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A *Survivin* Gene Signature Predicts Aggressive Tumor Behavior

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Abstract

Gene signatures that predict aggressive tumor behavior at the earliest stages of disease, ideally before overt tissue abnormalities, are urgently needed. To search for such genes, we generated a transgenic model of survivin, an essential regulator of cell division and apoptosis overexpressed in cancer. Transgenic expression of survivin in the urinary bladder did not cause histologic abnormalities of the urothelium. However, microarray analysis revealed that survivin-expressing bladders exhibited profound changes in gene expression profile affecting extracellular matrix and inflammatory genes. Following exposure to a bladder carcinogen, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (OH-BBN), survivin transgenic animals exhibited accelerated tumor progression, preferential incidence of tumors as compared with premalignant lesions, and dramatically abbreviated survival. Conversely, transgenic expression of a survivin Thr^{34}→Ala dominant-negative mutant did not cause changes in gene expression or accelerated tumor progression after OH-BBN treatment. Therefore, survivin expression induces global transcriptional changes in the tissue microenvironment that may promote tumorigenesis. Detection of survivin or its associated gene signature may provide an early biomarker of aggressive tumor behavior before the appearance of tissue abnormalities. (Cancer Res 2005; 65(9): 3531-4)

Introduction

"Molecular profiling" of tumors may help devise individualized anticancer therapies (1), and identify patients that require closer follow-up protocols (2), but gene signatures that predict clinical course at the earliest stages of disease have not been readily identified. A candidate tumor biomarker is survivin (3), a member of the Inhibitor of Apoptosis (IAP) gene family (4) overexpressed in virtually every human cancer, and implicated in protection from apoptosis and regulation of mitosis (3). Survivin expression has often been linked to accelerated relapses, refractory disease, and unfavorable outcome (3), but whether this reflects a direct participation in tumor formation, which could be exploited as a predictive biomarker, has remained elusive. Here, we used a transgenic mouse model to define the role of survivin in tumor formation, and test its relevance as a predictive biomarker. We chose bladder cancer because survivin is a diagnostic (5) and therapeutic (6) target in the disease, and because urothelial tumors pose formidable challenges for patient follow-up, as ~20% of seemingly superficial lesions will progress to metastatic disease, and cause ~12,000 deaths every year in the U.S. alone (7).

Materials and Methods

Generation of survivin transgenic mice. All experiments involving animals were approved by an Institutional Animal Care and Use Committee. The survivin transgene was constructed by subcloning the human survivin cDNA (wild-type or Thr^{34}→Ala mutant; ref. 8) downstream of the murine uroplakin II (UPII) promoter (refs. 9, 10; Fig. 1*A*). After purification by ion exchange chromatography (Qiagen, Valencia, CA), and confirmation by DNA sequencing, the constructs (200 ng/μL) were microinjected into C57BL embryos, which were transferred into pseudopregnant females. Littermates were screened by PCR of tail genomic DNA (11) with primers (10 pmol each) corresponding to UPII (5′-CCTGAAAAAACCTGTCTGGGGCCCCCTCCC-3′) and survivin (5′-GGAATTCCTCAATCCATGGCAG-3′). Colonies were maintained as described (11). Wild-type or p53−/− breeders were purchased from Taconic (Germantown, NY).

Gene expression profiling. Control or transgenic animals were allowed to age without intervention for 12 or 15 months. Bladders were removed and total RNA was isolated and processed using standard Affymetrix protocols. Samples were processed by the Genomics Core of the University of Massachusetts Medical School over a GeneChip Mouse Genome 430 2.0 Array. Data were analyzed using established algorithms (GeneChip Operating Software, version 1.2, Affymetrix, Santa Clara, CA), and a cutoff of 2.2-fold difference.

Chemical carcinogenesis model. Transgenic animals or control littermates (20 per group, 14 weeks of age) were treated weekly with intragastric administration of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (OH-BBN, Tokyo Kasei Kogyo, Tokyo, Japan) for 12 weeks (12, 13). At the end of treatment, mice were checked for development of bladder tumors and hematuria (Henry Schein) for an additional 12 weeks. Mice that developed palpable masses and exhibited weight loss were sacrificed. All remaining animals were sacrificed 35 weeks after the first OH-BBN administration and processed for histology.

Western blotting and histology. Western blotting and tissue staining were carried out as described (14).

Statistical analysis. Data were analyzed using the unpaired *t* test on a GraphPad software package for Windows (Prism). A *P* value of 0.05 was considered as statistically significant.

Results and Discussion

Expression of the UPII-survivin or UPII-survivin(T34A) transgene was shown by PCR (Fig. 1*B*), and in the urinary bladder by immunohistochemistry (Fig. 1*D* and *E*), and Western blotting (Fig. 1*F*). In contrast, nontransgenic littermates did not express survivin in the bladder (Fig. 1*C* and *F*). UPII-survivin or UPII-survivin(T34A) mice were grossly indistinguishable from nontransgenic littermates, and had no histologic abnormalities of the bladder (Fig. 1*D* and *E*). UPII-survivin mice crossed with

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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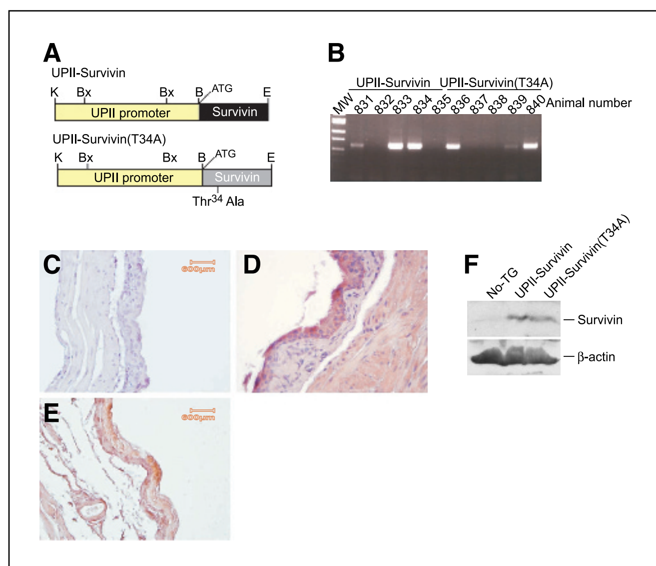

Figure 1. Characterization of survivin transgenic mice. *A,* survivin transgenic constructs. (K) *Kpn*I, (Bx) *Bxt*XI, (B) *Bam*HI, (E) *Eco*RI. The translational initiation codon (ATG) is indicated. *B,* genotyping. Genomic DNA isolated from transgenic mice was analyzed by PCR. *C-E,* immunohistochemistry. Bladders from nontransgenic (*C*), UPII-survivin (*D*), or UPII-survivin(T34A) (*E*) mice were stained with an antibody to survivin. Magnification (×200). *F,* immunoblotting. Bladders from nontransgenic (No-TG), UPII-survivin, or UPII-survivin(T34A) mice were analyzed by Western blotting.

p53−/− breeders to generate UPII-survivin-p53−/+ were also unremarkable, and these animals succumbed at 5.5 to 6 months of age due to hematologic tumors, without macroscopic alterations of the bladder (data not shown).

Despite the absence of histologic abnormalities, microarray analysis of bladders of UPII-survivin animals revealed striking changes in gene expression, as compared with nontransgenic littermates. After removal of unknown genes, expressed sequence tags, and low-abundance transcripts (<1 of 10 of the glyceraldehyde-3-phosphate dehydrogenase signal), 290 genes were found modulated by transgenic expression of survivin, with 102 and 188 genes down-regulated and up-regulated, respectively (Supplementary Table 1). Two discrete gene clusters were represented in a *survivin* gene signature (Table 1). The first cluster comprised up-regulated extracellular matrix constituents, including several collagen genes, fibulin, vimentin, dermatopontin, fibronectin, as well as modulation of genes controlling cell-extracellular matrix interactions, i.e., matrix metalloproteinase-2, small proteoglycans, connective tissue growth factor, and β-catenin (Table 1). The second cluster contained immune-inflammatory mediators, with up-regulation of several immunoglobulin and HLA genes, complement subunits, membrane myeloid/lymphoid antigens, cytokine and chemokine receptors and their ligands (Table 1). Three independent experiments validated the gene expression changes. First, using semiquantitative reverse transcription-PCR, mRNA levels of several randomly selected genes of the *survivin* gene signature were found to be up-regulated in the bladders of UPII-survivin mice, as compared with nontransgenic littermates (Fig. 2*A*). Second, using Western blotting, increased expression of the extracellular matrix protein fibronectin was observed in bladder samples of UPII-survivin mice as compared with control littermates (Table 1; Fig. 2*B*). Third, with immunohistochemistry, bladders of UPII-survivin mice (four of eight animals), but not control littermates, exhibited infiltration of mononuclear cells (Fig. 2*C* and *D*), which stained positive with an antibody to the T cell marker CD3 (Fig. 2*F* and *G*). In contrast, UPII-survivin(T34A) mice had no significant changes in gene expression by microarray analysis (Supplementary Table 2), and exhibited reduced inflammation of the bladder (two of eight animals, Fig. 2*E*).

Exposure of nontransgenic animals to OH-BBN resulted in frank hematuria and palpable abdominal masses starting at 3 to 4 weeks after suspension of treatment. By 12 weeks after the end of treatment, ∼30% of these animals exhibited abdominal masses (Fig. 3*A*) and shortened survival (Fig. 3*B*). UPII-survivin mice exhibited dramatic acceleration of lesion formation with ∼50% of the animals presenting with abdominal masses and hematuria within 3 weeks after the end of OH-BBN administration (Fig. 3*A*). After 12 weeks, 78% of UPII-survivin mice had abdominal tumors (Fig. 3*A*) and abbreviated survival (Fig. 3*B*). In contrast, UPII-survivin(T34A) mice were indistinguishable

Table 1. Survivin gene signature

Unigene	Gene name	Fold change
Mm.249555	Procollagen type III	↑4
Mm.271644	Fibrillin 1	↑3.8
Mm.277735	Procollagen, type I α1	↑3.8
Mm.130388	Procollagen type VIII, α1	↑3.6
Mm.16234	Integrin α5	↑3.6
Mm.10299	Procollagen type V, α2	↑3.4
Mm.193099	Fibronectin	↑3.4
Mm.29564	Matrix metalloproteinase-2	↑3.0
Mm.2608	Biglycan	↑2.8
Mm.258065	Laminin α4	↑2.6
Mm.268000	Vimentin	↑2.6
Mm.297992	Fibulin 1	↑2.4
Mm.28935	Dermatopontin	↑2.4
Mm.18888	Lumican	↑2.2
Mm.287146	Fibromodulin	↑2.2
Mm.291928	β-Catenin	↓3.0
Mm.1810	Connective tissue growth factor	↓3.0
Mm.351746	IgM Heavy chain	↑6.6
Mm.297582	Serum IgG_{2a}	↑6.2
Mm.1192	Immunoglobulin joining chain	↑5.4
Mm.2923	IL2 receptor γ chain	↑4.2
Mm.235338	Histocompatibility 2, class II antigen Aα	↑4.0
Mm.24130	CD52	↑3.6
Mm.276499	Ia-associated invariant chain	↑3.6
Mm.10116	Chemokine (CXC) ligand 13	↑3.4
Mm.45436	Lysozyme	↑3.2
Mm.254067	Histocompatibility 2, class II antigen Aβ1	↑3.0
Mm.42029	Chemokine (CC) ligand 8	↑3.0
Mm.3453	Complement component C1qγ	↑2.6
Mm.246497	Serum IgG_1	↑2.4
Mm.22119	Fcγ receptor IIB	↑2.4
Mm.2692	CD53	↑2.4

NOTE: Microarray analysis was done on bladders from UPII-survivin mice or control littermates at 12 to 15 months of age. Representative modulated genes are shown for the two gene clusters comprising the *survivin* gene signature (extracellular matrix and inflammatory clusters).

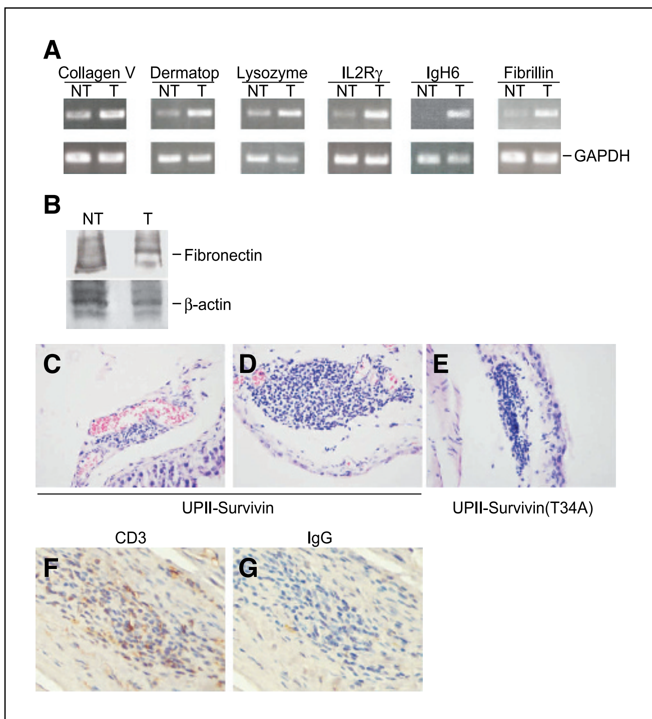

Figure 2. Validation of *survivin* gene signature. *A,* reverse transcription-PCR. RNA from UPII-survivin mice (T) or nontransgenic littermates (NT) was reverse-transcribed and amplified for the indicated gene products or glyceraldehyde-3-phosphate dehydrogenase by PCR. *B,* Western blotting. Bladders of UPII-survivin mice (T) or nontransgenic littermates (NT) were analyzed by Western blotting. *C-E,* histology. Bladders from UPII-survivin (*C* and *D*) or UPII-survivin(T34A) (*E*) mice were stained by H&E. Magnifications (×100, ×200). *F* and *G,* immunohistochemistry. Bladders of UPII-survivin mice exposed to OH-BBN were analyzed with an antibody to CD3 or control IgG. Magnification (×200).

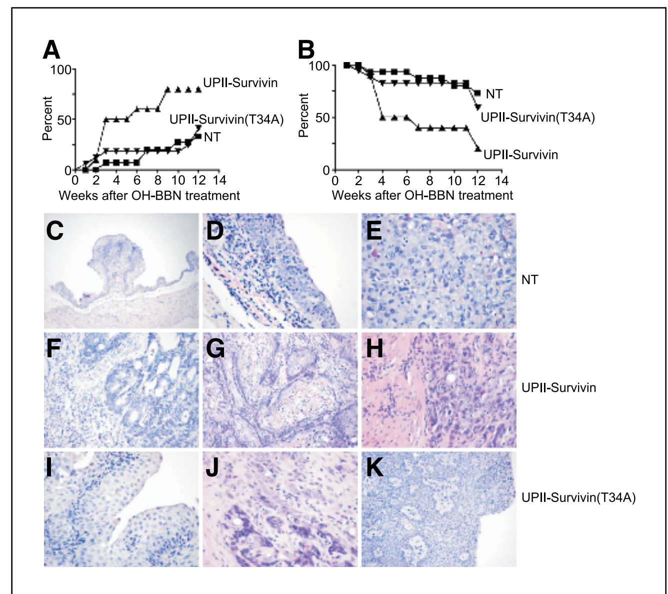

Figure 3. Transgenic survivin enhances OH-BBN-induced bladder cancer. *A,* onset of abdominal lesions. Nontransgenic (NT), UPII-survivin, or UPII-survivin(T34A) mice treated with OH-BBN were monitored for palpable abdominal masses. *B,* survival. The experimental conditions are as in (*A*), except that animal survival was monitored after OH-BBN treatment. *C-K,* histology. Tissue sections from nontransgenic (NT, *C-E*), UPII-survivin (*F-H*), or UPII-survivin(T34A) (*I-K*) mice were stained by H&E. The histologic diagnoses are as follows: *C,* papillary hyperplasia; *D,* dysplasia; *E,* undifferentiated carcinoma; *F,* transitional cell carcinoma; *G,* squamous cell carcinoma; *H,* undifferentiated carcinoma; *I,* papillary metaplasia; *J,* transitional cell carcinoma; *K,* papillary transitional cell carcinoma. Magnifications (×100, ×200).

from nontransgenic littermates for the appearance of abdominal masses (Fig. 3*A*) and survival (Fig. 3*B*). Histologically, control littermates treated with OH-BBN exhibited a field of urothelial lesions, including simple and nodular hyperplasia (9 of 15, 60%), dysplasia (3 of 15, 20%), squamous (2 of 15, 13%), and transitional cell carcinomas (2 of 15, 13%), often in the same sample (Fig. 3*C-E*). Conversely, OH-BBN-treated UPII-survivin animals presented a shift of urothelial lesions toward malignancy with reduced hyperplasia (3 of 9, 33%), no dysplasia (0 of 9) and increased incidence of squamous (3 of 9, 33%), and transitional (4 of 9, 44%) cell carcinomas (Fig. 3*F-H*). In contrast, UPII-survivin(T34A) mice had urothelial lesions similar to nontransgenic littermates with hyperplasia (10 of 16, 62%), dysplasia (3 of 16, 18%), and preferential occurrence of transitional cell carcinoma (5 of 16, 31%), as opposed to squamous cell carcinoma (1 of 16, 6%; Fig. 3*I-L*).

In summary, transgenic expression of survivin in the urinary bladder causes global changes in gene expression with up-regulation of extracellular matrix and inflammatory genes. This *survivin* gene signature was specific, i.e., transgenic expression of a loss-of-function survivin $Thr^{34}\rightarrow Ala$ mutant (8) had no effect, and was phenotypically linked to aggressive tumor behavior *in vivo*. Extracellular matrix genes are typically up-regulated in invasive bladder cancer (15), as well as metastatic cancer gene signatures (16), and inflammatory changes may promote transformation by suppressing apoptosis *in vivo* (17). In this context, survivin may contribute to tumorigenesis via inhibition of tumor cell apoptosis (14), and changes in gene expression (this study) that favor tumor cell invasiveness (18). Importantly, the *survivin* gene signature was detected in the absence of histologic abnormalities, thus potentially reflecting the "field" nature of bladder tumorigenesis (7). Therefore, detection of survivin or its gene signature could provide a predictive biomarker of underlying malignancy at the earliest stages of development, and with propensity for aggressive clinical course.

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