TRIO Amplification and Abundant mRNA Expression Is Associated with Invasive Tumor Growth and Rapid Tumor Cell Proliferation in Urinary Bladder Cancer

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Studies by comparative genome hybridization have suggested that 5p amplification is related to tumor progression in urinary bladder cancer. In this study seven genes (*TAS2R*, *ADCY2*, *DNAH5*, *CTNND2*, *TRIO*, *ANKH*, and *MYO10*) located to 5p15.31-5p15.1 were analyzed by fluorescence *in situ* hybridization using a tissue microarray containing samples from tumors and cell lines with known 5p amplification by comparative genome hybridization. Amplification frequency was highest for *TRIO*, which maps to 5p15.2 and encodes a protein with a putative role in cell-cycle regulation. To further investigate the role of *TRIO* amplification in bladder cancer, a tissue microarray containing samples from 2317 bladder tumors was used for fluorescence *in situ* hybridization analysis. *TRIO* amplification was strongly associated with invasive tumor phenotype, high tumor grade, and rapid tumor cell proliferation (Ki67 I) ($P < 0.0001$ each). Only 7 of 456 pTaG1/G2 tumors (1.5%) but 62 of 485 pT1-4 carcinomas (12.8%) had *TRIO* amplification. *TRIO* amplification was not associated with poor prognosis. Using a frozen bladder tumor tissue microarray RNA *in situ* hybridization confirmed that *TRIO* is up-regulated in amplified tumors. It is concluded that *TRIO* up-regulation through amplification has a potential role in bladder cancer progression. (Am J Pathol 2004, 165:63-69)

Studies by comparative genomic hybridization (CGH) demonstrated gains and amplifications at many different chromosomal loci mainly in invasive bladder cancer.¹ Gene copy number increase is an important mechanism leading to abnormal expression of known oncogenes such as *MYC* at 8q24, *CCND1* at 11q13, or *HER2* at 17q21.²⁻⁴ However, most of the chromosomal regions

found amplified in bladder cancer¹ do not contain known oncogenes, suggesting that many amplification target genes remain to be identified.

Amplifications of the short arm of chromosome 5 are of particular interest. In previous CGH studies 5p amplification was found to be one of the few alterations occurring more frequently in muscle invasive tumors (stage pT2 and higher) than in early invasive cancers (stage pT1).⁵ Our hypothesis of a role of 5p amplification for bladder cancer progression was further corroborated by the results of a second CGH study finding a significant association between 5p amplifications and an increased risk for tumor progression in a series of pT1 carcinomas.⁶ Because amplifications often cover large areas of 5p in bladder cancer, it is difficult to narrow down the region of amplification using positional cloning approaches. Based on our CGH studies, one of the most common sites of amplification is in the 5p15-p14 region.⁵ In an attempt to find oncogene candidates among known genes allocated in this chromosomal area, we became interested in *TRIO* located at 5p15.2.⁷ The *TRIO* protein contains a serine/threonine kinase domain and two guanine nucleotide exchange factor domains for the family of Rho-like GTPases, specific for Rac1 and RhoA.⁸ These functional domains suggest that this enzyme may play a role in signaling pathways controlling cell proliferation.

In this study we used a mini tissue microarray (TMA) containing 5p-amplified bladder tumors for gene pre-screening and a recently manufactured tissue microarray (TMA)^{9,10} composed of 2317 bladder cancers to examine *TRIO* copy number changes and its association with tumor phenotype, cell proliferation, and patient prognosis. The correlation of *TRIO* mRNA expression and amplification was analyzed in 80 arrayed frozen bladder tumors.

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Figure 1. CGH results in tumors with 5p amplification. CGH profile examples of two primary bladder cancers showing distinct peaks in the 5p15.31-p15.1 region suggesting amplification. The number of observations is shown on the **bottom** of each profile. The **vertical lines** indicate the base line ratio (1.0), and the threshold selected to define relative chromosomal gains (1.2, **right**) and losses (0.8, **left**).

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 16 primary tumors and 5 cell lines with 5p amplification. Examples of CGH profiles showing circumscribed 5p amplifications in the 5p15.31-5p15.1 area are shown in Figure 1.

Bladder Cancer Tissue Microarrays (TMAs)

Three different TMAs were used in this study. A mini TMA containing 10 5p-amplified bladder tumors and four cell lines (5-HTB, RT11-D21, RT112, and CRL-7930) served as a prescreening tool to determine the amplification frequency and copy numbers of seven 5p15 genes in these samples. The second bladder cancer prognosis TMA was previously described.^{9,10} One pathologist (G.S.) reviewed all slides of all tumors. Tumor stage and grade were defined according to International Union Against Cancer and World Health Organization classification.^{11,12} Time to recurrence and time to progression (to stage pT2 or higher) were selected as clinical endpoints for pTa and pT1 tumors, if regular follow-up cystoscopies had been performed at least at 3, 9, and 15 months, then annually until the endpoint 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.

An additional TMA composed of 80 histologically not further characterized frozen bladder carcinomas was manufactured as described.¹³ A home-made semiautomated tissue-arraying device equipped with a 0.6-mm drill for recipient hole making was used. An H&E-stained section of this frozen TMA is shown in Figure 3A.

Fluorescence in Situ Hybridization (FISH)

The tissue microarray sections were treated according to the Paraffin Pretreatment Reagent kit protocol (Vysis, Inc., Downers Grove, IL) before hybridization. FISH was performed with digoxigenated BAC probes specific for seven genes located to 5p15.31-5p15.1 (Table 1) (Invitrogen, Carlsbad, CA) and a Spectrum Red-labeled chromosome 6 centromeric probe as a reference (Vysis). Hybridization and posthybridization washes were according to the Locus Specific Identifier (LSI) procedure (Vysis). Probe visualization using fluorescein isothiocyanate (FITC)-conjugated sheep anti-digoxigenin (Roche Diagnostics, Rotkreuz, Switzerland) was as described.¹⁴ Slides were counterstained with 125 ng/ml of 4',6-diamino-2-phenylindole in an anti-fade solution. Amplification was defined as presence (in $\geq 5\%$ of tumor cells) of either more than 10 gene signals or more than three times as many gene than centromere 6 signals.

RNA in Situ Hybridization (RISH)

Four oligonucleotides of 40 bases specific for *TRIO* were designed using the Vector NTI program (www.vectornti.co.kr). Each of the oligonucleotides was labeled separately in a reaction volume of 4.5 μ l using [α -³²P]-dATP (Amersham Pharmacia Biotech, Buckinghamshire, UK) and terminal transferase (Catalys, Wallisellen, Switzerland). Unincorporated nucleotides were removed according to the manufacturer's instructions (Qiagen, Basel, Switzerland). Denhardt's hybridization solution (120 μ l) containing 50% formamide, 4 $\times$ standard saline citrate, 0.02 mol/L NaPO₄ (pH 7), 7% dextran sulfate, 5% sarcosyl, Cot1 DNA (40 μ g/ml), 0.15 mol/L dithiothreitol, and radiolabeled oligos (5,000,000 cpm/ml hybridization solution) were mixed and added to the microarray and hybridized overnight at 42°C in a moist chamber. Slides were washed for 4 $\times$ 15 minutes in 1 $\times$ standard saline citrate at 55°C, for 1 hour with 1 $\times$ standard saline citrate at room temperature, dehydrated in distilled water, 60%

Table 1. Amplification Frequency and Copy Numbers of 5p15 Genes in 5p-Amplified Bladder Cancers

Gene	Clone	Accession number	Cytoband	5p Locus (Mb)	Amplified tumors	Copy numbers	Amplified cell lines	Copy numbers
<i>ADCY</i>	CTD-2217J21	AC010437	5p15.31	7.4-7.9	1/10	11	1/3	7
<i>TAS2R1</i>	CTD-2143L24	AC026787	5p15.31	9.6-9.7	4/9	7-8	2/4	9
<i>CTNND2</i>	CTD-2154B17	AC012629	5p15.2	11-12	7/10	8-13	2/4	9
	CTD-2138K21	AC010626						
<i>DNAH5</i>	CTC-2322H10	AC016546	5p15.2	13.7-14	1/9	10	1/4	7
<i>TRIO</i>	CTD-2207B17	AC010434	5p15.2	14.2-14.6	8/9	8 $\rightarrow$ 20	4/4	8-12
	CTD-2022F20	AC010352						
<i>ANKH</i>	CTD-2219M19	AC010438	5p15.2	14.7-14.9	7/10	7 $\rightarrow$ 20	3/4	7-8
<i>MYO10</i>	CTD-2303G11	AC010473	5p15.1	16.7-17	3/10	7 $\rightarrow$ 20	2/4	8-10

ethanol, and 95% ethanol (30 seconds for each step) and air-dried for 10 minutes. Hybridization signals were visualized after 48 hours of exposure to a high-resolution screen with Cyclone Phosphor Imager (Canberra Packard, Zurich, Switzerland). Specificity of RISH was evaluated by treating the TMA with Hypercoat emulsion LM-1 according to the protocol of Amersham Pharmacia Biotech.

Northern Blot Analysis

Total RNA was extracted from three bladder cancer cell lines (3-HTB, 4-HTB, and 5-HTB) using Trizol (Invitrogen AG, Basel, Switzerland). Two μg of poly(A)⁺ mRNA of each cell line was selected with the DynaBead-kit (Dyna, Oslo, Norway), separated on a 1% agarose-formaldehyde gel and transferred to a nylon membrane (Amersham Pharmacia Biotech). The membrane was hybridized with [α -³²P]-dATP (Amersham Pharmacia Biotech)-labeled *TRIO*-specific oligonucleotides at 42°C for 5 hours in ExpressHyb solution (Clontech, Heidelberg, Germany). Northern analysis was performed with Cyclone phosphor imager (Canberra Packard).

Statistics

Only the first biopsy was used for statistical analyses in patients having more than one tumor on the TMA. Chi-square tests were applied to study the relationship between histological tumor type, grade, stage, and *TRIO* amplification. Student's *t*-tests were used to examine the associations of the Ki67 LI with *TRIO* amplification, tumor stage, and grade. Survival curves were plotted according to the Kaplan-Meier method and analyzed 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.

Results

FISH Prescreening

Prescreening and establishing optimal hybridization conditions was completed on a mini-TMA containing four cell lines and 10 primary tumors that had shown 5p amplification by CGH. FISH analysis of seven 5p15 genes on the mini-TMA revealed highest frequency of amplification for *TRIO* according to our definition. *TRIO* was amplified in all four cell lines and eight of nine interpretable primary tumors (Table 1). The average *TRIO* copy numbers in four of these tumors were 14, 16, 18, and more than 20 clustered signals. Lower amplification rates and copy numbers were detected for the remaining genes located in close vicinity to *TRIO*. Based on these results the role of *TRIO* in bladder cancer was further examined using a prognosis TMA.

Figure 2. *TRIO* analysis on a bladder cancer tissue microarray. **A:** *TRIO* amplification: tumor cell showing clusters of green *TRIO* signals and two red centromere 6 signals. **B:** Nonamplified tumor cell with two copies each of *TRIO* (green) and centromere 6 (red).

TRIO Gene Amplification

Large-scale TMA analysis showed *TRIO* amplification in a total number of 111 of 1636 interpretable tumors. FISH related problems (weak hybridization, background, tissue damage) were responsible for approximately two-thirds, TMA-linked problems (too few or absence of tumor cells on the TMA spot) were causing approximately one third of the noninformative cases. Examples of amplified and nonamplified tumors are shown in Figure 2, A and B. The associations with tumor phenotype are summarized in Table 2. *TRIO* amplification was not only frequent in muscle-invasive transitional cell carcinomas (TCC) (15.9%) but also in small cell (35.7%), sarcomatoid (two of eight amplified), and squamous cell cancers (5.7%) as well as in adenocarcinomas (one of four amplified). Within TCC, which is by far the most common bladder cancer subtype, *TRIO* amplification was strongly associated with tumor grade and stage ($P < 0.0001$ each). Most

Table 2. *TRIO* Amplification and Tumor Phenotype

	<i>TRIO</i> amplification		
	<i>n</i>	Amplified (%) [*]	<i>P</i> value [†]
Stage [‡]			
pTa	499	1.7	<0.0001
pT1	240	9.6	
pT2-4	245	15.9	
Grade [‡]			
G1	139	1.4	<0.0001
G2	489	2.7	
G3	360	15.6	
Stage/grade [‡]			
pTaG1	139	1.4	<0.0001
pTaG2	317	1.6	
pTaG3	43	4.9	
pT1G2	104	2.9	
pT1G3	136	14.7	
pT2-4G2	65	7.7	
pT2-4G3	180	18.8	
Histologic type [§]			
Transitional cell	245	18.9	
Squamous	37	5.7	
Adeno	4	25	
Small cell	14	35.7	
Sarcomatoid	8	25	

^{*} For definition see text; TCC with *TRIO* gains excluded.

[†] Chi-square test.

[‡] Only first biopsies of patients with TCC included.

[§] Only first biopsies of patients with muscle invasive carcinomas (pT2-4) included.

Figure 3. *TRIO* expression and amplification in bladder cancer. **A:** H&E-stained section of a TMA containing 80 bladder tumors from fresh frozen tissues. **B:** RISH phosphor image of the TMA hybridized with *TRIO*-specific oligomers after 48 hours of exposure time. **C:** Autoradiography of a bladder tumor array element showing tumor cells with high *TRIO* mRNA expression. **D:** Schematic illustration of the RISH result. **E:** Schematic illustration of the FISH result obtained from a consecutive TMA section. **F:** Northern blot analysis of three bladder tumor cell lines. Abundant *TRIO* mRNA expression (9.4 kb) in 5p amplified 5-HTB and low expression in the not amplified cell lines 3-HTB and 4-HTB. The Northern blot was rehybridized with GAPDH probe as a control.

strikingly, *TRIO* amplification was rare in pTaG1/G2 tumors (7 of 456; 1.5%) whereas 12.8% (62 of 485) of the invasively growing TCC (pT1-4) were amplified.

TRIO mRNA Expression in Bladder Cancer

To determine whether *TRIO* amplification leads to elevated *TRIO* mRNA expression, RISH and FISH was performed on two consecutive sections of a microarray containing 80 fresh frozen bladder tumors. *TRIO* mRNA expression and DNA copy number analyses was evaluated in 59 tumors. There was a high correlation between overexpression and *TRIO* amplification. Fifteen of 17 tumors (88%) with strong *TRIO* mRNA expression had more than six *TRIO* gene copies. Five of 28 tumors (18%) with moderate and only 1 of 14 tumors (7%) with low or no detectable *TRIO* mRNA expression were amplified (Figure 3; B to E). Northern blot hybridization of mRNA ex-

tracted from three bladder tumor cell lines confirmed the binding specificity of the oligo probes to *TRIO* mRNA (Figure 3F).

TRIO Amplification and Tumor Cell Proliferation [Ki67 Labeling Index (LI)]

TRIO amplification was significantly associated with rapid tumor cell proliferation ($P < 0.0001$). The separate analyses of tumors of identical grades and stages lead to significant differences in the proliferation between *TRIO* nonamplified and amplified tumors (Table 3).

TRIO Amplification and Prognosis

TRIO amplification was not associated with poor tumor-specific survival neither if all patients were included in the

Table 3. *TRIO* Amplification in Relation to Ki-67 LI

Stage/ grade*	FISH		<i>P</i> value†
	Normal	Amplified‡	
pTa	490 10.5 ± 9.3§	9 13.3 ± 9	0.3852
pT1	217 24.5 ± 14	23 34.4 ± 17.8	0.0018
pT2-pT4	206 30.1 ± 16.6	39 38.2 ± 17.6	0.006
G1	137 6.4 ± 6.3	2 7 ± 2.9	0.883
G2	476 14.1 ± 11	13 21.1 ± 14.1	0.0237
G3	304 30.2 ± 15.8	56 37.7 ± 17.8	0.0014

* Only first biopsies of patients with TCC included.

† For definition see text; TCC with *TRIO* gains excluded.

‡ Student's *t*-test.

§ Mean percentage value of Ki67-positive cells with standard deviation.

analysis nor within the subgroup of pT2-4 TCCs. *TRIO* amplification provided no prognostic significance among pTa/pT1 tumors, neither for recurrence nor for progression.

Discussion

In this study, TMA technology was used to screen oncogene candidates located within the 5p15.31-5p15.1 region and to examine gene amplification and mRNA expression of *TRIO* on chromosome 5p15.2 in urinary bladder cancer. Besides bladder cancer, overrepresentations of chromosome 5p have been found in various other human tumor types including breast,¹⁵ colon,¹⁶ head and neck,¹⁷ lung cancer,¹⁸ and osteosarcoma.¹⁹ Although frequently altered in these tumor types, for example 70% of non-small-cell lung tumors showed 5p gains,¹⁸ potential oncogene candidates residing in this chromosomal area have hardly been described in the literature. The limited resolution of the CGH method (>10 Mb), and the large size of most CGH peaks on 5p make it difficult to exactly define the chromosomal location of interest. However, previous CGH analysis at almost 300 bladder carcinomas allowed us to identify several cases with more distinct peaks around 5p15.31-5p15.1.

No known oncogene has previously been assigned to chromosome 5p15.31-p15.1. A rapid search on the publicly available genome databases (<http://www.ensembl.org/>) revealed that *TRIO*, a large gene composed of 58 exons distributed throughout more than 250 kb, has been mapped to 5p15.2. The gene codes for a protein, which contains two guanine nucleotide exchange factor (GEF) domains for the family of Rho-like GTPases and a serine/threonine kinase domain.⁸ These functional domains suggest that the *TRIO* protein may play a key role in several pathways that control cell cycle, migration, and cell-cell interactions. It is conceivable that up-regulation of GEFs by *TRIO* amplification may lead to an increased activation of Rho-like GTPases, thus driving cell motility and invasion.²⁰ Increased amounts of Rho-like GTPases were

described in head and neck,²¹ breast, colon, lung,²² pancreas,²³ and testicular germ cell tumors,²⁴ as well as T-lymphoma²⁵ and melanoma.²⁶ It is also well known that dysregulation of serine/threonine kinases may lead to uncontrolled cell proliferation and hence to cancer. For example, amplification of serine/threonine kinase genes such as *CDK2* at 12q13, *CDK4* at 12q14, *PS6K* at 17q23, and *STK15* at 20q13 were observed in tumors of the ovary,²⁷ breast,^{28,29} colon,³⁰ brain,^{31,32} and cervix.³³ The strong association of *TRIO* amplification with high grade, advanced stage, and tumor cell proliferation suggests *TRIO* as appropriate oncogene candidate when amplified and overexpressed in bladder tumors.

Preferential amplification with high copy numbers in all four cell lines and eight of nine interpretable tumors with 5p amplification by CGH provided evidence for an involvement of *TRIO* in bladder cancer and prompted us to further analyze its role in urinary bladder tumors. Using a TMA containing 2317 bladder carcinomas, *TRIO* amplification was found in 9.6% of minimally invasive (pT1) tumors and in 15.9% of muscle-invasive (pT2-4) tumors. This result is consistent with a previous CGH study in which 5p aberrations occurred significantly more frequent in pT2-4 tumors than in pT1 tumors.⁵ The rate of muscle-invasive tumors with *TRIO* amplifications was somewhat higher than that of pT2-4 tumors analyzed with CGH. It is likely that in some cases regional amplifications were interpreted as gain rather than amplification because of technical or DNA quality reasons. Additional RISH and FISH analyses on 80 arrayed frozen bladder tumors confirmed high correlation of *TRIO* amplification and strong mRNA expression.

In this study a very conservative cutoff was used to define amplification. Therefore, the group of nonamplified tumors contained a fraction of cancers with *TRIO* gains that were cautionary not considered amplified for statistical calculations. However, the portion of pT2-4 tumors with *TRIO* copy numbers ≥3 was 30%, which is similar to that observed by CGH analysis.^{5,34} It is of note that 5p gains detected by CGH were associated with high risk of progression in early invasive (pT1) bladder cancer in one study.⁶ This finding was interpreted with caution because only 5 of 54 pT1 tumors analyzed demonstrated 5p gains. Although the rate of *TRIO*-amplified pT1 tumors with clinical follow-up information (9.6%) was comparable to the results obtained by CGH, there was no significant correlation with tumor progression. This holds also true if tumors with average *TRIO* copy numbers of less than seven were included in the statistical analysis.

Oncogenes such as *CCND1*, *ERBB2*, and *MYC* are commonly highly amplified in different human tumors and appear as tight clustered signals representing up to a few hundred gene copies.³⁵ In contrast, 91% of *TRIO*-amplified bladder tumors showed signals ranging between 7 and 14 gene copies, which were more or less equally distributed within tumor cell nuclei. This observation suggests a mechanism of gene amplification that differs to that seen for most of the known oncogenes. Cytogenetic analysis demonstrated that 5p might be involved in translocations and/or formation of isochromosomes in a sub-

stantial number of bladder tumors.³⁶ An accumulation of 5p isochromosomes because of increasing genomic instability described in invasively growing tumors³⁶ may account for the *TRIO* amplification pattern seen in most amplified bladder cancers.

In summary, we show that *TRIO* amplification is strongly linked to high-grade, advanced stage, bladder tumors and rapid tumor cell proliferation in urinary cancer. Preferential amplification within 5p15.31-5p15.1 and the strong association between amplification and mRNA abundance suggests *TRIO* as an oncogene candidate within this amplicon.

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