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E2F3 amplification and overexpression is associated with invasive tumor growth and rapid tumor cell proliferation in urinary bladder cancer

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E2F3 is located in the 6p22 bladder amplicon and encodes a transcription factor important for cell cycle regulation and DNA replication. To further investigate the role of E2F3 in bladder cancer, a tissue microarray containing samples from 2317 bladder tumors was used for gene copy number and expression analysis by means of fluorescence *in situ* hybridization (FISH) and immunohistochemistry (IHC). E2F3 amplification was strongly associated with invasive tumor phenotype and high tumor grade ($P<0.0001$ each). None of 272 pTaG1/G2 tumors, but 35 of 311 pT1-4 carcinomas (11.3%), had E2F3 amplification. A high E2F3 expression level was associated with high grade, advanced stage, and E2F3 gene amplification ($P<0.0001$ each). To evaluate whether E2F3 expression correlates with tumor proliferation, the Ki67 labeling index (LI) was analysed for each tumor. There was a strong association between a high Ki67 LI and E2F3 expression ($P<0.0001$), which was independent of grade and stage. We conclude that E2F3 is frequently amplified and overexpressed in invasively growing bladder cancer (stage pT1-4). E2F3 expression appears to provide a growth advantage to tumor cells by activating cell proliferation in a subset of bladder tumors.
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Introduction

Gene amplification plays an important role in the progression of bladder cancer. More than 30 different genomic loci have been identified that recurrently harbor DNA amplifications (Mitelman, 1994; Kallioniemi *et al*., 1995; Voorter *et al*., 1995; Richter *et al*., 1997, 1998; Simon *et al*., 1998; Simon *et al*., 2000). Several of these amplifications contain known oncogenes such as HER2 at 17q21, CCND1 at 11q13, MYC at 8q24, EGFR at 7p13, or MDM2 and CDK4 at 12q13-15 (Dalla-Favera *et al*., 1982; Kondo and Shimizu, 1983; Popescu *et al*., 1989; Xiong *et al*., 1992; Reifenberger *et al*., 1994). The target genes are unknown for the majority of amplicons, such as 1q21-31, 2q13, 3p22-24, 6p22, 8p11, 8q21, 9p21, 10p13-14, 13q13, 13q31-33, 18p11, 20q, 21p11, 22q11-13, Xp11-13, and Xq21-22.2 (Kallioniemi *et al*., 1992; Mitelman, 1994; Sauter *et al*., 1994; Kallioniemi *et al*., 1995; Voorter *et al*., 1995; Richter *et al*., 1997, 1998; Simon *et al*., 1998; Richter *et al*., 1999; Terracciano *et al*., 1999; Zhao *et al*., 1999).

Based on our comparative genomic hybridization (CGH) data of more than 300 bladder carcinomas (Richter *et al*., 1997, 1998; Simon *et al*., 1998; Richter *et al*., 1999; Terracciano *et al*., 1999; Zhao *et al*., 1999; Simon *et al*., 2000), one of the most common sites of high-level amplification is 6p22, which was present in 10 of 172 advanced-stage tumors of our patients. One of the potential candidate genes at the 6p22 region is E2F3 (Veltman *et al*., 2003), which belongs to a family of cell cycle regulatory transcription factors that are controlled by the retinoblastoma tumor suppressor (Lees *et al*., 1993; Leone *et al*., 1998; Humbert *et al*., 2000; Leone *et al*., 2001; Wu *et al*., 2001). Heterodimers of E2F1, E2F2, or E2F3 with DP1 serve as transcriptional activators of genes that promote cell cycling, whereas complexes of E2F/DP with pRb repress transcription and inhibit cell growth (Leone *et al*., 1998; Nevins, 1998; Trimarchi and Lees, 2002). Recent reports have indicated that different members of the E2F gene family could play specific and diverse roles in tumorigenesis of various human malignancies. For example, increased copy numbers and overexpression of E2F1 were found in an erythroleukemia cell line (Saito *et al*., 1995), and E2F5 was amplified and upregulated in 4.2% of breast cancers (Polanowska *et al*., 2000). Decreased expression of E2F1 was to be associated with an increased risk of progression to metastases in bladder cancer (Rabbani *et al*., 1999).

Whereas almost all research on E2F3 has been performed in cell line or mice models so far, little is known about the potential role of E2F3 in human cancers. Based on the rate-limiting role of E2F3 for cell proliferation (Humbert *et al*., 2000), it is possible that a

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disturbed regulation of E2F3 could facilitate cell cycle progression and an increased cell proliferation rate. In turn, overexpression of E2F3 could provide a growth advantage to cells exhibiting this alteration, resulting in clonal selection explaining the presence of 6p22 amplifications in advanced-stage tumors. In this study, we applied the tissue microarray (TMA) technology (Kononen *et al*., 1998) to study E2F3 alterations in urinary bladder cancer. A TMA containing 2317 bladder cancers was used to examine the impact of E2F3 gene copy number changes on the protein expression level, tumor phenotype, and clinical outcome.

Results

Technical aspects

Immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) analyses were performed in a blinded way on different TMA sections. Owing to technical reasons, the number of interpretable samples varies between the individual analyses and comparisons.

E2F3 gene amplification

FISH analyses were performed in two steps: prescreening and establishing optimal hybridization conditions was carried out on a mini-TMA containing specimens with 6p22 amplification (as identified by CGH), followed by a large-scale TMA analysis of 2317 clinical specimens. The 6p22-amplification-specific mini-TMA revealed E2F3 amplification in all three cell lines and seven primary tumors that had shown 6p22 amplification by CGH. The average E2F3 copy numbers in these tumors ranged between 7 and 31.

FISH was successful in 875 of 2317 (38%) samples of the large TMA. FISH-related problems (weak hybridization, background, tissue damage) were responsible for about 80%, TMA-linked problems (too few or absence of tumor cells on the TMA spot) were causing about 20% of the noninformative cases. Statistical analysis was limited to 696 tumors, representing the initial biopsies of 696 patients. E2F3 amplification was detected in 49 these 696 tumors. Examples of amplified and nonamplified tumors are shown in Figure 1. The associations with tumor phenotype are summarized in Table 1. Within urothelial carcinoma, which is by far the most common bladder cancer subtype, E2F3 amplification was strongly associated with high tumor grade and advanced stage ($P<0.0001$ each). Most strikingly, E2F3 amplification was absent in pTaG1/G2 tumors (0 of 272), while 11.3% (35 of 311) of the invasively growing urothelial carcinoma (pT1-4) had E2F3 amplification. E2F3 amplification was most frequent in the histological subgroups of muscle invasive urothelial carcinoma (14.3%) and small cell carcinomas (16.7%).

E2F3 expression in bladder tumor cell lines

To estimate the influence of E2F3 amplification on protein expression, Western blot analysis was performed on three amplified (CRL1479, HTB5, HTB9) and two nonamplified bladder tumor cell lines (CRL7882, RT112). All three amplified, but none of the nonamplified cell lines, showed strong E2F3 protein expression (Figure 2).

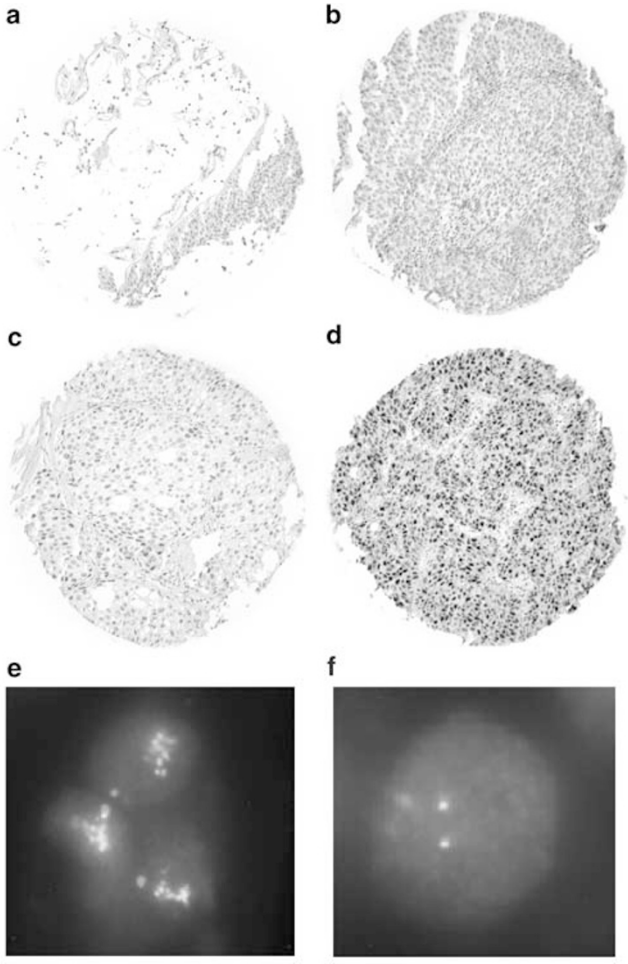

Figure 1 E2F3 alterations in bladder cancer. (**a**) Normal urothelium appeared negative for E2F3 IHC under the selected experimental conditions. (**b–d**) Bladder cancer tissue spots showing different levels of immunostaining. (**d**) Tissue spot of an E2F3-amplified cancer sample showing strong E2F3 expression. All tissue spots (**a–d**) are located on the same TMA slide and have been subjected to identical experimental conditions. (**e**) E2F3 gene amplification as detected by FISH analysis. Red fluorescence signals represent centromere 6 copies, massive increase of green E2F3 signals indicate gene amplification. (**f**) Tumor cell nuclei with two E2F3 and centromere 6 copy numbers, representing the normal copy number state

E2F3 IHC on tumor microarrays

E2F3 IHC was analysed within 1334 first biopsy tumor tissue spots on the bladder cancer TMA. Nearly one-fifth of the tumors exhibited varying degrees of positive staining. No E2F3 staining was observed in normal urothelium (Figure 1a). Examples of E2F3-positive and -negative tumors are shown in Figure 1b–d. E2F3 expression by IHC was significantly linked to E2F3 copy

Table 1 E2F3 alterations and tumor phenotype in urinary bladder cancer

	E2F3 FISH			*E2F3 IHC*		
	n[a]	*Amplified (%)*	P[b]	n[a]	*Positive (%)*	P[b]
All tumors	696	7.0		1334	17.3	
Histology[c]						
Urothelial carcinoma	140	14.3		337	21.1	
Squamous cell carcinoma	14	7.1		38	18.4	
Small cell carcinoma	12	16.7		18	55.6	
Sarcomatoid carcinoma	6	0.0		10	0.0	
Adenosquamous carcinoma	1	0.0		1	0.0	
Stage[d]						
pTa	301	0.3		539	11.1	
pT1	171	8.8	*P<0.0001*[e]	271	20.7	*P<0.0001*[e]
pT1-	27	18.5		46	26.1	
pT2–4	140	14.3		337	21.1	
Grade[d]						
G1	73	0.0		163	8.0	
G2	315	1.3	*P<0.0001*	527	10.8	*P<0.0001*
G3	254	14.6		507	26.0	
Stage and grade[d]						
pTa G1	73	0.0		162	8.0	
pTa G2	199	0.0		330	10.9	
pTa G3	29	3.4		47	23.4	
pT1 G2	70	1.4		107	9.3	*P<0.0001*
pT1 G3	101	13.9	*P<0.0001*	164	28.0	
pT2–4 G2	35	5.7		79	8.9	
pT2–4 G3	105	17.1		258	24.8	
Growth pattern[c,d]						
Papillary	58	13.8	*0.4292*	126	20.6	*0.6486*
Solid	81	14.8		210	21.4	

[a]Number of tumors with interpretable results (only first biopsies are considered). [b]Chi-square test. [c]Only pT2-4. [d]Only urothelial ca. [e]pT1-excluded

number changes ($P<0.0001$). E2F3 positivity was found in 24 of 34 amplified urothelial carcinomas (70.6%), but in only 82 of 456 (18.0%) nonamplified tumors. This association held also true within the group of 269 pT1-4 urothelial carcinomas that could be analysed by both FISH and IHC ($P<0.0001$). E2F3 positivity was significantly more frequent in small cell carcinomas (55.6% positive) than in other histologic subtypes (Table 1). Within urothelial carcinomas, E2F3 expression was linked to advanced stage and high grade ($P<0.0001$ each). The frequency of E2F3-positive tumors increased from 10% in pTaG1/G2 tumors (49 of 492) to 20.9% in invasively growing pT1-4 urothelial carcinomas (127 of 608).

E2F3 expression and tumor cell proliferation (Ki67 labeling index (LI))

Both gene amplification and protein overexpression were significantly associated with rapid tumor cell proliferation ($P<0.0001$ each). Analysis of variance (ANOVA) analysis including E2F3 expression/amplification and either tumor stage or grade showed that both E2F3 expression and amplification were independent predictors of rapid tumor cell proliferation ($P<0.0001$ each). Accordingly, the separate analyses of tumors of identical grades and stages lead to either significant differences in the proliferation between E2F3-negative and -positive tumors or at least to a clear tendency towards a higher Ki67 LI in E2F3-positive tumors (Table 2).

E2F3 alterations and prognosis

E2F3 expression was associated with poor tumor-specific survival if all patients were included in the analysis ($P<0.05$,. Figure 3). There was no association between E2F3 expression and tumor-specific survival within the subgroup of pT2-4 urothelial carcinomas, or between E2F3 alterations and tumor recurrence or tumor progression among pTa/pT1 tumors. There were too few E2F3-amplified tumors with available survival data to allow statistically meaningful analysis.

Discussion

Previous CGH studies have repeatedly highlighted 6p22 as an amplification site in bladder cancer (Richter *et al*., 1998, 1999). E2F3, a key gene for G1/S transition (Leone *et al*., 1998), has been mapped to 6p22. Studies by array CGH have suggested that E2F3 can be

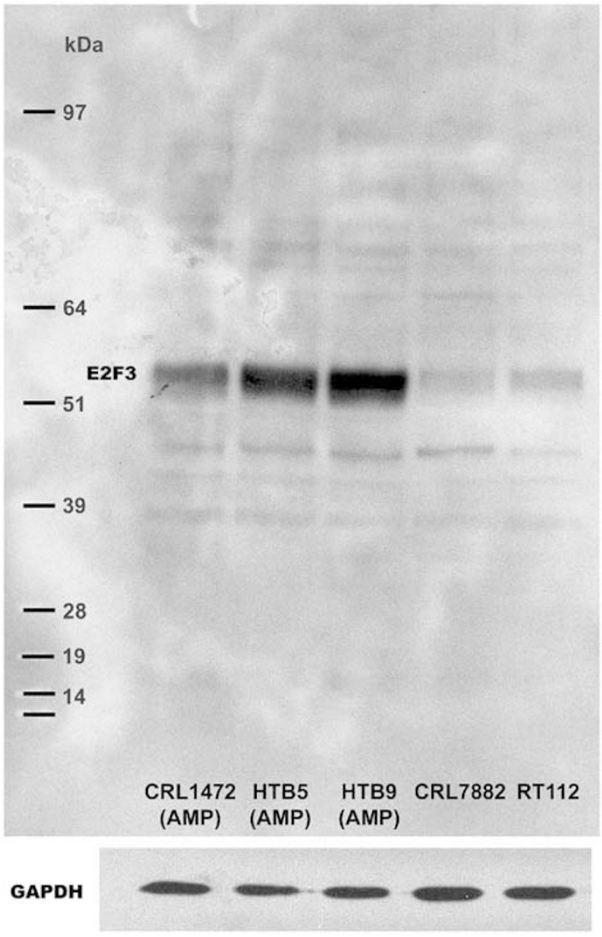

Figure 2 Western blot analysis of E2F3 in 6p22-amplified bladder cancer cell lines (CRL1472, HTB5, HTB9) and in nonamplified cell lines (CRL7882, RT112). Amplified cell lines show a massive increase of E2F3 protein expression as compared to nonamplified bladder cancer cell lines. Weak nonspecific bands are seen in both amplified and nonamplified cell lines

included in the 6p22 amplicon in at least a fraction of bladder tumors (Veltman *et al*., 2003). In an attempt to further investigate the importance of E2F3 in primary bladder tumors and in bladder cancer cell lines, we first analysed a mini-TMA composed of 10 tumors with known 6p22 amplification (by CGH). Our finding of an involvement of E2F3 in the 6p22 amplicons of all 10 amplified tumors provided strong evidence for a major role of E2F3 in bladder cancer. Western blot analysis targeting the E2F3 protein showed strong E2F3 overexpression in all amplified cell lines demonstrating a functional relevance of E2F3 amplification. Based on these data, we proceeded to analyse the prevalence and significance of E2F3 amplification/expression in our previously constructed bladder cancer TMA-containing tissues from 2317 different tumors (Richter *et al*., 2000).

The TMA approach allowed the rapid analysis of more than 800 carcinomas on the DNA and protein level. The findings suggest an important role of E2F3 amplification/overexpression in invasive bladder cancer. A total of 14.3% of muscle invasive bladder cancers showed E2F3 amplification. This makes E2F3 one of the most frequently amplified genes in invasive bladder cancer. Using the same methodological criteria, including FISH protocols, copy number cutoff levels, and TMA resources, we have observed similar frequencies for amplifications of HER2 (14%) (Simon *et al*., 2003) and CCND1 (15%) (Zaharieva *et al*., 2003) in pT2-4 bladder cancer. Remarkably, E2F3 amplification was not found in any of 272 pTaG1/G2 tumors, which is consistent with models suggesting that pTaG1/G2 tumors are genetically stable neoplasias with a much lower likelihood to acquire chromosomal rearrangements than invasively growing tumors (Richter *et al*., 1997, 1998; Simon *et al*., 1998; Richter *et al*., 1999; Zhao *et al*., 1999; Simon *et al*., 2001, 2002). Recently, chromosomal alterations have been successfully used for the detection of bladder cancer in voided urine cells (Halling *et al*., 2000; Bubendorf *et al*., 2001) and a commercial FISH test has been approved by the US Food and Drug Administration in 2001. Our data raise the possibility that 6p22 amplification detection could have clinical utility for distinction of invasive and noninvasive bladder tumors in urine cells.

Given the well-known function of E2F3 for S-phase induction (Leone *et al*., 1998), it could be expected that E2F3 overexpression exerts an oncogenic function through cell cycle activation. This hypothesis is largely supported by the strong association of E2F3 amplification and expression with tumor cell proliferation (Ki67 LI), which was also found in all large subgroups of tumors with identical grade and stage. Despite this strong link between E2F3 positivity and a high Ki67 LI, no association was found between E2F3 overexpression and prognosis if tumors with comparable tumor stages were examined. This result is not completely unexpected. Although there are studies suggesting a prognostic role of tumor cell proliferation in urinary bladder cancer (Lipponen *et al*., 1993; Liukkonen *et al*., 1999), there are also reports that fail to reproduce these data (Nakopoulou *et al*., 1998; Pfister *et al*., 1998). Our own previous analyses on the same set of carefully staged tumors were unable to show a convincing association of Ki67 LI with prognosis in pTa, pT1, or pT2-4 cancers (Nocito *et al*., 2001). Although accelerated cell proliferation is an important prerequisite for tumor growth, it is probably other factors, like invasive growth and the metastatic potential, which are the key determinants for the clinical outcome of neoplastic diseases.

The comparison of E2F3 FISH and IHC data revealed a good but not a perfect correlation. A strong E2F3 expression by IHC was seen in 70% of E2F3-amplified, but in only 18% of nonamplified tumors. These data seem to suggest that E2F3 overexpression is not present in all amplified tumor samples. However, at least some of the discrepant results might be caused by technical reasons. For example, none of the currently commercially available antibodies targeting E2F3 (including that one used in our study) is specifically recommended for IHC on formalin-fixed tissues. It is possible that technical shortcomings of our IHC procedure resulted in a fraction of IHC false-negative

Table 2 E2F3 amplification/overexpression in relation to Ki-67 LI

	E2F3 FISH			*E2F3 IHC*		
	Nonamplified	*Amplified*	P[a]	*Negative*	*Positive*	P[a]
pTaG1	73[b] 5.7[c] (4.5–6.6)[d]	0		147 6.3 (5.4–7.2)	13 7.4 (4.4–10.3)	0.4929
pTaG2	199 10.1 (9.0–11.2)	0		294 10.2 (9.2–11.1)	36 13.8 (11–16.6)	0.015
pTaG3	28 22.0 (17.2–26.8)	1 35.3 (9.9–60.6)	0.2984	36 22.8 (18.4–27.2)	11 24.2 (16.3–32.1)	0.7512
pT1G2	69 19.1 (16.2–22.0)	1 14.9 (−9.2 to 38.8)	0.7252	96 19.1 (16.7–21.5)	10 24.7 (17.3–32.1)	0.1567
pT1G3	86 28.9 (25.6–32.2)	14 45.9 (37.7–54.1)	0.0002	118 28.8 (26–31.6)	46 37.6 (33.1–42.1)	0.0012
pT2G2	33 21.4 (17.0–25.8)	2 36.5 (18.7–54.2)	0.1019	72 21.9 (18.9–24.8)	7 35.9 (26.5–45.4)	0.0059
pT2G3	87 35.0 (31.4–38.5)	18 44.4 (36.6–52.2)	0.0305	192 31.4 (29.1–33.7)	63 44.1 (40.2–48.1)	<0.0001

Only first biopsies of patients with TCC ($n = 1853$) included. Nonamplified cases include gains. [a]Student's *t*-test. [b]Mean Ki-67 LI. [c]95% CI of Ki-67 LI. [d]Number of samples

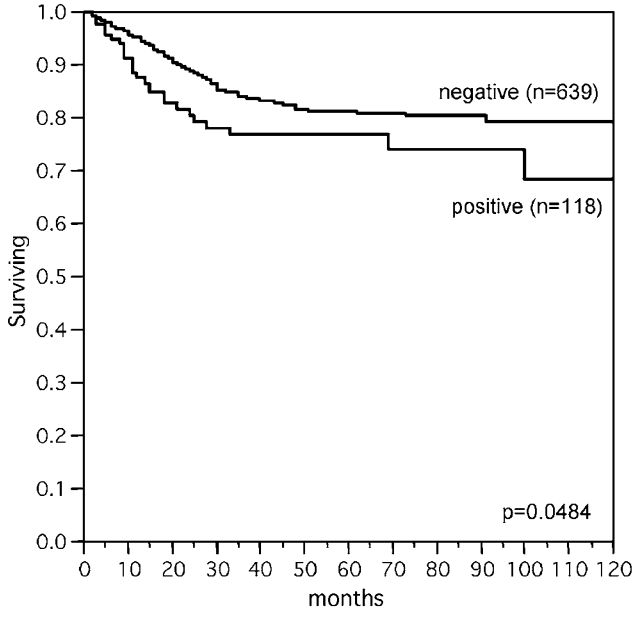

Figure 3 Survival analysis (Kaplan–Meier plot) in a subset of 757 urothelial carcinoma samples with data on E2F3 IHC and patient outcome. Immunohistochemically positive cancers show shortened tumor-specific survival

samples. Alternatively, our data could indicate that E2F3 is not the (only) amplification target at 6p22. Overexpression of one or several other genes in the same amplicon could be required to drive the growth advantage of amplified cells in 6p22-amplified tumors without E2F3 protein overexpression. There is a growing evidence indicating that the molecular mechanism of gene amplification does not follow the simple one gene–one amplicon concept. Amplification may be a mechanism that is particularly effective to simultaneously overexpress multiple adjacent genes, which may jointly provide a growth advantage to amplified tumor cells. For example, neighboring oncogenes that sometimes undergo coamplification have recently been identified at various genomic regions such as MDM2, GLI, CDK4, and SAS at 12q13-q15 (Reifenberger *et al*., 1994) or CCND1, EMS1, and INT2 at 11q13 (Hui *et al*., 1997).

Only few known genes are known to be located in direct genomic neighborhood of E2F3. A gene of unknown function (GenBank: NM_017774) maps closely centromeric to E2F3. The 579 amino-acid protein contains a domain that is present in several other proteins associated with the translation machinery and in a family of small, uncharacterized archaeal proteins that are predicted to play a role in the regulation of tRNA modification or translation (Anantharaman *et al*., 2001). SOX4, a transcription factor that is a member of the high mobility group (HMG)-box family of DNA binding protein (Farr *et al*., 1993), and PRL (the gene encoding Prolactin) map to region between 1 and 2 Mb centromeric to E2F3. Prolactin (PRL) is a protein hormone closely related to growth hormone and mainly secreted in the anterior pituitary lactotrope and the

decidualized stromal cell of the human endometrium (DiMattia *et al*., 1990). However, Northern analysis demonstrated no correlation between amplification and overexpression of these genes in eight cell lines (Bruch *et al*., 2000).

In summary, E2F3 is regularly included in a bladder cancer amplicon at 6p22. Taking together the known cell cycle activating role of E2F3, its overexpression in amplified tumors, and the association with cell proliferation *in vivo*, it appears that E2F3 could be an important target gene inside the 6p22 amplification whose overexpression gives growth advantage to amplified tumor cells. This study also demonstrates how a combination of genomic technologies, microarray discovery platforms, and bioinformatics resources can be used to rapidly identify, validate, and characterize target genes for genetic alterations and associate these changes to specific medical conditions.

Materials and methods

CGH

A review of the CGH profiles of 278 primary bladder carcinomas and 20 cell lines previously examined in our laboratory revealed 10 tumors and three cell lines with distinct peaks around 6p22. Examples of CGH profiles are shown in Figure 4.

Bladder cancer tissue microarray (TMA)

The composition of our bladder cancer TMA containing 2317 formalin-fixed paraffin-embedded tissues from 1853 bladder cancer patients was previously described (Richter *et al*., 2000; Simon *et al*., 2002). Some of the clinical data were updated for this study. All slides of all tumors were reviewed by one pathologist (GS). Tumor stage and grade were defined according to UICC and WHO (Mostofi, 1973; UICC, 1992). Stage pT1 was defined by 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 were available from 1123 patients. The medium follow-up period was 42 months (range 1–236 months). Time to recurrence and time to progression (to stage pT2 or higher) were selected as study end points for patients with pTa and pT1 tumors.

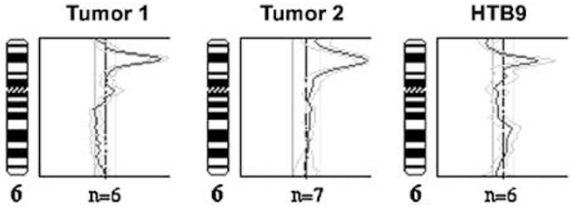

Figure 4 Examples of 6p22 amplifications in bladder cancer as detected by CGH. The central line indicates the fluorescence ration of balanced DNA sequence copy number state (1.0), lines to the left and right represent the 0.8 and 1.2 thresholds for losses and gains. 6p22 amplifications are indicated by strong shifts of the fluorescence ratio profile to the right in the respective chromosomal regions

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 is given in Table 3.

FISH

The tissue microarray sections were treated according to the Paraffin Pretreatment Reagent Kit protocol (purchased from Vysis, Downers Grove, IL, USA) before hybridization. FISH was performed with a digoxigenated PAC probe (PAC

Table 3 Histological and clinical parameters of 2317 arrayed bladder cancer samples

	Tumors (n = 2317)	*Patients (n = 1853)*	*Patients with clinical data (n = 1123)*
Stage			
pTa	951	768	502
pT1	515	425	263
pT1-	101	80	34
pT2–4	737	571	320
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	70
pT2–4G3	551	431	250
Histology			
Transitional cell carcinoma	2108	1678	1032
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	868
Solid	633	472	249
No. of tumors per patient			
One	1533	1533	914
Two or more	784	320	209
Clinical end points	—	—	
Tumor-specific survival (pT2-4)	—	—	320
Time to progression (pTa and pT1)	—	—	482
Time to recurrence (pTa and pT1)	—	—	535

dJ177P22, Sanger Centre, UK) containing the E2F3 gene and a Spectrum Red-labeled chromosome 6 centromeric probe (CEP6) as a reference (purchased from Vysis). Hybridization and posthybridization washes were according to the 'LSI procedure' (Vysis). Probe visualization using fluorescent isothiocyanate (FITC)-conjugated sheep anti-digoxigenin (Roche Diagnostics, Rotkreuz, Switzerland) was as described (Wagner *et al*., 1997). Slides were counterstained with 125 ng/ml 4′,6-diamino-2-phenylindole in an antifade solution. Amplification was defined as presence (in $\geqslant 5\%$ of tumor cells) of at least three times as many E2F3 gene signals than centromere 6 signals.

IHC

Standard indirect immunoperoxidase procedures were used for IHC (ABC-Elite, Vector Laboratories, Burlingame, CA, USA). The monoclonal antibody E2F3 Ab-4 (Lab Vision Corporation, CA, USA) was tested on array sections containing formalin-fixed paraffin-embedded, E2F3-amplified and nonamplified bladder tumors. Optimal staining of the cell nuclei (1 : 100 dilution of primary antibody) could best be achieved after pretreatment in 1 mM EDTA at 99°C for 40 min for antigen retrieval. The primary antibody was omitted for negative controls. Diaminobenzidine was used as a chromogen. Some cytoplasmic staining was seen in most tissue spots but only nuclear staining was scored. The IHC staining intensity (scored in a four step scale including 0, 1+, 2+, and 3+) and the fraction of positive tumor cells was recorded for each tissue spot. Based on these values, a final IHC result was calculated according to the following criteria: Negative: no staining at all, or 1+ staining intensity in no more than 10% of tumor cells; positive: at least 2+ staining intensity in more than 10% of tumor cells.

The rabbit monoclonal antibody MIB1 (1 : 800, Dianova, Hamburg, Germany) was employed to detect Ki67 protein (expressed in all cells in G1, S, G2, and M phase) as previously described (Moch *et al*., 1997). Nuclei were considered Ki67 positive if any nuclear staining was seen. The Ki67 LI (percentage of Ki67-positive cells) was determined on each arrayed tumor sample by scoring at least 300 cells each. Tumors with Ki67-negative mitoses were excluded from analyses.

Western analysis

Protein was extracted from about 2×10^6 cells from bladder cancer cell lines CRL1472, HTB5, HTB9, CRL7882, and the RT112 cell line as described (Leone *et al*., 1998). In all, 10 μg protein of each sample was subjected to SDS–PAGE on 10% polyacrylamide gels. Proteins were transferred onto PVDF membrane (Bio-Rad, Glattbrugg, Switzerland). The membrane was blocked in TBS (25 mM Tris at pH 7.4, 137 mM NaCl, 2.7 mM KCl) containing 10% skim milk at RT for 2 h. Blots were then incubated with mouse monoclonal E2F3 Ab-4 antibody (5 μg/ml) (Lab Vision, CA, USA), which is directed against the E2F3 full-length protein, in TBS containing 5% skim milk overnight at 4°C, and washed subsequently in TBS containing 0.1% Tween 20 for 30 min. Blots were incubated with a second antibody (1 : 2000) (goat anti-mouse IgG, Fc, AP127P; Juro Supply AG, Lucerne, Switzerland) for 1 h at RT, and washed for 30 min. Blots were processed with the ECL system (Amersham Pharmacia Biotech, Dubendorf, Switzerland).

Statistics

All tissue samples on the TMA were utilized for comparisons of amplification and overexpression of E2F3. Only the first biopsy was used for further statistical analyses in patients having more than one tumor on the TMA. Contingency table analysis and Chi-square tests were applied to study the relationship between histology tumor type, grade, stage, and E2F3 expression/amplification. Student's *t*-tests were employed to examine the associations of the Ki67 LI with E2F3 expression/amplification. ANOVA was utilized to determine the parameters with greatest influence on tumor cell proliferation. Survival curves were plotted according to the Kaplan–Meier method and analysed for statistical differences using a log rank test. Patients with pTa/pT1 tumors were censored at the time of their last clinical control showing no evidence of disease or at the date when cystectomy was performed. Patients with pT2-4 carcinomas 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.

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