild type or control. This equal expression was caused by the presence of a *TP53* mutation in the original JO'N cell line. The observation that bladder cancer cells with a *TP53* mutation can only grow in 3-D when FGFR3 is not expressed is in line with the previously reported exclusiveness of these mutations in clinical samples of bladder cancer [3, 4], and thus provides a model to study this exclusiveness.

Another candidate for the induction of apoptosis was Stat1 [31]. Legeai-Mallet *et al.* showed that *FGFR3* mutant cells from a patient with thanatophoric dysplasia (TD) had ligand-independent activation of the Stat1 signaling pathway, higher expression of Bax, and a decrease in Bcl-2, which corresponded to an increased number of apoptotic cells in TD fetuses [29]. Furthermore, normal and TD cells showed the same proliferation rate. These similarities to findings in our transfected cell lines lead us to investigate Stat1 activation. We found that mutant

JO'N cells from adherent plastic culture lack activation of Stat1; however, we subsequently showed that under these conditions they were not apoptotic. The mutant FGFR3 signaling that leads to apoptosis in our transfected JO'N cell line in Matrigel therefore requires further study.

The apoptotic index of mutant spheres in 3-D culture was about 30% higher than that of the wild type or control colonies. This percentage corresponded to the 30% of cells lacking integrin expression, suggesting that they may represent the same population. Integrins are the major receptors for cell adhesion to the ECM, and in normal epithelial cells loss of ECM adhesion induces the cells to undergo apoptosis, a process called anoikis. JO'N cells transfected with mutant FGFR3 receptor indeed attached very poorly to the plastic substrate in regular cell culture, which corresponds with the loss of integrin expression; however, they are not apoptotic. Cancer cells enhance the expression of integrins that favor their survival and tend to lose the integrins that have the opposite effect, leading to anoikis resistance [32]. Functional inactivation of the tumor suppressor p16 is common in cancer, and it has been shown that re-expression of p16 in cancer cells restores anoikis sensitivity by enhancing expression of $\alpha_5\beta_1$ [33]. Indeed, we did not find elevated expression of p16 in our mutant cells, and a substantial subset of these cells lacked expression of β_1, indicating that the mutant FGFR3 expressing cells are probably not sensitive to anoikis. Furthermore, apoptosis of mutant cells on plastic substrate culture is absent. These results suggest that the cells lacking integrin expression have escaped anoikis and are able to survive in cell culture. In 3-D culture, however, these cells can not escape contact with the ECM since they are surrounded by Matrigel, and apoptosis is subsequently induced.

When cells grown on plastic substrate were harvested for integrin expression analysis, floating cells were washed away and only adherent cells remained. However, one third of this adherent mutant cell population appeared to have lost integrin expression. Because the mutant cells grew in heaps instead of a monolayer, it may be that the cells that lost their integrins grew on top of the subpopulation of cells that retained integrin expression, allowing for integrin-mediated contact with the ECM. Mutant FGFR3 cells lacking integrin might adhere to this population of plastic adherent cells using other adhesion molecules such as E-cadherin, preventing the activation of the apoptotic pathway in these overlying integrin-negative cells. Cells transfected with wild type FGFR3 displayed the same growth pattern in adherent plastic culture, suggesting that these cells also have lost part of their integrin expression. This is also indicated by the elevated loss of $\alpha_6\beta_4$ (15%) and β_1 (4.5%) integrin expression, which we observed in FACS

analysis. These cells, however, have not lost their integrin expression completely, compared to the mutant cells, which comprise a subpopulation that is completely negative for β_1 integrin expression (Figure 5B). These results suggest that signal transduction mediated by FGFR3 leads to a dose-dependent loss of integrin expression, and that a slight increase in FGFR3 expression already results in a multilayered growth pattern.

The $\alpha_6\beta_4$ integrin is expressed in normal human urothelial cells where it is the principal integrin involved in substratum adhesion, showing a polarized expression in basal cells at their junction with the lamina propria [34]. However, $\alpha_6\beta_4$ is overexpressed in the majority of bladder cancer tissues, showing the strongest expression in invasive bladder cancers [35]. Integrin $\alpha_6\beta_4$ promotes cell migration and invasion, which is supported by the observation that bladder cancer patients with strong overexpression throughout the tumor cells had a significantly worse survival [36]. Because FGFR3 mutant cells lost $\alpha_6\beta_4$ expression and therefore the ability to attach to the ECM, this is another indication that *FGFR3* mutations protect a noninvasive tumor from invading into the basement membrane.

When we analyzed mutant spheres for FGFR3 expression by IHC, we found that the few large colonies that were present had selectively lost FGFR3 protein expression. Colonies in Matrigel were grown under G418 selection, yet the inability of mutant cells to grow in Matrigel created a second form of selection in which only cells that were able to inactivate FGFR3 protein expression and retain expression of the neomycin gene were able to survive. Small, apoptotic spheres that consist of only a few cells occur in Matrigel cultures of all cell lines, but they are dominantly present in mutant cultures (50%). However, they differ regarding FGFR3 protein expression: all single-cell apoptotic spheres in wild type and control cultures were FGFR3 negative, whereas in the mutant cultures these colonies were all positive. Our current hypothesis is that all cells from the mutant clone express the mutant FGFR3 receptor, i.e. have high FGFR3 protein expression. When enough cells have attached to the ECM to form a stable basis, the rest of the cells that have lost integrin expression will grow by attaching to these cells using E-cadherin. This shift to E-cadherin as the major receptor for adhesion corresponds with the invasion pattern of stage T1 bladder tumors with an *FGFR3* mutation: van der Aa *et al.* showed that the majority of mutant *FGFR3* tumors display microinvasion into the lamina propria, with usually only a single cluster of tumor cells invading the lamina propria, suggesting that these cells prefer to attach to each other. In contrast, almost all wild type tumors (93%) show extensive multifocal infiltration, and single tumor cells were observed in the stroma, showing that these cells are inclined

to contact the ECM [37]. It is tempting to compare the morphological analogy of the mutant FGFR3 transfected cells, particularly the multilayered growth, with the papillary formations observed particularly in *FGFR3* mutant urothelium; however, further studies are necessary to validate our results in other model systems, and to fully understand the role of FGFR3 in papillary bladder carcinogenesis.

In conclusion, we have shown that noninvasive bladder cancer cells that overexpress mutant FGFR3 retain their epithelial properties such as CK19 and E-cadherin expression. Mutant and wild type cells both grow three-dimensionally in culture, however only a subpopulation of the mutant cells showed complete loss of integrin expression, suggesting a gradual loss of attachment to the ECM. Mutant cells are only able to survive in Matrigel when they lose FGFR3 protein expression, which may explain why *FGFR3* mutated bladder tumors have reduced invasive properties.

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CHAPTER 8

General discussion

Molecular genetic studies conducted over the last decades have greatly increased our knowledge of genetic alterations in bladder cancer. Particularly, the combination of histopathological findings and molecular genetic events has led to the concept of the two-pathway model for bladder carcinogenesis, with *TP53* responsible for the pathway leading to dysplasia, carcinoma *in situ* (CIS) lesions and invasive tumors [1, 2]. The other arm of the model is represented by *FGFR3*: the identification of mutations in the *FGFR3* gene in a substantial proportion of primary bladder tumors by Cappellen *et al.* [3] has been the most exciting discovery in the recent years. Further studies provided some striking results: *FGFR3* mutations were common in bladder tumors of low stage and grade [4] and associated with a good prognosis in bladder cancer patients [5]. Mutations in *FGFR3* were also found in seborrheic keratosis, a papilloma of the skin that never progresses [6, 7]. These lesions resemble noninvasive pTa papillary carcinomas of the bladder, which should in fact be defined as papillomas since they do not pass the basal membrane. Thus, mutations in *FGFR3* are associated with benign neoplasms of epithelial cells.

The main goals of this thesis were 1) to explore the potential use of *FGFR3* mutations in bladder cancer diagnosis, prognosis, and in surveillance of patients with bladder cancer, and 2) to understand the functional role of mutant FGFR3 in bladder carcinogenesis.

FGFR3 mutations in bladder carcinogenesis

The association of *FGFR3* mutations with a large percentage of TaG1-2 tumors suggested that *FGFR3* mutation is an early event in the development of papillary tumors. This view was confirmed in this thesis by the study of flat urothelial hyperplasia, a lesion thought to be the obligatory precursor of TaG1-2 tumors [8-10]. *FGFR3* mutations were frequently found in flat urothelial hyperplasias adjacent to papillary tumors (**Chapter 3**). Furthermore, we looked at the differentiation of bladder tumors by staining with cytokeratin 20 (CK20). In normal urothelial tissue, CK20 is exclusively expressed in the upper urothelial cell layer that consists of the umbrella cells. An abnormal staining pattern, defined by CK20 expression in all urothelial cell layers or by absence of expression, indicates tissue that is less well differentiated (**Chapter 5**). This abnormal CK20 staining thus signifies a disordered differentiation of the urothelial cells. For the pTaG1 subset of papillary UCC, *FGFR3* mutations were present in 82% of tumors, whereas only 59% presented with an abnormal CK20 staining pattern. Together, these observations indicate that mutations in the *FGFR3* gene occur prior to this early sign of maturation disturbance of the urothelial cells. In **Chapter 3** we demonstrated that

LOH of chromosome 9 is present in the majority of flat urothelial hyperplasias with an *FGFR3* mutation, and that more than half of the hyperplasias with LOH 9 is wild type for *FGFR3*. This would indicate that loss of chromosome 9 still remains the earliest known event in bladder carcinogenesis. Furthermore, LOH 9 may already be detected in the histologically normal urothelial mucosa in patients with bladder cancer. On the other hand, only 50% of bladder tumors display loss of chromosome 9, suggesting that other early events have to be present which lead to carcinogenesis in bladder tumors without LOH 9.

Although LOH 9 is the earliest known genetic event in bladder cancer, it is still unclear whether genetic instability drives tumor development. Bartkova *et al.* [11] suggested that LOH at fragile sites (including chromosome 9q) induces the DNA damage response leading to cell-cycle blockade (senescence) or apoptosis, thereby preventing tumor progression and genetic instability. Similarly, these authors surmised that activating mutations in *FGFR3* might play the same role, i.e. induction of a DNA damage response [11]. In **Chapter 4** we found a low number of genetic alterations per tumor for *FGFR3* mutated tumors, suggesting that the *FGFR3* mutation would indeed protect cells against genetic instability, e.g. by mediating senescence or apoptosis. This paradoxical view stands in contrast with the alleged growth stimulatory role of mutant FGFR3.

Upper urinary tract tumors are genetically different from bladder tumors: they have more frequent MSI, and promoter hypermethylation is more frequent and more extensively present. Nevertheless, we showed in **Chapter 6** that the frequency of *FGFR3* mutations, LOH 9, and LOH 17 is the same for bladder and upper tract tumors.

FGFR3 mutations in bladder cancer prognosis and surveillance

The initial study on *FGFR3* mutations and prognosis in bladder tumors suggested that the presence of *FGFR3* mutations in bladder cancer is associated with a good prognosis, since patients with an *FGFR3*-mutated tumor were shown to have a lower risk of tumor progression and a better survival. However, *FGFR3* mutation status was not an independent predictor for progression-free and disease-specific survival but had to be combined with Ki-67 staining and classified into three different molecular grades to reach independence [5]. We conducted a separate study using a tissue microarray comprising 255 primary tumors from unselected patients, described in **Chapter 5**. In our study, we found that four markers including *FGFR3* were all strong, but not independent predictors of disease-specific survival. These results corresponded to the previous study, however the previously

reported results for molecular grading could not be confirmed in disease-specific survival analysis. Strikingly, the identification of an *FGFR3* mutation in bladder cancer virtually excluded the presence of associated carcinoma *in situ*. This may be of potential clinical interest since the finding of carcinoma *in situ* is an adverse prognosticator. In **Chapter 6**, we analyzed tumors from the entire urothelial tract (i.e. bladder, ureter, and renal pelvis), and we found that *FGFR3* mutation status is an independent predictor for progression and disease-specific survival in tumors from the ureter. Because of their location and their genetically different background it might be possible that tumors from the upper urinary tract present different biological behavior compared to bladder tumors. In **Chapter 5 and 6** we also demonstrated that the presence of an *FGFR3* mutation in combination with either a normal CK20 staining pattern or an unmethylated *RASSF1A* gene were able to define the group of low-grade noninvasive papillary tumors with a very low risk of progression. The combination of *FGFR3* with one of these molecular markers is therefore recommended to optimize prognostication of patients presenting with superficial bladder cancer.

The majority of patients presents with pTa/T1 bladder tumors of low grade. These tumors have a low risk of progression, but the recurrence rate in these patients is high. Recently, a 25-year prospective study suggested that the intensity of bladder cancer surveillance by cystoscopy can be reduced in bladder cancer patients with TaG1 tumors, and that patients can be discharged when they stay recurrence-free for 5 years after the primary tumor has been removed [12]. Cystoscopy is an invasive technique, and for this reason much effort is undertaken to find alternative methods to detect bladder cancer recurrences in voided urine specimens. Thus, it can be envisaged that a proportion of the cystoscopies during the first few years can be replaced by a test on voided urine, provided that a urine test is available with comparable sensitivity and specificity as the current golden standard, cystoscopy. Since *FGFR3* mutations are found in a large proportion of low grade, low stage bladder cancers, the development of a robust test detecting these mutations would be the most obvious step. The results in this thesis further emphasize that *FGFR3* would be a useful molecular marker in bladder cancer diagnosis and prognosis, and could – in theory – provide an excellent urine marker for follow-up of patients with low grade and low stage bladder cancer. Furthermore, the observation that *FGFR3* mutations occur at the same frequency in upper tract tumors is important, since it has been reported that patients with Ta bladder cancer with two or more recurrences with intervals of less than a year are at higher risk for developing a tumor in the ureter or renal pelvis [13]. Since

surveillance for upper tract tumors is more difficult and even less comfortable than surveillance for bladder cancer, *FGFR3* mutation analysis in urine could also be very useful for patients with upper urinary tract tumors. Urine samples of patients with upper tract tumors have to be analyzed to verify that these tumors can indeed be identified.

All *FGFR3* mutation analyses in this thesis were done using the new assay we have developed, which is described in **Chapter 2**. This PCR-based assay is able to analyze nine *FGFR3* mutations in bladder cancer simultaneously. Recently, we added primers for a tenth mutation (G382R) that represents the most commonly mutated nucleotide in humans, but has only been found once in bladder cancer, and the latest mutation (S373C) that had already been described in the skeletal dysplasia TD1 but has only recently been identified in bladder cancer. Therefore, the assay now comprises all eleven known *FGFR3* mutations in bladder cancer [14]. Our aim was to eventually use the new *FGFR3* mutation detection assay in urine diagnostics, reducing the frequency of surveillance cystoscopies. However, a first study showed that the sensitivity of our *FGFR3* assay in urine to detect mutant *FGFR3* bladder cancer recurrences was only 62% (**Chapter 2**). Currently, the signal for our assay has been improved such that the sensitivity is increased. Studies on its use for the detection of recurrences in urine are ongoing.

The functional role of mutant FGFR3 in bladder cancer

As mentioned above, mutations in *FGFR3* are specific for the pathway of papillary tumorigenesis, whereas *TP53* mutations cause carcinoma *in situ* (CIS) and subsequent development of invasive tumors. However, 30-40% of papillary tumors are *FGFR3* wild type but present with the same phenotype as mutant tumors. A substantial part of these *FGFR3* wild type tumors present with mutations in one of the *Ras* genes, and from all low grade (G1) papillary tumors 82% could be defined by a mutation in either *FGFR3* (71%) or *Ras* (11%) [15]. FGFR3 and Ras are in the same signal transduction pathway, which might be a possible explanation for the mutual exclusiveness of mutations in these genes. However, *Ras* mutations are not the same as *FGFR3* mutations, since they are not associated with stage or grade.

To unravel the functional mechanism that stands at the basis of the difference in prognosis between mutant and wild type *FGFR3* bladder tumors, we set out to study the biological consequences of the introduction of the mutant receptor in urothelial cells otherwise lacking this mutation (**Chapter 7**). Our initial aim was to use normal urothelial cells for these experiments, since bladder cancer

cell lines have many unknown abnormalities. However, normal urothelial cells cannot be cultured without immortalization, and this also leads to several random genetic aberrations. For our model we therefore chose a human bladder cancer cell line (JO'N) that retained its urothelial morphology and that is not invasive in vivo. In this model, we confirmed previous predictions [15] that mutant activated FGFR3 enhanced the MAPK pathway. The most striking difference between cells expressing wild type and mutant FGFR3 receptor was that mutant cells display loss of integrin expression and show increased apoptosis in Matrigel. This indicates that noninvasive bladder cancer cells expressing mutant FGFR3 are not able to survive when they make contact with the extracellular matrix (ECM), suggesting that they are not able to invade. These results correlate with the "protective" effect of the *FGFR3* mutation, which demonstrates itself in a low progression rate in bladder tumors with an *FGFR3* mutation (**Chapter 5 and 6**), and in a low number of genetic alterations per tumor for mutated tumors (**Chapter 4**). Nevertheless, wild type and mutant cells both display the same three-dimensional growth pattern in plastic substrate culture, suggesting that FGFR3 expression leads to a gradual loss of integrin expression.

The model we used in our study has its limitations. A major disadvantage of using cell lines is that many unknown genetic aberrations may be present, as became manifested by the unexpected discovery of a *TP53* mutation in the JO'N cell line; however, many other aberrations will remain unnoticed. Since *FGFR3* and *TP53* mutations are almost exclusive in bladder tumors, the effect of the *TP53* mutation might interfere with the effect of FGFR3 activation. From the JO'N cell line we created various stable FGFR3-expressing cell lines. Stable cell lines are not an ideal model because of clonal variation. Clone-related results can be reduced by studying several stable cell lines from the same transfection; however, from all our stable mutant FGFR3 cell lines only one expressed mutant FGFR3 (**Chapter 7**). The expression of FGFR3 in this one clone was very high, which might result in artificial effects in the model. Our results have therefore to be validated in other models, e.g. by using other bladder cancer cell lines, or by using an inducible expression system, in which the expression of wild type and mutant FGFR3 can be switched on or off. Future studies should also be aimed at the analysis of signal transduction proteins in wild type and mutant *FGFR3* bladder tumors, which can be achieved by dissection of fresh tumor tissue followed by protein expression analysis on Western blot and by using proteomics. With this method we can study signal transduction pathways in bladder cancer in more detail, and it will be more clear which pathways are differentially activated or enhanced in mutant FGFR3

expressing tumors.

Since 50% of primary bladder tumors carry an *FGFR3* mutation, several studies have been aimed at the use of FGFR3 as a therapeutic target in bladder cancer. Two studies have already displayed growth inhibition of bladder cancer cell lines *in vitro* expressing mutant FGFR3 when the receptor is blocked by either siRNA or FGFR inhibitor [16, 17]. However, the mechanism of FGFR3-induced bladder carcinogenesis needs to be unraveled further before this kind of therapy should be considered for clinical use. The transforming properties of mutant FGFR3 have still to be studied in urothelial cells. It is possible that another common genetic event for both wild type and mutant tumors initiates tumor growth, and that mutant FGFR3 induces loss of invasion ability. Inhibition of FGFR3 expression could therefore have an adverse effect, as exemplified in our Matrigel studies (**Chapter 7**). In vivo studies have not been possible up until now, since bladder tumors could not be induced by targeted expression of FGFR3 to mouse urothelium.[1] This suggests that FGFR3 alone is not enough to induce bladder tumor growth. Furthermore, the bladder cancer cell line used by Tomlinson *et al.* that expressed FGFR3-S249C, 97-7, does not produce tumors as subcutaneous xenografts in immunodeficient mice. The latter correlates with the lack of growth of FGFR3-R248C expressing bladder cancer cells in Matrigel. Mutations in another oncogene, *HRAS*, have also been found in benign tumors, suggesting that Ras alone is not sufficient to induce cancer [18]. It has now been demonstrated that in benign tumors, activation of Ras creates a negative feedback loop leading to oncogene-induced senescence [19]. However, activation of FGFR3 did not lead to induction of senescence in our model. An important goal for further research on both mechanisms and therapies for superficial bladder cancer will be to assess tumorigenicity in the bladder *in vivo*. These *in vivo* studies might then be able to clarify whether *FGFR3* mutations are tumorigenic, and whether inhibition of FGFR3 expression in bladder tumors will indeed be beneficial.

Is *FGFR3* an oncogene?

Since *FGFR3* mutations were not associated with LOH at 4p16, it is unlikely that *FGFR3* functions as a tumor suppressor gene [20]. The question if *FGFR3* functions as an oncogene, however, has not been fully answered yet. *FGFR3* and *RAS* mutations are mutually exclusive in both MM and bladder tumors, suggesting that FGFR3 functions via the Ras/MAPK pathway. The detection of MAPK activation in MM, and the elevated MAPK signaling we showed in the JO'N

[1] F Radvanyi, personal communication

bladder cancer cell line that was transfected with mutant FGFR3 (**Chapter 7**) are in line with this idea. As a result, *FGFR3* mutations induce proliferation in MM, and probably also in bladder tumors. This has been demonstrated by the inhibition of FGFR3 by siRNA, which leads to growth inhibition of bladder cancer cell lines expressing mutant FGFR3 [17].

Mutant FGFR3 is able to transform NIH-3T3, 293T, and murine bone marrow cells. The transforming properties of FGFR3 in epithelial or urothelial cells, however, have not been tested yet. Targeted expression of mutant FGFR3 to the urothelium did not induce bladder tumors in mice, and bladder cancer cells expressing mutant FGFR3 did not produce tumors as subcutaneous xenografts in immunodeficient mice, suggesting that FGFR3 alone is not enough to induce bladder tumor growth.

In summary, activation of FGFR3 provides an important growth advantage in bladder tumors. However, to date there is no evidence that FGFR3 is able to induce these tumors. *FGFR3* should therefore not be considered an oncogene.

Conclusion

This thesis describes both clinical and functional aspects of the activation of FGFR3 in bladder cancer. The results confirm the association of FGFR3 with well-differentiated, noninvasive bladder tumors with few genetic alterations and a good prognosis. Furthermore, important questions on the function of mutant FGFR3 expression in bladder cancer cells have been raised that merit further study.

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SUMMARY

The identification of frequent *FGFR3* mutations in superficial bladder cancer suggests that mutation of the *FGFR3* gene is a key genetic event in the development of noninvasive bladder tumors. Furthermore, *FGFR3* mutations were associated with a good prognosis, suggesting that the activation of FGFR3 has a beneficial effect during urothelial tumor formation. In this thesis, two aspects of *FGFR3* mutations in bladder cancer have been investigated. First, the potential use of *FGFR3* mutations in bladder cancer diagnosis, prognosis, and in surveillance of patients with bladder cancer has been explored. The second, more important aim was to get insight in the functional role of mutant FGFR3 in bladder carcinogenesis.

For clinical studies, a simple and fast method for *FGFR3* mutation detection was developed. In **Chapter 2**, a new assay for the analysis of all known *FGFR3* mutations in bladder cancer is described that is fast, easy, more sensitive and less laborious than previous techniques. The test can easily be adjusted when additional mutations in the *FGFR3* gene are discovered. With this technique, several studies described in this thesis were done on the role of FGFR3 in bladder carcinogenesis and patient outcome.

Since *FGFR3* mutations occur in low grade, low stage bladder tumors, mutation of the *FGFR3* gene is considered an early event in bladder carcinogenesis. We investigated this by analyzing *FGFR3* mutations in urothelial hyperplasia, a precursor of low-grade papillary carcinoma, and found that *FGFR3* mutations are already present in hyperplastic lesions (described in **Chapter 3**). Furthermore, we reported a low number of chromosomal aberrations in bladder tumors with an *FGFR3* mutation (**Chapter 4**), which suggests that *FGFR3* mutated tumors are genetically stable, in contrast to most tumors that do not carry this mutation. In **Chapter 5** we showed that this genetic stability of *FGFR3* mutant tumors was paralleled by a normal differentiation of urothelial cells, since a normal staining pattern for cytokeratin 20, a marker for terminal differentiation of urothelium, was correlated with bladder cancers carrying the *FGFR3* mutation.

In **Chapter 5** we also described the relation of four molecular markers (*FGFR3*, CK20, Ki-67, P53) to bladder cancer patient outcome. The subset of *FGFR3* mutated tumors with a normal CK20 staining pattern rarely progressed, providing a combination of two molecular markers that is able to define the group of prognostically favorable low-grade noninvasive papillary tumors. Urothelial cell carcinomas not only occur in the bladder but also in the upper urinary tract (*i.e.* in

the ureter and renal pelvis). These upper tract tumors may be genetically different from bladder tumors. The *FGFR3* mutation frequency was, however, equally high (~50%) in all urothelial tract tumors, and *FGFR3* mutation status was an independent predictor for progression and disease-specific survival in tumors from the ureter (**Chapter 6**). Furthermore, a second combination of molecular markers (*i.e. FGFR3* mutation status and *RASSF1A* methylation status) was identified that was also able to define low-risk noninvasive bladder cancer: only one of 25 tumors with an *FGFR3* mutation and absence of *RASSF1A* methylation progressed.

The effect of mutant FGFR3 receptor on the phenotype of urothelial cells *in vitro* has never been described, nor is their any information available on FGFR3 signaling pathways involved in urothelial or epithelial cells. In **Chapter 7**, we report the results of an experimental study analyzing the expression of a mutant FGFR3 receptor transfected in a human bladder cancer cell line. The most striking effect was that cells expressing the mutant FGFR3 receptor display both loss of integrin expression and increased apoptosis when cultured in Matrigel. This would suggest that interaction of bladder cancer cells expressing mutant FGFR3 with Matrigel (*i.e.* basement membrane substances) does not permit their survival. The latter would also explain the clinical finding that *FGFR3* mutant bladder cancers have a comparatively low tendency to become invasive.

In conclusion, this thesis describes both clinical and functional aspects of the activation of FGFR3 in bladder cancer. The results confirm the association of mutant FGFR3 with well-differentiated, noninvasive bladder tumors with few genetic alterations and a good prognosis. Furthermore, important questions on the function of mutant FGFR3 expression in bladder cancer cells have been raised that merit further study.

SAMENVATTING

De frequente aanwezigheid van fibroblast groeifactor receptor 3 (*FGFR3*) mutaties in oppervlakkige blaaskanker wijst erop dat de mutatie van het *FGFR3* gen een sleutelrol speelt in de ontwikkeling van niet-invasieve blaastumoren. Daarnaast heeft de activatie van de FGFR3 receptor waarschijnlijk een gunstig effect tijdens de groei van blaastumoren, aangezien *FGFR3* mutaties geassocieerd worden met een goede prognose in blaaskankerpatiënten. In dit proefschrift zijn twee kanten van de aanwezigheid van *FGFR3* mutaties in blaaskanker belicht. Als eerste hebben we de mogelijkheid onderzocht om *FGFR3* mutaties te gebruiken in de diagnose, prognose en controle van blaaskankerpatiënten. Belangrijker was echter nog om inzicht te verkrijgen in de functionele rol van de mutant receptor in het ontstaan van blaaskanker.

Voor de klinische studies hebben we een gemakkelijke en snelle methode voor het analyseren van *FGFR3* mutaties ontwikkeld. In **Hoofdstuk 2** wordt deze nieuwe techniek beschreven die alle bekende *FGFR3* mutaties kan herkennen en die gevoeliger en minder bewerkelijk is dan voorgaande technieken. De test kan gemakkelijk aangepast worden wanneer er eventueel nieuwe *FGFR3* mutaties worden gevonden. Met behulp van deze techniek hebben we verschillende studies naar de rol van FGFR3 in het ontstaan van blaaskanker en in het ziekteverloop van blaaskankerpatiënten kunnen doen.

Omdat *FGFR3* mutaties in laaggradige tumoren van een laag stadium voorkomen wordt mutatie van het FGFR3 gen beschouwd als een vroege gebeurtenis in het ontstaan van blaaskanker. Dit hebben we onderzocht door hyperplasie van het urotheel, een voorloperlaesie van laaggradige papillaire tumoren, te screenen voor *FGFR3* mutaties, en we vonden dat *FGFR3* mutaties al in deze laesies aanwezig zijn (beschreven in **Hoofdstuk 3**). Vervolgens vonden we ook dat blaastumoren met een *FGFR3* mutatie minder chromosomale afwijkingen hebben (**Hoofdstuk 4**), wat erop wijst dat *FGFR3* gemuteerde tumoren genetisch stabiel zijn, in tegenstelling tot de meeste tumoren die deze mutatie niet hebben. In **Hoofdstuk 5** laten we zien dat deze genetische stabiliteit van *FGFR3* mutant tumoren overeenkomt met een normale differentiatie van de urotheelcellen, aangezien een normaal patroon van CK20 aankleuring, wat een marker is voor terminale differentiatie van urotheel, gecorreleerd is aan blaastumoren met een *FGFR3* mutatie.

In **Hoofdstuk 5** wordt ook de relatie van vier verschillende moleculaire markers (*FGFR3*, CK20, Ki-67, P53) met het ziekteverloop van blaaskankerpatiënten

beschreven. Progressie kwam zelden voor in de subgroep van *FGFR3* gemuteerde tumoren met een normaal CK20 patroon, wat een combinatie van twee moleculaire markers oplevert die in staat is om de groep laaggradige niet-invasieve tumoren met een gunstige prognose te definiëren. Urotheelcelcarcinomen komen niet alleen in de blaas voor, maar ook in de hogere urinewegen (te weten de urineleider en het nierbekken). Deze hogere urinewegtumoren zijn mogelijk genetisch verschillend van blaastumoren. Desondanks was de mutatiefrequentie in het *FGFR3* gen gelijk (~50%) in alle urinewegtumoren, en was *FGFR3* zelfs een onafhankelijke voorspeller voor progressie en overlevingskans in urineleider tumoren (**Hoofdstuk 6**). Daarnaast werd een tweede combinatie van moleculaire markers (te weten *FGFR3* mutatie en *RASSF1A* methylatie) ontdekt die ook in staat was om de groep van niet-invasieve blaaskanker met een laag risico te definiëren: slechts één van de 25 *FGFR3* gemuteerde tumoren zonder RASSF1A methylatie vertoonde progressie.

Het effect dat de mutant FGFR3 receptor heeft op het fenotype van urotheelcellen *in vitro* is nog nooit beschreven, noch is er iets bekend over FGFR3 signaaltransductieroutes in urotheel- of epitheelcellen. In **Hoofdstuk 7** beschrijven we de resultaten van een experimentele studie waarin de expressie van een mutant FGFR3 receptor in een humane blaaskankercellijn onderzocht wordt. Het meest opvallende effect was dat cellen die de mutant receptor tot expressie brengen verlies van integrines vertonen en in apoptose gaan zodra ze gekweekt worden in Matrigel. Dit zou erop kunnen wijzen dat cellen die mutant FGFR3 tot expressie brengen niet kunnen overleven zodra ze contact maken met Matrigel (ofwel met componenten afkomstig van de basaalmembraan). Dit laatste zou ook een verklaring kunnen zijn voor onze klinische bevindingen, waarin blaastumoren met een *FGFR3* mutatie relatief minder geneigd zijn om invasief te worden.

Dit proefschrift beschrijft zowel de klinische als functionele aspecten van FGFR3 activatie in blaaskanker. De resultaten bevestigen de associatie van mutant FGFR3 met goed gedifferentieerde, niet-invasieve blaastumoren met weinig genetische veranderingen en een goede prognose. Daarnaast zijn er een aantal belangrijke vragen opgeroepen omtrent de functie van mutant FGFR3 expressie in blaaskankercellen die verdere aandacht verdienen in toekomstige studies.

CURRICULUM VITAE

Joke van Oers werd op 13 maart 1981 geboren te Roosendaal en Nispen. Van 1992 tot 1996 doorliep zij de eerste vier klassen van het VWO aan het Onze Lieve Vrouwelyceum in Breda, om aansluitend de laatste twee klassen te volgen aan het Sint-Maartenscollege in Voorburg, alwaar zij in 1998 haar gymnasiumdiploma behaalde. Aansluitend begon zij aan een studie Biologie aan de (Rijks) Universiteit Leiden waar zij in augustus 2002 haar doctoraaldiploma behaalde. Als onderdeel van deze studie liep zij stage op de afdeling Klinische Oncologie van het Leids Universitair Medisch Centrum. Onder begeleiding van Ilse Slager verrichte zij daar onderzoek naar de activatie van T-cellen tegen het melanoom antigen CAMEL door gebruik te maken van dendritische cellen getransduceerd met de Ad5F35-CAMEL vector. Op 29 oktober 2002 begon zij haar promotieonderzoek aan de Erasmus Universiteit Rotterdam op de afdeling Pathologie van het Erasmus MC, waarvan de resultaten staan beschreven in dit proefschrift. Het onderzoek naar de activatie van FGFR3 in blaaskanker werd begeleid door Prof.dr. Theo van der Kwast en Dr. Ellen Zwarthoff. Vanaf september 2007 zal zij werkzaam zijn als postdoc op de afdeling Celbiologie van het Albert Einstein College of Medicine, Bronx, New York, in de groep van Prof.dr. Winfried Edelmann.

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-x- Joke