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High-Throughput Tissue Microarray Analysis of 3p25 (RAF1) and 8p12 (FGFR1) Copy Number Alterations in Urinary Bladder Cancer

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ABSTRACT

Studies by comparative genomic hybridization revealed that the chromosomal regions 3p25 and 8p11-p12 are recurrently amplified in bladder cancer. To investigate the prevalence of DNA copy number alterations in these chromosomal regions and study their clinical significance, we used probes for the *RAF1* (3p25) and *FGFR1* (8p12) genes for fluorescence *in situ* hybridization. A tissue microarray containing 2317 tumors was analyzed. The analysis revealed *RAF1* amplification in 4.0% and *FGFR1* amplification in 3.4% of interpretable tumors. In addition, deletions were found at the 3p25 locus in 2.2% and at the 8p11–12 locus in 9.9% of interpretable tumors. Both amplifications and deletions of *RAF1* and *FGFR1* were significantly associated with high tumor grade ($P < 0.0001$), advanced stage ($P < 0.0001$), and poor survival ($P < 0.05$) if tumors of all of the stages where analyzed together. *RAF1* amplifications were associated with subsequent tumor progression in pT1 carcinomas ($P < 0.05$). The marked differences in the frequency of all of the analyzed changes between pTa grade 1/grade 2 and pT1–4 carcinomas support the concept of these tumor groups representing different tumor entities.

INTRODUCTION

Urinary bladder cancer is the fifth most common malignancy in men in Western societies. Progression of bladder cancer is often accompanied by gene amplification, which is suggested to represent a common way of oncogene activation. More than 30 different chromosomal loci have been found highly amplified in bladder cancer including 1p22-p32, 1q21-q24, 2q32, 3pter-p23, 3p11, 3q26, 5p11-p13, 5p15, 5q21, 6p22, 7q21-q31, 7q36, 8p11-p12, 8q21-q22, 8q24, 9p21, 9p24, 10p11-p12, 10p13-p15, 10q22-q23, 10q25, 11q13, 12q13-q15, 12q14-q21, 13q34, 16q21-q22, 17q11-q21, 17q22-q23, 17q24-q25, 18p11, 20q12-q13, 22q11-q13, Xp21, and Xq21 (1–10). Some of these amplification sites contain known oncogenes such as *EGFR* at 7p13, *MYC* at 8q24, *CCND1* at 11q13, *MDM2* and *CDK4* at 12q13–15, or *ERBB2* at 17q21, but the target genes of most amplifications are still unknown. Previous studies (1–4, 6, 10) using CGH[2] have repeatedly revealed amplifications at 3p25 and 8p11-p12 in bladder cancer. These loci harbor the *RAF1* oncogene (3p25) and the *FGFR1* gene (8p11.2-p12), both of which have been suggested to have an oncogenic function (11, 12). Amplification of *RAF1* has been shown previously in 7 of 54 non-small cell lung cancers (13) and 1 of 50 urothelial cancers (14). *FGFR1* amplification was found to correlate with poor outcomes in node-positive breast carcinomas (15). *FGFR1* is known to be expressed in normal urothelium (16). The same loci where *RAF1* and *FGFR1* reside have also been found to be recurrently deleted in urinary bladder cancer (3, 4). In this study, we used FISH on a large TMA to simultaneously investigate gene amplifications and deletions for the *RAF1* and *FGFR1* loci in a large set of well-characterized bladder tumors. In a previous study (17) investigating cyclin E gene alterations, we found that our bladder cancer TMA containing 2317 specimens technology was highly suitable to find associations between rare genetic events and phenotype or clinical outcome in bladder tumors (18).

MATERIALS AND METHODS

Bladder Cancer TMA. The construction and composition of our bladder cancer TMA containing 2317 formalin-fixed paraffin-embedded tissues was described previously (18). An overview of an H&E-stained microarray section is shown in Fig. 1*A*. All of the slides of all of the tumors were reviewed by one pathologist (G. S.). Tumor stage and grade were defined according to the Union Internationale Contre le Cancer and WHO (19, 20). Stage pT1 was defined by the presence of both unequivocal tumor invasion of the suburothelial stroma and tumor-free fragments of the muscular bladder wall. Carcinomas with stroma invasion but absence of muscular bladder wall in the biopsy were classified as at least pT1 (pT1−). Clinical data of 1123 patients were retrospectively evaluated. The medium follow-up period was 42 months (range, 1–236 months). For patients with pTa and pT1 tumors with clinical follow-up information available, time to recurrence and time to progression (to stage pT2 or higher) were selected as study end points. The follow-up information was considered complete enough to include a pTa/pT1 cancer patient in the study if cystoscopies had been performed at least at 3, 9, and 15 months, then annually until the end point of this study (recurrence, last control). To include a patient for analyses of time to progression, longer intervals between controls were accepted if the last follow-up control ruled out progression. Recurrences were defined as cystoscopically visible tumors. Tumor progression was defined as the presence of muscle invasion (stage pT2 or higher) in a subsequent biopsy. An overview of the histological and clinical data are given in Table 1.

FISH. The TMA sections were treated according to the Paraffin Pretreatment Reagent Kit protocol (Vysis, Downers Grove, IL) before hybridization. FISH was performed with Spectrum Orange-labeled *RAF1* and *FGFR1* probes and Spectrum Green-labeled chromosome 3 and 8 centromeric probes as reference (Vysis). Hybridization and posthybridization washes were according to the "LSI procedure" (Vysis). In brief, 4-μm paraffin sections were mounted on superfrost plus microscopic slides and deparaffinized in Hemo-De (Fisher #15-152-507A). Slides were dehydrated in 100% ethanol and pretreated with pretreatment solution (Vysis; 30 min at 80°C) and protease digestion (10 min at 37°C). After applying the probes, slides and probes were denatured simultaneously (72°C for 5 min) and hybridized overnight at 37°C in a moist chamber. Posthybridization washes were carried out in 2 × SSC for 2 min at 72°C. Slides were then counterstained with 125 ng/ml 4′,6-diamino-2-phenylindole in an antifade solution.

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[2] The abbreviations used are: CGH, comparative genomic hybridization; FGFR1, fibroblast growth factor receptor 1; FISH, fluorescence *in situ* hybridization; TMA, tissue microarray.

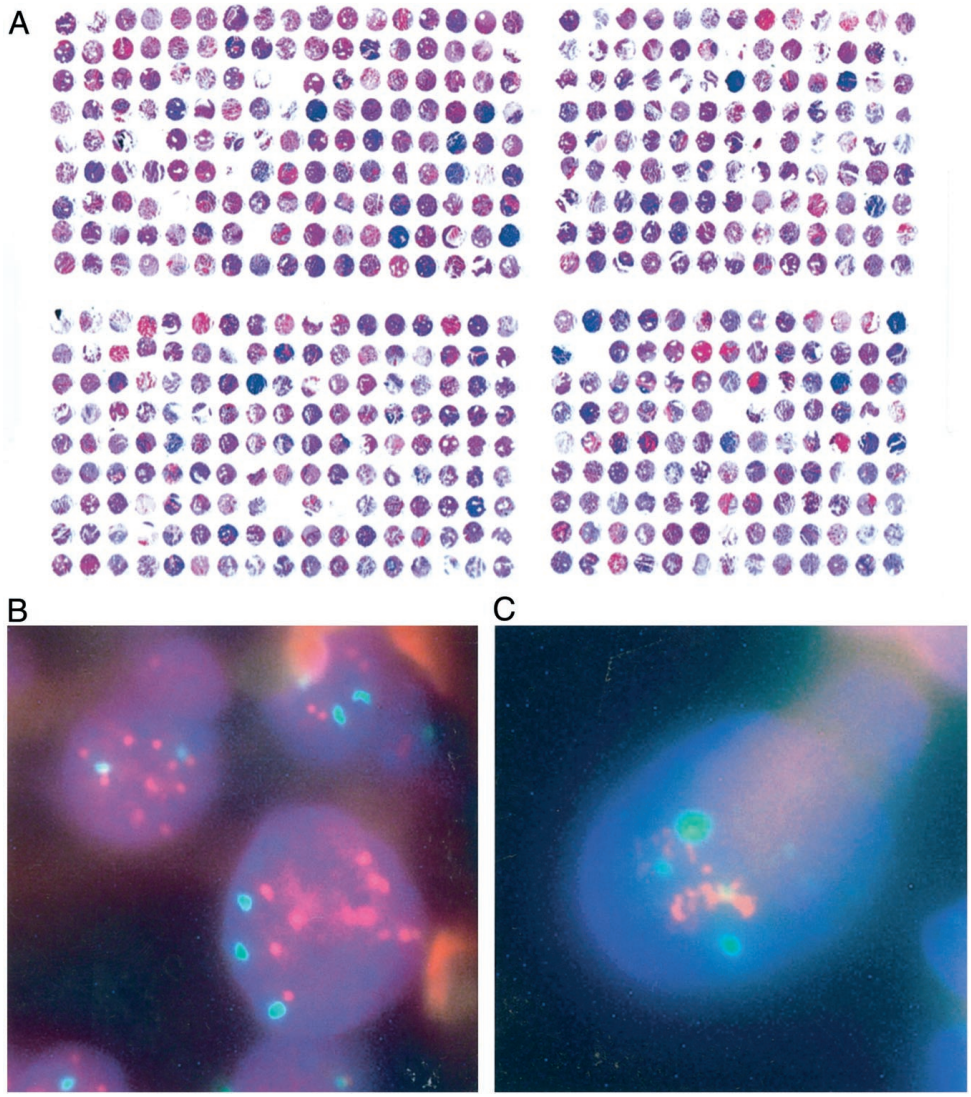

Fig. 1. Bladder cancer TMA. *A*, overview (H&E; 4.5-fold magnification) of one of the five array blocks containing 540 samples. *B*, *RAF1* amplification. Amplified cells contain 10–20 *RAF1* signals (*red*) and three centromere 3 signals (*green*) each. *C*, *FGFR1* amplification. These cells contain 20–30 *FGFR1* signals (*red*) and three centromere 8 signals (*green*) each.

Scoring of FISH Signals. For rapid evaluation of the array, signal numbers were estimated for each tissue spot, applying predefined criteria. Amplification was defined as presence of at least three times more gene signals than centromere signals in at least 10% of nuclei as described previously (18, 21). A deletion was assumed if at least 50% of cells had fewer gene signals than centromere signals. A clearly higher threshold (50%) was selected for deletions than for amplification because some artificially deleted cells are always found in tissue sections because of truncation of nuclei and in case of inefficient hybridization. High threshold levels can be used on TMAs because very little heterogeneity is usually seen on small arrayed tissue samples usually showing amplification or deletion in all or none of the cells.[3] All of the tumors not meeting these criteria were considered normal.

Immunohistochemistry. Immunohistochemistry was performed for FGFR1 only, because a RAF1-specific antibody for formalin-fixed tissues was not available. A small TMA containing 15 tissue samples that did show high-level *FGFR1* amplification by FISH and an additional 15 samples with normal *FGFR1* copy number was constructed. Sections of this array were used to immunostain with anti-FGFR1 (Research Diagnostics, NJ) at dilutions of 1:800, 1:1600, 1:3200, and 1:6400.

Statistics. Only the first biopsy was used for additional statistical analyses on patients having more than one tumor in the array. Contingency table analysis and χ^2 tests were used to study the relationship between histological tumor type, grade, stage, and *RAF1* or *FGFR1* amplification. Survival curves were plotted according to Kaplan-Meier (22). A log rank test was applied to examine the relationship between grade, stage, or *RAF1*- and *FGFR1*-alterations and clinical outcome. For analysis of recurrence or progression, patients were censored at the time of their last clinical control showing no evidence of disease or at the date when cystectomy was performed. For survival analysis, patients were censored at the time of their last clinical control or at the time of death if they died from causes not related to their tumor. Twenty-five patients with an unclear cause of death were excluded from survival analysis.

RESULTS

***RAF1* and *FGFR1* Gene Copy Number.** FISH analysis was successful in 1494 of 2317 (64.5%) arrayed tumors for *RAF1* and in 1515 of 2317 (65.4%) tumors for *FGFR1*. FISH-related problems (weak hybridization, background, and tissue damage) were responsible for about two-thirds of the noninformative cases, whereas the rest of the reasons for analysis failure were attributable to the TMA technology, such as missing samples or too few tumor cells in a tissue spot. Amplifications were seen for *RAF1* in 48 of 1194 (4.0%) tumors and for *FGFR1* in 41 of 1221 (3.4%) tumors (only first biopsies of patients having more than one tumor on the array were enclosed). Examples of *RAF1*- and *FGFR1*-amplified cells are shown in Fig. 1, *B* and *C*. In addition, deletions were found at the *RAF1* locus in 2.2% and at the *FGFR1* locus in 9.9% of the tumors. The relationship of *RAF1* and *FGFR1* amplifications and deletions with the tumor phenotype is given in Table 2. Both amplifications and deletions of *RAF1* and

[3] Unpublished observations.

Table 1 *Histological and clinical parameters of 2317 arrayed bladder cancer samples*

		Tumors $n = 2317$	Patients $n = 1853$	Patients with clinical data $n = 1122$
Stage	pTa	951	768	502
	pT1	515	425	263
	pT1−	101	80	34
	pT2–4	737	571	319
Grade	G1	282	230	157
	G2	987	792	467
	G3	1048	831	498
Stage/grade	pTaG1	277	226	155
	pTaG2	567	461	291
	pTaG3	107	81	56
	pT1G2	206	170	98
	pT1G3	309	255	165
	pT2–4G2	186	140	69
	pT2–4G3	551	431	250
Histology	Transitional cell carcinoma	2108	1678	1031
	Squamous cell carcinoma	73	59	34
	Small cell carcinoma	31	25	12
	Adenocarcinoma	22	17	8
	Adenosquamous carcinoma	2	2	1
	Sarcomatoid carcinoma	24	17	8
Growth pattern	Papillary	1665	1367	867
	Solid	633	472	249
No. of tumors/patient	One	1533	1533	914
	Two or more	784	320	208
Clinical endpoints	Tumor-specific survival (pT2–4)			319
	Time to progression (pTa and pT1)			482
	Time to recurrence (pTa and pT1)			535

FGFR1 were significantly associated with high tumor grade and advanced stage ($P < 0.0001$). *RAF1* amplifications and *FGFR1* deletions were also significantly more frequent in noninvasive high-grade carcinomas (pTa grade 3) than in noninvasive low-grade carcinomas (pTa grade 1 and 2; $P \leq 0.0005$ each; Fig. 2). There was no association between amplifications of *RAF1* and *FGFR1*.

FGFR1 Expression. FGFR1 immunostaining at deletions of <1: 1600 always showed a positive staining in all of the 30 examined tumors. Differences in FGFR1 immunostaining intensity were best seen at a dilution of 1:3200, showing presence or absence of FGFR1 expression in arrayed samples rather than quantitative differences. There was no association between presence of FGFR1 immunostaining and *FGFR1* gene amplification. Eleven of 15 amplified tumors and 13 of 15 nonamplified tumors were FGFR1 immunohistochemistry positive. At the same concentration, a positive staining was also seen in normal urothelium.

Prognostic Relevance of *RAF1* and *FGFR1*. Copy number changes of *RAF1* and *FGFR1* were examined for their prognostic significance regarding the clinically most relevant end points. These included tumor-specific survival in all of the patients, tumor specific survival in the subgroup of muscle invasive carcinomas, tumor progression in pT1 carcinomas, and recurrences in pTa tumors. Additional subgroup analyses were not done because of the small total number of amplifications and deletions. *RAF1* amplifications were

Table 2 *RAF1 and FGFR1 alterations and tumor phenotype*

	RAF1					*FGFR1*				
	All	Amplified	*P*	Deleted	*P*	All	Amplified	*P*	Deleted	*P*
Tumor samples successfully analyzed[a]	1194	4.0%		2.2%		1221	3.4%		9.9%	
Histology[b]										
Transitional cell carcinoma	250	8.0%		6.4%		250	6.0%		22.0%	
Squamous cell carcinoma	26	0.0%		0.0%		28	10.7%		10.7%	
Small cell carcinoma	13	0.0%		15.4%	0.21[c]	13	7.7%		0.0%	
Sarcomatoid carcinoma	10	0.0%		0.0%		9	0.0%		22.2%	
Adenocarcinoma	5	0.0%		0.0%		4	25.0%		0.0%	
Stage[d]										
pTa	532	0.6%		0.0%		550	1.5%		3.5%	
pT1	275	7.3%	<0.0001[e]	0.4%		281	3.2%	<0.0001[e]	10.3%	<0.0001[e]
pT1−	47	10.6%		10.6%	<0.0001[f]	46	6.5%		21.7%	
pT2–4	250	8.0%		6.4%	<0.0001[g]	250	6.0%		22.0%	
Grade[c]										
G1	159	0.0%		0.0%		152	0.7%		0.7%	
G2	509	1.6%	<0.0001[h]	0.8%	<0.0001[h]	541	1.5%	<0.0001[h]	5.5%	<0.0001[h]
G3	440	9.1%		4.1%		439	6.2%		18.7%	
Growth pattern[b,c]										
Papillary	101	5.9%		3.0%		96	6.3%		20.8%	
Solid	146	8.9%	0.3311[i]	8.9%	0.0577[i]	152	5.9%	0.9039[i]	23.0%	0.7861[i]

[a] Only first biopsies.
[b] Only pT2–4.
[c] Small cell *versus* transitional cell cancer.
[d] Only transitional cell carcinomas.
[e] pTa *versus* pT1.
[f] pT1 *versus* pT1−.
[g] pT1 *versus* pT2–4.
[h] G1 *versus* G2 *versus* G3.
[i] Papillary *versus* solid.

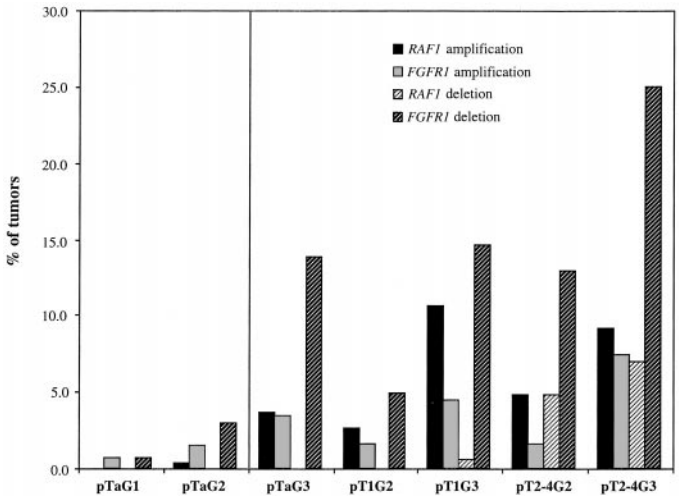

Fig. 2. Frequency of amplifications and deletions of *RAF1* and *FGFR1* in bladder cancer samples of different stage and grade.

associated with short tumor-specific survival if all of the patients were analyzed ($P = 0.0022$; Fig. 3*A*) but not in a separate analysis of patients with pT2–4 tumors ($P = 0.95$; Fig. 3*B*). *RAF1* amplification was associated with tumor progression in the combined groups of pTa and pT1 carcinomas tumors ($P < 0.0001$; Fig. 3*C*) and also in the subgroup of pT1 tumors ($P = 0.0412$; Fig. 3*D*). *RAF1* amplification was not significantly associated with recurrence in pTa tumors ($P = 0.07$). The prognostic significance of 3p25 (*RAF1*) deletions was not analyzed because of the low frequency of this alteration.

FGFR1 amplification was linked to poor tumor-specific survival if all of the patients were included in the analyses ($P = 0.0427$; Fig. 3*E*) but not in a separate analysis of patients with pT2–4 tumors ($P = 0.5$; Fig. 3*F*). *FGFR1* amplification was not significantly linked to prognosis in pTa or pT1 tumors. *FGFR1* deletions (8p12) were strongly linked to poor tumor-specific survival in all of the patients ($P = 0.0006$; Fig. 3*E*) but not in the subgroup of muscle invasive carcinomas ($P = 0.47$; Fig. 3*F*). Also, *FGFR1* deletions were not significantly associated with tumor recurrence in pTa tumors ($P = 0.057$) or tumor progression in pT1 carcinomas ($P = 0.48$).

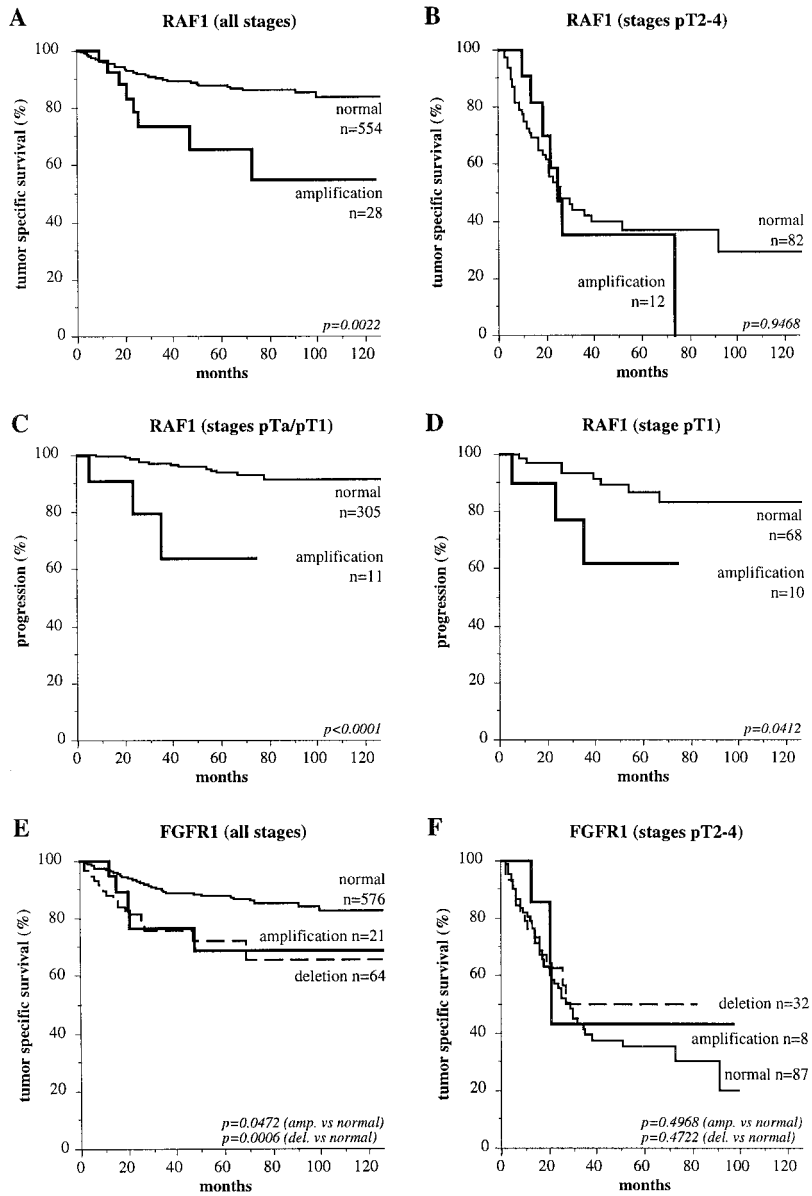

Fig. 3. Prognostic relevance of *RAF1* and *FGFR1* alterations. *RAF1* amplification and survival in all of the tumors (*A*) and in pT2–4 carcinomas (*B*). *RAF1* amplification and tumor progression in pTa/pT1 tumors (*C*) and in pT1 carcinomas (*D*). *FGFR1* alterations (deletion, amplification) and survival in all of the tumors (*E*) and in pT2–4 carcinomas (*F*).

DISCUSSION

In this study, we applied our recently developed TMA technology to evaluate the significance of copy number changes of 3p25 (*RAF1*) and 8p11–12 (*FGFR1*) in urinary bladder cancer. The data show that both amplifications of *RAF1* and *FGFR1* and deletions of these loci are associated with advanced bladder cancer.

Amplifications and deletions of both genes and loci were strikingly more frequent in invasively growing bladder carcinomas (pT1–4) and in pTaG3 tumors than in low-grade papillary bladder tumors (pTaG1/G2). The association of all of the examined structural chromosomal aberrations with invasive or high-grade tumor phenotype is consistent with previous observations (2, 4) suggesting major genetic differences between noninvasive and invasive tumors. In general, pTaG1/G2 carcinomas are thought to represent genetically stable tumors that usually accumulate few cytogenetic changes only rarely including high level gene amplifications. In contrast, invasively growing bladder neoplasms (pT1–4) are genetically unstable, often characterized by a high number of cytogenetic alterations. The similar high number of gene amplifications and deletions in pTaG3 tumors as in invasive cancers is consistent with the hypothesis that these tumors are genetically related to the group of invasively growing carcinomas (10), potentially representing one of their precursor lesions. Given the known survival differences between the pTa/pT1 tumors and muscle invasive carcinomas, it is not surprising that all of the molecular changes analyzed in this study were linked to poor prognosis if tumors of all of the stages were simultaneously analyzed. Analysis of tumors of identical stages did not reveal a prognostic relevance of genomic alterations, with the only exception of an association of *RAF1* amplifications with progression in pT1 tumors. Interestingly, gains of 3p were also linked to subsequent tumor progression in pT1 carcinomas in a previous CGH study (6). Taken together, these findings raise the possibility that activation of *RAF1* or one or several other genes in the 3p25 region may play a role in bladder cancer progression.

It is important to realize that the occasional presence of DNA amplification does not provide absolute proof of an involvement of these genes in bladder cancer biology because overexpression could not be linked to amplification. For RAF1, no immunohistochemical examination could be done because of the lack of a suitable antibody. The immunohistochemical analysis for FGFR1 confirmed the known abundant expression of FGFR1 in urothelium (16), but a dilution series failed to reveal an expression difference between amplified and nonamplified tumors. This suggests that FGFR1 is generally expressed in normal urothelium and in bladder cancer and that gene amplification may not lead to a significant further increase of the expression level. Therefore, it is possible that cells showing *FGFR1* amplification in our analysis obtain a growth advantage through coamplification and consecutive overexpression of a neighboring gene rather then by *FGFR1* activation. It is well known that amplified DNA sequences often contain multiple genes, only some of which are clearly overexpressed in amplified tumors (23–26).

The high prevalence of 3p25 and 8p11–12 deletions further emphasizes that alterations on the DNA level are not equivalent to a relevant functional alteration of the involved genes. On the basis of their known function, it is highly unlikely that *RAF1* and *FGFR1* are deletion target genes, of which underexpression or inactivation provides a growth advantage to affected cells. It is much more likely that *RAF1* and *FGFR1* were included by chance in large 8p and 3p deletions that were detected in our FISH analysis. Deletions of both 3p and 8p have been described previously (1–6, 8, 10) in bladder cancer. Whereas 3p25 (*RAF1*) deletions were seen in only 6.4% of the invasively growing transitional cell carcinomas, this alteration was observed in 2 of 13 (15.4%) of the small cell carcinomas of the bladder. This is consistent with the results of previous CGH studies finding 3p deletions in 3 of 10 small cell carcinomas (1) but in only 2.2% of invasive transitional cell carcinomas (3). Because 3p deletions are also common in small cell lung cancer (27–29), it was suggested that this region could contain a gene the alterations of which are relevant to small cell carcinomas of different sites of origin (1). 8p12 deletions were observed in 22% of our muscle invasive transitional cell carcinomas. This was clearly less than in previous studies where 8p deletions were seen in 22–45% by loss of heterozygosity analyses (30–32) or in 54% by FISH analysis (33). Most likely, this discrepancy was at least partly attributable to the proximal location of the *FGFR1* gene at 8p12. In our previous FISH study (33), we had found only about half as many deletions at 8p12 than at 8p22, suggesting that the putative 8p tumor suppressor gene might be located distally of 8p12.

This study provides another example on how efficiently molecular data can be collected on TMAs derived from large sets of well-characterized tumors. Although amplifications of both examined loci were infrequent, associations could be established with tumor phenotype and prognosis because of the analysis of more than 1000 different bladder tumors. The possibility to miniaturize tissue analyses will substantially facilitate translational and clinical cancer research. Most of all, the TMA format greatly increases the number of targets that can be analyzed from one set of tumors. Tens of thousands of TMA sections can be generated from paraffin blocks containing a 10 × 10-mm tumor area. This is dramatically more than could be accomplished using traditional techniques of sectioning entire tumor blocks, where only about 200 sections can be generated before the blocks are exhausted. The TMA format will be the only way to analyze thousands of molecular targets from one set of hundreds or thousands of tissue specimens. TMAs would theoretically make it possible to analyze the expression of all of the human genes in one set of well-characterized tissue specimens.

In summary, the association of 3p25 and 8p11–12 amplifications/deletions with tumor phenotype provides further evidence for major genetic differences between pTaG1/G2 and invasively growing tumors (pT1–4). Although the examined genes may not be the actual target of amplifications and/or deletions at these loci, they may serve as markers for a chromosomal region that has importance in bladder cancer.

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