

Molecular Profiling of ADAM12 in Human Bladder Cancer

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Abstract Purpose: We have previously found ADAM12, a disintegrin and metalloprotease, to be an interesting biomarker for breast cancer. The purpose of this study was to determine the gene and protein expression profiles of ADAM12 in different grades and stages of bladder cancer.

Experimental Design: ADAM12 gene expression was evaluated in tumors from 96 patients with bladder cancer using a customized Affymetrix GeneChip. Gene expression in bladder cancer was validated using reverse transcription-PCR, quantitative PCR, and *in situ* hybridization. Protein expression was evaluated by immunohistochemical staining on tissue arrays of bladder cancers. The presence and relative amount of ADAM12 in the urine of cancer patients were determined by Western blotting and densitometric measurements, respectively.

Results: ADAM12 mRNA expression was significantly up-regulated in bladder cancer, as determined by microarray analysis, and the level of ADAM12 mRNA correlated with disease stage. Reverse transcription-PCR, quantitative PCR, and *in situ* hybridization validated the gene expression results. Using immunohistochemistry, we found ADAM12 protein expression correlated with tumor stage and grade. Finally, ADAM12 could be detected in the urine by Western blotting; ADAM12 was present in higher levels in the urine from patients with bladder cancer compared with urine from healthy individuals. Significantly, following removal of tumor by surgery, in most bladder cancer cases examined, the level of ADAM12 in the urine decreased and, upon recurrence of tumor, increased.

Conclusions: ADAM12 is a promising biomarker of bladder cancer.

Bladder cancer is a common malignant disease in both men and women; with estimates projecting that >60,000 new cases would be diagnosed in the U.S. in 2006 (1). The majority of patients diagnosed with bladder cancer present with non-muscle-invasive tumors. However, despite intensive surveillance, these patients have a risk of progression of the disease to muscle-invasive cancer of up to 60% at long-term follow-up (2, 3). The current procedure for detecting bladder tumors with potential progression is difficult and error-prone, and new biomarkers are needed to optimize the molecular characterization of tumors (4, 5). In this study, we propose that ADAM12, a disintegrin and metalloprotease, is a new and promising biomarker for bladder cancer.

ADAM12 is a protease, and proteases have multiple functions in normal and pathophysiologic conditions. Matrix metal-

loproteases have been studied extensively, and increased activity of these proteolytic enzymes has been shown to be associated with the malignant phenotype (6). More recently, the ADAM family of proteins, including ADAM9, 12, and 28, has been implicated in cancer (7–12). The ADAMs, like the matrix metalloproteases, belong to the superfamily of zinc-dependent metzincin proteases, and consist of >35 members that are multidomain transmembrane proteins with protease, cell adhesive, and signaling activities. Thus, ADAMs may play diverse roles in different tissues. They induce ectodomain shedding of growth factors, cytokines, and their receptors, and they bind to integrins and syndecans, influencing cell-cell and cell-matrix interactions (13). The prototype ADAM contains, from the NH₂ terminus, a signal peptide, a prodomain, a metalloprotease domain, a disintegrin domain, a cysteine-rich domain, an epidermal growth factor-like domain, a transmembrane domain, and a cytoplasmic domain. Four ADAMs (ADAM9, 11, 12, and 28) exist in alternatively spliced secreted (-S) forms that do not contain transmembrane and cytoplasmic domains (14–17).

ADAM12 is highly expressed in rapidly growing tissues such as the placenta (8) and malignant tumors (7, 8, 18–20). In 1999, we reported that ADAM12 is highly expressed by the malignant tumor cells in several different forms of cancer (8). We also reported that ADAM12 mRNA was almost undetectable in normal livers, but increased in hepatocellular carcinomas (a 6-fold increase) and liver metastases from colonic carcinomas (up to a 60-fold increase; ref. 18). Kodama et al. (10) recently examined the expression of 13 different ADAMs in human malignant astrocytomas, and found that ADAM12 is selectively overexpressed in glioblastomas, with a direct

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correlation between the level of ADAM12 mRNA expression and cell proliferation activity. *In situ* hybridization and immunohistochemical analysis showed that ADAM12 is produced by the glioblastoma cells. We and others have studied ADAM12 in breast cancer (8, 19, 21, 22). Interestingly, we found that urinary levels of ADAM12 correlate with breast cancer status and stage (22). Most recently, we showed that ADAM12 enhances mammary tumor progression in a transgenic mouse model (21). When ADAM12 expression was increased, the time of tumor onset was decreased, and tumor burden, metastasis, and grade of malignancy were increased. We also provided evidence that ADAM12 decreases the apoptosis of tumor cells and enhances the apoptosis of stromal cells.

In the present study, we investigated the potential of ADAM12 as a biomarker in bladder cancer. This report is, to our knowledge, the first to show that both the mRNA and protein expression levels of ADAM12 correlate with the stage of bladder cancer. We also showed that ADAM12 levels in the urine from bladder cancer patients are significantly increased as compared with urine from healthy individuals. Importantly, we found that the level of ADAM12 in the urine decreased following tumor removal and increased upon tumor recurrence, suggesting that ADAM12 could become an important biomarker for bladder cancer diagnostics and surveillance.

Materials and Methods

Microarray gene expression profiling. In this study, we analyzed 21 normal bladder biopsies and biopsies from 31 T_a tumors, 20 T₁ tumors, and 45 T₂₋₄ tumors by microarray analysis. Bladder tumor biopsies were obtained directly from surgery after removal of the necessary amount of tissue for routine pathology examination. Normal bladder tissue biopsies were obtained from individuals with no history of bladder tumors. Tissue samples were frozen at -80°C in a guanidinium thiocyanate solution for preservation of the RNA. Informed consent was obtained from all patients, and the protocols were approved by the scientific ethical committee of Aarhus County. RNA extraction, sample labeling, hybridization to customized Affymetrix GeneChip Eos Hu03 (Affymetrix, Santa Clara, CA), and generation of expression intensity measures was done as described (23, 24).

Reverse transcription-PCR and quantitative PCR. Total RNA was extracted and isolated as described (23). One microgram of RNA was treated with DNase I (Invitrogen, Carlsbad, CA) and reverse-transcribed using random hexamer primers and the Transcriptor First Strand cDNA synthesis kit (Roche, Indianapolis, IN). As a positive control, RNA was isolated from human rhabdomyosarcoma cells, RD (ATCC no. CCL-136; American Type Culture Collection, Manassas, VA). In addition, plasmids containing the cDNA sequence of ADAM12-L or -S were used as positive controls. Intron-spanning primers for ADAM12-L and -S were designed as follows: primers targeting ADAM12-L (forward, 5'-CAGCCAAGCCTGCACTAG-3'; reverse, 5'-AGTGAGCCGAGTTG-TTCTGG-3') produced a 101 bp fragment, and primers targeting ADAM12-S (forward, 5'-GCTTTGAGGAAGCACAGAC-3'; reverse, 5'-TCAGTGAGCAGTAGACGCA-3') produced a 135 bp fragment. Primers targeting the reference gene glyceraldehyde-3-phosphate dehydrogenase (forward, 5'-AAGTCCATCCCAGAGCTGAACG-3'; reverse, 5'-TGTCATACCAGGAATGAGC-3') produced a 292 bp fragment. The PCR program consisted of 5 minutes at 95°C, followed by 35 cycles of 15 seconds at 94°C, 20 seconds at 55°C (glyceraldehyde-3-phosphate dehydrogenase) or 60°C (ADAM12-L and -S), 1 minute at 72°C, and a final extension step for 2 minutes at 72°C. Products were confirmed on a 2% agarose gel.

Quantitative PCR (qPCR) was done using the LightCycler FastStart DNA Master SYBR Green I and the LightCycler QPCR machine (Roche). Primers targeting the reference gene 18S rRNA (forward, 5'-CGCCGCTAGAGGTGAAATTC-3'; reverse, 5'-TTGGCAAATGCTTTCGCTC-3') produced a 62 bp fragment (18). The qPCR program consisted of 10 minutes at 95°C, followed by 35 cycles of 0 seconds at 95°C, 8 seconds at 60°C, and 22 seconds at 72°C, followed by the measurement of fluorescence at 82°C for ADAM12-L and -S for 0 seconds. The qPCR program was followed by a melting point program to check the purity of PCR products. The data were analyzed using the 2(-ΔΔC(T)) method (25). qPCR products were purified, TA cloned into pTZ57R/T (Fermentas International, Inc., Burlington, Ontario, Canada), transformed into DH5α cells, and plated on Luria-Bertani agar plates containing carbenicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Isolated plasmids were sequenced using M13 reverse (-49) primers at MWG Biotech (Ebersberg, Germany).

In situ hybridization for ADAM12. Breast tumor sections from ADAM12-MMTV-PyMT and control MMTV-PyMT mice (a mouse breast cancer model) and human bladder cancer tissue arrays were used for ADAM12 mRNA *in situ* hybridization as described (26). A human ADAM12 PCR product (representing nucleotides 2208 to 2397 in the cysteine-rich and epidermal growth factor-like domains) was generated using full-length human ADAM12-L as a template (GenBank no. AF023476). The forward primer was 5'-GGATCCAATAATACGACTCAC-TATAGGGAGAGGCCACAAAGTGTGCAGATG-3' containing a T₇ RNA polymerase recognition site (*italics*) and an ADAM12 mRNA sequence (underlined) and the reverse primer was 5'-GAGAATTCATTAACCCCTCACTAAAGGGGAGTCTGTGCTTCCTCCAAAGC-3' containing a T₃ RNA polymerase recognition site (*italics*) and an ADAM12 mRNA complementary sequence (underlined). The resulting PCR fragment was excised from a Tris-acetate 1% Seakem agarose gel (BMA Product, Rockland, ME) and purified by Spin-X (Costar, Cambridge, MA) as described by the manufacturer. Single-stranded sense and antisense ([α-³⁵S]UTP)-labeled RNA probes (190 bp) were generated by *in vitro* transcription of the purified cDNA fragment using T₇ and T₃ RNA polymerase (Roche). The labeled probes were purified on S-200 microspin columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden); 2 × 10⁶ cpm were used per section. Paraffin sections were deparaffinized and treated with 1.25 μg/mL of proteinase K for 5 minutes (mouse sections) or 5 μg/mL of proteinase K for 10 minutes (human sections) in 50 mmol/L of Tris-HCl, 5 mmol/L of EDTA (pH 8.0). Before use, the probes were denatured by heating to 80°C for 3 minutes. The hybridization buffer consisted of 0.3 mol/L of NaCl, 10 mmol/L of Tris-HCl, 10 mmol/L of NaH₂PO₄, 5 mmol/L of EDTA, 0.02% (w/v) Ficoll 400, 0.02% (w/v) polyvinyl pyrrolidone-40, 0.02% (w/v) bovine serum albumin fraction V (pH 6.8), 50% formamide, 10% dextran sulfate, 0.92 mg/mL of t-RNA, and 8.3 mmol/L of DTT. In all steps, diethyl pyrocarbonate-treated water was used. The sections were incubated overnight at 55°C with sense or antisense probes in a moist chamber containing diethyl pyrocarbonate-treated water. After hybridization, the sections were washed under increasing stringency at 55°C in 2× SSC, 1× SSC, and 0.2× SSC containing 0.1% SDS, and 10 mmol/L DTT. The sections were treated with RNase A (20 μg/mL) for 10 minutes in NTE buffer [0.5 mol/L NaCl, 10 mmol/L Tris-HCl (pH 7.2), 1 mmol/L EDTA], washed in 0.2× SSC, 10 mmol/L of DTT, and dehydrated in ethanol with 0.3 mol/L of ammonium acetate. The sections were coated in liquid photo emulsion from Ilford (Marly, Switzerland) and stored in the dark at 4°C. After 3 weeks, the sections were developed using D-19 (Sigma, St. Louis, MO) and counterstained with Mayer's hematoxylin (Sigma).

Tissue arrays and other tissue samples. Four commercially available bladder cancer tissue arrays were examined. To correlate the expression of ADAM12 with tumor grade, three tissue arrays (BC12011, BL801, and BC12012) were obtained from Biomax, Inc. (Rockville, MD). A total of 155 cases (age range, 38-88 years old; 46 females and 109 males) were examined: 18 grade 1 tumor cases, 54 grade 2 tumor cases, and 83 grade 3 tumor cases. The histopathologic entities included 152

transitional cell carcinomas, 1 squamous carcinoma, and 2 adenocarcinomas. To correlate the expression of ADAM12 with tumor stage, an AccuMax array (A215-urinary bladder cancer tissues) was obtained from ISU ABXIS, Co. (Stretton Scientific, Ltd., Derbyshire, United Kingdom). This array contained 45 cancer cases, with two spots for each cancer case, and four nonneoplastic cases with one spot each. Forty of the cases were classified according to the tumor-node-metastasis system, and found to be T_a (8 cases), T₁ (14 cases), T₂ (7 cases), T₃ (6 cases), and T₄ (5 cases). Histologic grading of these 40 cases showed 5 grade 1 tumor cases, 14 grade 2 tumor cases, and 21 grade 3 tumor cases. The pathologic entities included 34 transitional cell carcinomas, 4 squamous carcinomas, and 2 adenocarcinomas. Two cases were not classified according to tumor-node-metastasis, and three cases were diagnosed as carcinoma *in situ*. For the 40 classified cases, there were 10 female and 30 male patients (age range, 33-87 years old). Tissue specimens were fixed in formalin, embedded in paraffin, and spots 1 mm in diameter used for tissue arrays. Adjacent nontumorous tissue present in some of the cases on the arrays was also examined, as were tissue specimens of normal bladder mucosa from 10 persons without bladder cancer.

Antibodies. Antibodies against human ADAM12 (14, 27, 28) used in this study were a rabbit antiserum against the recombinant cysteine-rich domain (rb122), a rabbit antiserum against the recombinant prodomain (rb132), a rabbit antiserum against a carboxyl-terminal ADAM12-S peptide (rb116), a rabbit antiserum against a carboxyl-terminal ADAM12-L peptide (rb109), a rat monoclonal antibody recognizing the disintegrin domain of ADAM12 (2E7), and mouse monoclonal antibodies recognizing ADAM12 (6E6, 8F8, and 6C10). Antibodies to uroplakin 3 (AU1) were obtained from American Research Products (Belmont, MA).

Immunostaining. Tissue sections were deparaffinized, treated with 0.1% hydrogen peroxide for 10 minutes to inhibit endogenous peroxidase, treated with 5 µg/mL of proteinase K for 10 minutes in 50 mmol/L Tris-HCl (pH 7.5), and incubated with polyclonal antibodies to human ADAM12 or uroplakin 3 (1:200 in Dulbecco's PBS with no calcium and magnesium) in a moist chamber for 1 hour at room temperature. Urine samples were mixed with equal amounts of 99% ethanol, centrifuged for 2 minutes in a Cytospin microfuge (Shandon, Pittsburgh, PA) to collect cells onto glass slides, and the cells air-dried. Cells were subsequently permeabilized with 0.2% Triton X-100 in PBS for 5 minutes at room temperature and incubated with rb122 (1:200) or uroplakin 3 (1:150) for 1 hour at room temperature. Detection was done using the DakoChemMate detection kit (DAKO, Glostrup, Denmark), which is based on an indirect streptavidin-biotin technique using a biotinylated secondary antibody. As a negative control, primary antibodies were either omitted or replaced with nonimmune rabbit or mouse serum as described (8). All such control sections were negatively stained. Tumor cells were rated ADAM12-positive when the immunostaining reaction was clearly above the negative background. Cells were examined using a Zeiss Axioplan microscope connected to an AxioCam camera using the AxioVision software.

Western blotting of urine samples. Urine samples from patients with bladder cancer whose tumors had been analyzed by microarray were also analyzed by Western blotting. Urine was collected from 11 patients with T_a tumors (one grade 1 tumor case, nine grade 2 tumor cases, and one grade 3 tumor case), 4 patients with T₁ (all being grade 3 cases), and 17 patients with T₂₋₄ tumors (16 grade 3 tumor cases and one grade 4 tumor case). In addition, urine from six patients with non-muscle-invasive bladder tumors was collected at three time points: (a) prior to transurethral resection, (b) during the surveillance period in which no tumor could be detected, and (c) when recurrence of invasive tumor was diagnosed. Urine samples were collected immediately into sterile containers before surgery or control cystoscopy and centrifuged, and the pellets and supernatants frozen at -80°C. Samples containing blood were excluded. Cytology specimens were assessed and considered positive only when malignant cells were present. Urine samples from eight volunteers (Caucasians) with no history of bladder tumors (age

range, 25-65 years old) served as normal standards. Normal standard specimens were selected to evaluate the specificity of the Western blot and included five cases of benign prostatic hyperplasia and two cases of pregnancy. To reduce the amount of albumin, all urine samples were absorbed with Fast flow Cibacron blue 3GA (Sigma) for 3 hours at 4°C before analyses. Protein concentration was measured using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Urine samples (40 µg) or purified ADAM12-S (28, 29) were boiled in SDS sample buffer with (reducing) or without DTT (nonreducing) and resolved by NuPAGE 12% Bis-Tris gels (Invitrogen), followed by electrophoretic transfer to Immobilon-P membranes (polyvinylidene difluoride membranes from Millipore Corp. Billerica, MA). Membranes were blocked overnight with 5% nonfat dried milk at 4°C, then incubated with primary polyclonal or monoclonal antibodies against human ADAM12. Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were used as secondary antibodies. Chemiluminescent detection of horseradish peroxidase was done by standard methods (Amersham Pharmacia Biotech, Uppsala, Sweden). The densities of the observed 68 kDa band were estimated from films using the NIH Image 1.61 program (<http://rsb.info.nih.gov/nih-image>). Urine from each of the volunteers was pooled and used as a normal

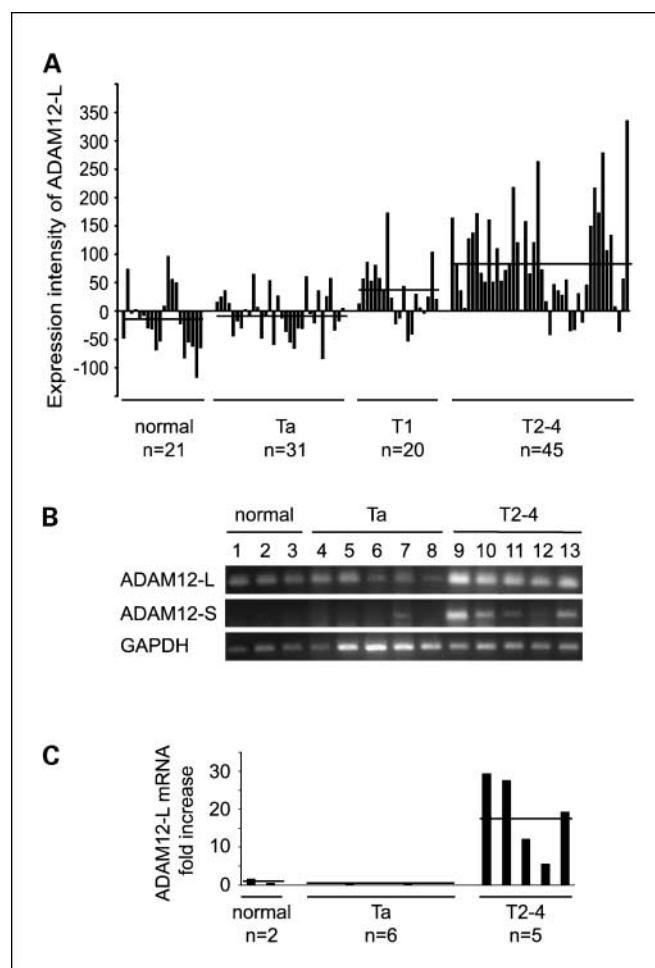


Fig. 1. Gene expression profiling of ADAM12 in bladder cancer. **A**, microarray analysis of ADAM12-L gene expression levels in 21 normal bladder mucosa samples, 31 T_a tumors, 20 T₁ tumors, and 45 T₂₋₄ tumors. **B**, reverse transcription-PCR analysis of mRNA expression of human ADAM12-L, ADAM12-S, and glyceraldehyde-3-phosphate dehydrogenase in normal bladder mucosa tissue (lanes 1-3), T_a (lanes 4-8), and T₂₋₄ (lanes 9-13) bladder cancer. **C**, qPCR analysis of ADAM12-L mRNA expression in two normal bladder mucosa samples, six T_a, and five T₂₋₄ tumors. Horizontal lines, the average expression intensity in each group (A and C).

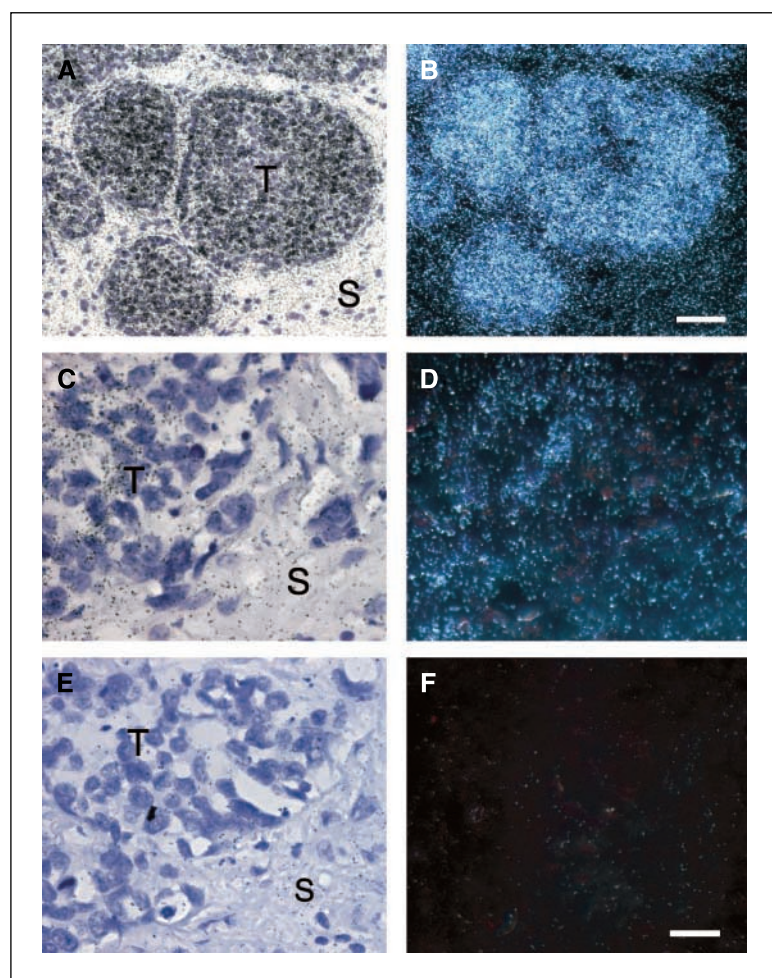


Fig. 2. *In situ* hybridization of ADAM12 in bladder cancer. *A*, tumor sections from ADAM12-MMTV-PyMT mouse breast cancer tissue. Strong hybridization signal with ADAM12 T₃ antisense probe is present as dark grains over the tumor islets and only very weak signals are seen over the surrounding stroma. *B*, dark-field image of the same tumor area as in (*A*). *C*, tumor sections from human bladder cancer (grade 2) with positive signal over the tumor cell (*T*) using T₃ antisense probe. *D*, dark-field image of the same tumor as in (*C*). *E*, adjacent tumor sections from the same bladder tumor as in (*C* and *D*) with ADAM12 T₃ sense probe hybridization with little or no signal. *F*, dark-field image of the same tumor area as in (*E*). Bar, 20 μ m (*B*); bar, 8 μ m (*F*). *A* and *B* use the same magnification and *C* to *F* use the same magnification.

standard on each of the Western blots. The densitometric score of the pooled normal standard was used to estimate the apparent amount of the ADAM12 68 kDa band in urine from normal individuals and patients with cancer. In some experiments, the immunoprecipitation of 500 μ L aliquots of urine supernatant was done as described using a mixture of mouse monoclonal antibodies (6E6, 8F8, and 6C10; refs. 30, 31) and subjected to Western blot as described above.

Statistical analysis. Statistical analysis was done using the Mann-Whitney test, the Student's *t* test, or the χ^2 (Pearson). *P* < 0.05 values were considered statistically significant.

Results

ADAM8, 10, and 12 gene expression in bladder cancer correlates with disease status. Gene expression profiling was done using a customized Affymetrix GeneChip array. This GeneChip contained probe sets for the specific detection of 18 different ADAM transcripts (ADAM2, 3a, 5, 8, 9, 10, 11, 12, 15, 19, 20, 22, 23, 28, 29, 30, 32, and 33). We found that only ADAM8, 10, and 12 had a positive correlation between gene expression and the disease stage of bladder cancer (Fig. 1A; Supplemental Fig. S1). In the present study, we subsequently focused only on the expression of ADAM12 in bladder cancer. The GeneChip contained transcript variants of both ADAM12-L and ADAM12-S. ADAM12-L was expressed at low levels in normal bladder biopsies and T_a tumors (average expression intensity: -17 and -6, respectively), higher levels in T₁ tumors

(average expression intensity, 33), and at the highest levels in T₂₋₄ tumors (average expression intensity, 89; Fig. 1A). We found a highly significant difference between the expression of ADAM12-L in normal tissue and T_a tumors compared with T₁ tumors (*P* = 0.00074, Student's *t* test) and to T₂₋₄ tumors (*P* = 1.0×10^{-10}). ADAM12-S transcripts were not detected in the bladder tumors using this array.

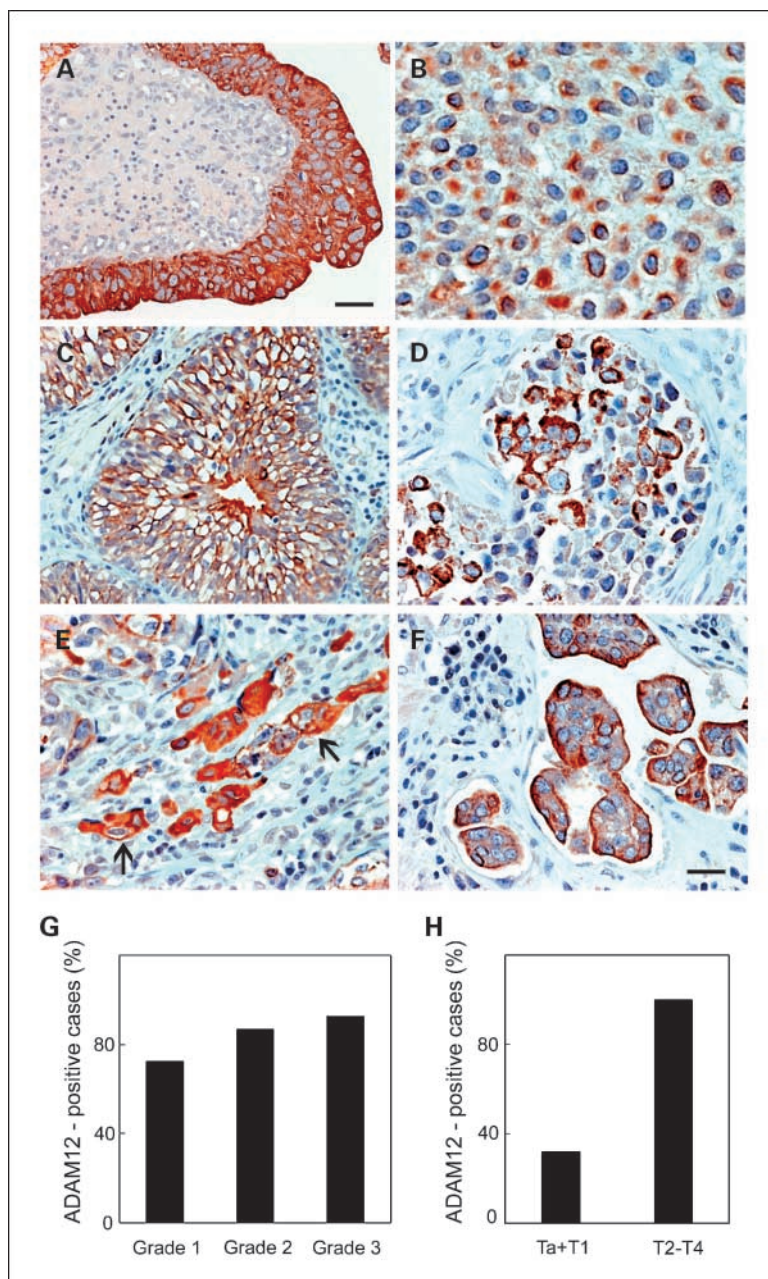
To determine and quantitate the presence of ADAM12-L and -S mRNA in tumor tissue from a subset of the patients analyzed by microarray (three normal, five T_a, and five T₂₋₄), reverse transcription-PCR, and qPCR were done. Using reverse transcription-PCR, ADAM12-L was detected in all samples and ADAM12-S was largely present in the T₂₋₄ tumor samples (Fig. 1B). The PCR products were sequenced, and comparison of the sequences to the GenBank verified the identity of nucleotides 2378-2512 in ADAM12-S (AF023477) and nucleotides 2816-2916 in ADAM12-L (AF023476). We developed a method for qPCR for ADAM12-L and used the method to analyze a subset of the patients analyzed by microarray (two normal, six T_a, and five T₂₋₄). ADAM12-L mRNA was expressed at ~15-fold higher levels in T₂₋₄ tumors compared with normal tissue (Fig. 1C; *P* = 0.017, Student's *t* test).

ADAM12 gene expression in bladder cancer is concentrated in tumor cells. Single-stranded sense and antisense ³⁵S-labeled RNA probes were generated by *in vitro* transcription of ADAM12 cDNA and used for *in situ* hybridization on tumors

obtained from the MMTV-PyMT mouse breast cancer model in which transgenic human ADAM12 is expressed (21). Intense positive signals for ADAM12 were found in the murine breast carcinoma cells with the antisense probes (Fig. 2A and B). The sense probes gave only a background signal (data not shown). This result confirmed the specificity of the probes for human ADAM12. These probes were subsequently used to examine ADAM12 mRNA expression in human bladder cancer tissue (Fig. 2C-F). Positive signals for ADAM12 were found in the tumor cells in all grades with the antisense probes, whereas lower signals were observed in the surrounding stroma (Fig. 2C and D). Much lower levels of signals were found with the sense probes in either the tumor cells or in the surrounding stroma (Fig. 2E and F). These results confirm that ADAM12 mRNA is expressed in human bladder cancer and is located primarily in the tumor cells.

ADAM12 immunostaining correlates with tumor grade and stage. The distribution of ADAM12 protein in bladder cancer tissue was evaluated by immunohistochemistry on tissue arrays (Fig. 3A-F). In most cases, tumor cells exhibited strong immunostaining. Areas representing apparent invasive fronts seemed to be the most intensely stained (Fig. 3E), and strongly positively stained tumor cells could be seen in small blood vessels (Fig. 3F). A few occasional stromal cells exhibited immunostaining. To evaluate the correlation between ADAM12 protein expression and tumor grade (histologic criteria), 155 cases of bladder carcinomas from three different tissue arrays were immunostained. Samples from a great majority of the cases (87%, 135 cases) exhibited positive ADAM12 immunostaining. More specifically, 93% (77 cases) of grade 3, 85% (46 cases) of grade 2, and 72% (12 cases) of grade 1 tumor

Fig. 3. ADAM12 immunostaining of bladder cancer tissue arrays. Tissue sections were incubated with a polyclonal antibody to human ADAM12 (rb122), then detection was done with a streptavidin-biotin technique. *A*, non-muscle-invasive papillary bladder cancer (T₁, grade 1) with strong positive staining for ADAM12 in most of the tumor cells. *B*, non-muscle-invasive papillary bladder cancer (T₁, grade 2) with uniform ADAM12 cytoplasmic immunostaining confined to the perinuclear Golgi-like area. *C*, invasive bladder cancer (T₂, grade 2) with ADAM12 immunostaining localized mostly along the plasma membranes. *D*, invasive bladder cancer (T₃, grade 3) with ADAM12 immunostaining in the cytoplasm in some cells whereas other tumor cells are less intensively stained. *E*, invasive bladder cancer (T₃, grade 3) with strong ADAM12 staining of tumor cells along the invasive front of the tumor. *F*, invasive bladder cancer (T₃, grade 3) with strong ADAM12 immunostaining of tumor cells located inside the blood vessels. *G*, ADAM12 immunostaining and correlation with tumor grades 1 to 3 (histopathologic diagnosis). The number of grade 3 tumor cases (%) positive for ADAM12 staining is significantly higher than the number of grade 1 tumor cases positive for ADAM12, $P < 0.005$ (χ^2 ; Pearson). *H*, ADAM12 immunostaining and correlation to tumor stage (tumor-node-metastasis). The number of T₂ to T₄ tumor cases (%) positive for ADAM12 staining is significantly higher than the number of T_a to T₁ tumor cases positive for ADAM12, $P < 0.00001$ (χ^2 ; Pearson). Sections were counterstained with hematoxylin. Bar, 20 μ m (*A*); bar, 10 μ m (*F*). *A* and *C* use the same magnification; *B*, *D* to *F* use the same magnification.



samples were positive for ADAM12 (Fig. 3G). The difference between the number of grade 3 and the number of grade 1 tumors positive for ADAM12 staining was found to be statistically significant ($P < 5 \times 10^{-3}$; χ^2 ; Pearson). To evaluate the correlation between ADAM12 expression and tumor stage, a tissue array with 40 cases staged according to the tumor-node-metastasis system was evaluated (Fig. 3H). All the T₂₋₄ tumors (18 cases) exhibited ADAM12-positive staining, whereas only 32% of the T_a + T₁ tumors (22 cases) were immunoreactive for ADAM12 ($P < 1 \times 10^{-5}$; χ^2 ; Pearson).

Distinct ADAM12 immunostaining of umbrella cells in the normal mucosa. ADAM12 protein expression was examined in adjacent nontumorous mucosa and mucosa from patients without bladder cancer. In most cases, the normal urothelium stained very weakly (Fig. 4A). Interestingly, the so-called umbrella cells often exhibited intensely positive ADAM12 staining. ADAM12 was located both intracytoplasmically and

along the cell membrane in these cells (Fig. 4C). The identity of these cells as umbrella cells was confirmed by immunostaining with antibodies to uroplakin 3 (Fig. 4D), an umbrella cell marker (32, 33). Umbrella cells shed into the urine were also immunoreactive with antibodies to ADAM12, whereas squamous epithelial and other urothelial cells were negative or only weakly positive (Fig. 4E and F). Interestingly, urothelium with atypical or dysplastic characteristics showed increased positive cytoplasmic ADAM12 immunoreactivity (Fig. 4G-I). Finally, we found that “umbrella-like” differentiated tumor cell in the bladder cancer tissue (34) exhibited striking ADAM12 immunoreactivity (Fig. 4J).

ADAM12 is detected in the urine from patients with bladder cancer. Purified human ADAM12-S appears as two separate bands on SDS-PAGE. The 68 kDa band represents the metalloprotease, disintegrin, cysteine-rich, and epidermal growth factor-like domains, and the 27 kDa band represents the

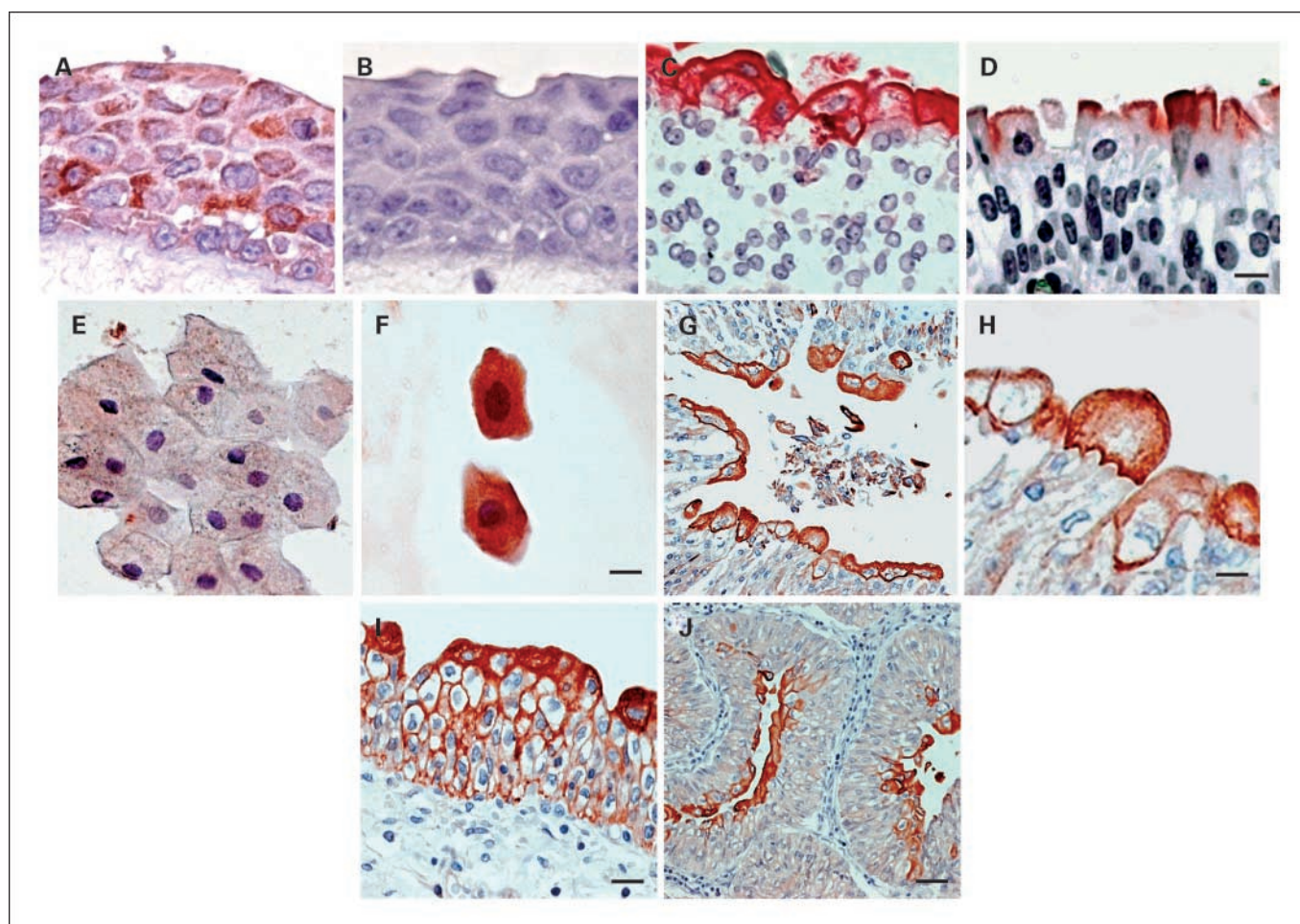


Fig. 4. ADAM12 immunostaining of normal and dysplastic bladder mucosa. Tissue sections were incubated with polyclonal antibodies to human ADAM12, then detection was done with a streptavidin-biotin technique. *A*, normal bladder urothelium exhibited weak ADAM12-S staining with rb116 and (*B*) no ADAM12 immunoreactivity with preimmune serum. *C*, the umbrella cells exhibited strong ADAM12-positive staining. *D*, the apical surface of umbrella cells also stained with antibodies to uroplakin 3, an umbrella cell marker. *E*, squamous epithelial cells isolated from the urine did not exhibit ADAM12 immunostaining, whereas (*F*) the umbrella cells in the urine exhibited strong ADAM12 immunostaining (rb122). Note the larger nuclei of the umbrella cells compared with the squamous cells. *G*, atypical hyperplasia showed strong ADAM12 immunostaining in the umbrella cells, whereas the underlying epithelium exhibited only weak staining. *H*, larger magnification of one of the umbrella cells in (*G*). Note the strong immunoreaction, particularly along the cell periphery. *I*, carcinoma *in situ* exhibited intense ADAM12 immunostaining of the epithelial cells. *J*, transitional cell carcinoma (grade 2) showed the strongest ADAM12 immunostaining in the most non – muscle-invasive tumor cells that mimic the morphology of umbrella cells (named “umbrella-cell differentiation”). This staining pattern was found in 23 out of 155 cases of bladder tumors (14.8%) examined in this study. Sections were counterstained with hematoxylin. Bar, 7 μ m (*D*); bar, 5 μ m (*F*); bar, 7 μ m (*H*); bar, 8 μ m (*I*); bar, 20 μ m (*J*). *A*, *B*, *E*, *F* use the same magnification; *C* and *D* use the same magnifications; and *G* and *J* use the same magnifications.

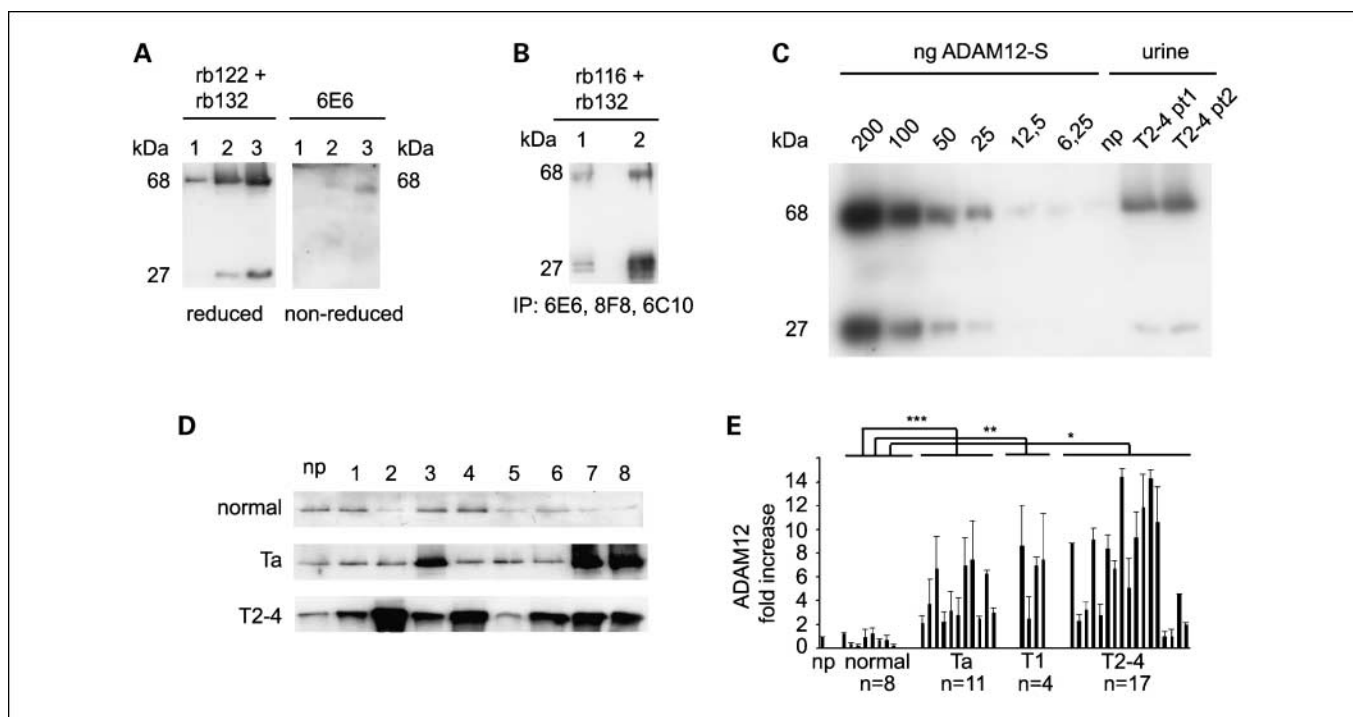


Fig. 5. Western blotting analysis of ADAM12 in urine from normal controls and patients with bladder cancer. *A*, urine from normal controls (lane 1) and from two patients with a T₂₋₄ tumor (lane 2 and 3) prepared using reducing or nonreducing conditions were loaded onto SDS-PAGE gels, transferred to Immobilon-P, and probed with a mixture of polyclonal antibodies against human ADAM12 [one directed against the cysteine-rich domain (rb122) and the other the prodomain (rb132)], or a monoclonal antibody against ADAM12 (6E6). The 68 and 27 kDa bands represent the mature form and the prodomain of ADAM12, respectively. *B*, immunoprecipitate of urine supernatant using a mixture of monoclonal antibodies (6E6, 8F8, and 6C10) against ADAM12 (lane 1) and purified ADAM12-S (lane 2) were immunoblotted with a mixture of antibodies against the carboxyl-terminus and the prodomain of ADAM12-S (rb116 and rb132). *C*, estimate of the relative amount of ADAM12 in urine supernatant using purified ADAM12-S as standard. Forty micrograms of protein were loaded per lane [for the pool of normal urine (np) 6 μ L was loaded, and for the two T₂₋₄ patients (pt 1 and pt 2) 12 and 4 μ L were loaded, respectively]. Urine samples were immunoblotted using a mixture of polyclonal antibodies rb122 and rb132. *D*, representative urine samples (40 μ g protein per lane) from normal controls (top), patients with T_a tumors (middle), and patients with T₂₋₄ tumors (bottom) were immunoblotted with rb122. The protein band represents the mature form of ADAM12-S at 68 kDa. On all Western blots, a pool of normal urine is presented in the first lane (np). *E*, densitometric quantitation of the ADAM12 68 kDa band signal present in urine from 8 normal volunteers, 11 patients with T_a tumors, 4 patients with T₁ tumors, and 17 patients with T₂₋₄ tumors. The pool of normal urine (np) was used to normalize the apparent amount of ADAM12 in normal and cancer urine. Points, mean of triplicate experiments; bars, SE; *, $P = 0.0004$; **, $P = 0.0001$; ***, $P = 0.00021$ (Student's t test).

prodomain that remains noncovalently associated with the body of the molecule following furin cleavage (29, 35). Urine from patients with bladder cancer was subjected to Western blotting analysis using a series of different ADAM12 domain-specific antibodies. Polyclonal antibodies to the cysteine-rich domain (rb122) recognized the 68 kDa band, whereas polyclonal antibodies to the prodomain (rb132) recognized the 27 kDa band (Fig. 5A). Under nonreducing conditions, a monoclonal antibody against ADAM12 (6E6) detected a protein band migrating slightly faster than the 68 kDa protein as previously reported (35). In addition, monoclonal antibodies to the disintegrin domain (2F7) reacted with the 68 kDa band and occasionally to a 50 kDa band that seems to be a degradation product (data not shown). Immunoprecipitation of urine supernatant using monoclonal antibodies against ADAM12, followed by immunoblotting with polyclonal antibodies specific for the carboxyl-terminus of ADAM12-S (rb116) and for the prodomain rb132 detected ADAM12-S in the urine of patients with bladder cancer (Fig. 5B).

To determine the approximate level of ADAM12 in urine from healthy individuals and cancer patients, we compared the amount of ADAM12 in urine with a standard of purified ADAM12-S (Fig. 5C). Using densitometric quantitation of the 68 kDa band, we found ~4 to 10 μ g of ADAM12 per milliliter of urine from patients with cancer. In normal urine, ADAM12

was only weakly detected, i.e., <1 μ g/mL urine (Fig. 5D and E). To further quantitate the relative amount of ADAM12 in cancer urine compared with urine from healthy controls, we examined 32 samples (11 T_a, 4 T₁, and 17 T₂₋₄) of cancer urine and eight samples of healthy control urine by Western blotting and densitometric quantitation (Fig. 5E). Importantly, the relative amount of ADAM12 protein was significantly higher in urine from patients with a T_a tumor (~4-fold increase; $P = 0.0002$, Student's t test), T₁ tumor (~6-fold increase; $P = 0.0001$, Student's t test), or with an invasive bladder tumor (T₂₋₄; ~7-fold increase; $P = 0.0004$, Student's t test) than in urine from normal individuals. We also compared the relative level of ADAM12 mRNA from the microarray experiments with the apparent level of ADAM12 protein in the urine, but found no correlation (data not shown).

Routine cytology was done on 29 bladder cancer cases, and identified 86% of the bladder cancers (Table 1). The level of ADAM12 in the urine of these 29 cases was examined by Western blot. We chose to use a >2-fold increase in the relative level of ADAM12 compared with normal control by Western blot as "positive." The relative levels of ADAM12 alone detected 97% (28 of 29) of the bladder cancers. In combination with cytology, the relative level of ADAM12 detected 100% of the tumor cases. Importantly, ADAM12 detected 100% of the T_a and T₁ tumor cases, as well as 100% of the grade 2 tumors, whereas

Table 1. Sensitivity of the relative level of ADAM12 and cytologic analysis of voided urine by stage and grade of bladder cancer

	Cytologic analysis of voided urine		>2-fold increase in ADAM12 levels compared with normal control	
	No. detected/total cancers	Sensitivity (%)	No. detected/total cancers	Sensitivity (%)
Cancer stage				
T _a	7/9	78	9/9	100
T ₁	3/4	75	4/4	100
T ₂₋₄	15/16	94	15/16	94
Overall	25/29	86	28/29	97
Grade				
2	7/9	78	9/9	100
3	13/15	87	15/15	100
4	5/5	100	4/5	80
Overall	25/29	86	28/29	97

cytology only detected 78% of T_a, 75% of T₁, and 78% of grade 2 tumors. To evaluate the specificity of the Western blot, we analyzed the urinary levels of ADAM12 obtained from five cases of benign prostatic hyperplasia and two cases of pregnancy. The level of ADAM12 in the urine of these cases did not differ from the control healthy individuals (data not shown).

ADAM12 in the urine of bladder cancer patients following surgical removal of tumor correlates with the presence of tumor. We analyzed two cases of T_a and four cases of T₁ tumors that all eventually progressed to the T₂₋₄ stage. In all tested cases, ADAM12 was detectable in the urine prior to surgery. Figure 6 illustrates a patient follow-up with decreasing urinary levels of ADAM12 after removal of T_a tumor and increasing levels with recurrence of invasive tumor. In both T_a tumor cases and in one T₁ case, the level of urine ADAM12 decreased following removal of the tumor, and increased again with the appearance of invasive tumor (Table 2, cases A, B, C). In one case, the urinary level of ADAM12 did not decrease during the period of surveillance; however, selected site biopsies from this patient showed carcinoma *in situ* (Table 2, case D).

Discussion

There is a tremendous need to identify comprehensive biomarkers to predict, diagnose, and monitor disease, including cancer (4, 5). ADAM proteins are members of the metzincin superfamily of zinc-dependent proteases and have been implicated in normal and abnormal growth during development and in disease, such as cancer (13, 36). We have recently proposed that ADAM12 could be a valuable marker of breast cancer (22). In the present study, we report that the levels of ADAM8, 10, and 12 mRNAs are significantly up-regulated in human bladder cancer, and we examined, in more detail, the hypothesis that ADAM12 could be a valuable biomarker for bladder cancer.

A number of previous studies have shown that human tumor cells produce ADAM12 (7, 8, 10, 18–20, 22). In this study, ADAM12 mRNA expression was assessed by microarray analysis for the first time. Using microarrays, we found that bladder cancers express increased amounts of ADAM12 mRNA and that the level strongly correlates with disease status. We established a qPCR method for ADAM12 that confirmed the increase of ADAM12 mRNA in bladder cancer. Furthermore, *in situ*

hybridization showed that the bladder cancer cells are the site of ADAM12 gene expression. Immunohistochemistry showed that the protein expression pattern of ADAM12 correlates with tumor grade and stage. Finally, we documented that ADAM12 is present in increased amounts in urine from patients with bladder cancer when compared with the levels found in the urine of healthy controls.

Non-muscle-invasive bladder tumors can be successfully removed by transurethral resections, but the recurrence rate is high (30-70%), and the progression rate of superficially invasive cancer (T₁) to muscle-invasive cancer (T₂₋₄) is up to 60% in long-term follow-up (2, 3, 37). Extensive research has been undertaken to define biomarkers in urine that could either add to or replace cytology in follow-up for low-grade/stage bladder tumors (4, 5). Bladder tumor antigen, nuclear matrix

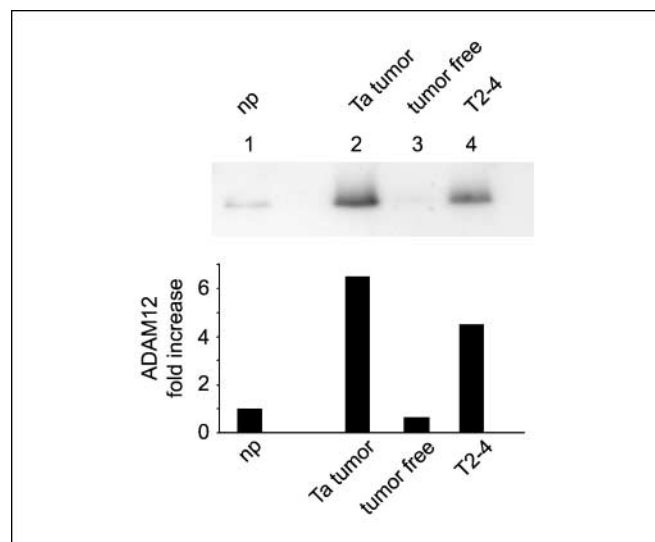


Fig. 6. Western blotting analysis of ADAM12 in urine of patients with bladder cancer who underwent surgical removal of tumor. Urine samples were subjected to immunoblotting using rb122 and densitometric quantitation of the resulting 68 kDa ADAM12 band was done. Top, urine from a pool of normal controls (*np*, lane 1), and from a patient (case A) with non-muscle-invasive bladder cancer prior to transurethral resection (T_a tumor, lane 2), during the surveillance period in which no tumor could be detected (*tumor-free*, lane 3), and when recurrence of invasive tumor was diagnosed (T₂₋₄, lane 4). Forty micrograms of total protein were applied per lane. Bottom, the pool of normal urine was used to normalize the apparent amount of ADAM12 in the cancer urine.

Table 2. Relative level of ADAM12 in urine (fold increase compared with normal)

Case	Superficial tumor	Surveillance period	Recurrence of tumor	Time from first diagnosis to recurrence of tumor (mo)
A	T _a , gr2		T ₂₋₄ , gr3-4	
	6	1	5	26
B	6	2	3	7
	T ₁ , gr3			
C	7	1	2	7
D	3	3*	2	6
E	7	5	1	13
F	9	10	10	17
Average	6.3 ± 2	3.6 ± 3	3.8 ± 3	12.6 ± 8

NOTE: Averages are presented as means ± SD.

*Carcinoma *in situ* was observed.

protein 22, fibronectin and its fragment, and cytokeratin 8, 18, 19, and 20 are among the most commonly evaluated markers; however, none of these are yet well-established in the clinical setting (5). In the present study, we found that whereas the urine level of ADAM12 was low in all healthy individuals, the urine levels of ADAM12 significantly increased in all patients with superficial noninvasive tumors (T_a), superficial invasive (T₁), and were highest in patients with invasive cancers (T₂₋₄). We also analyzed two cases of T_a tumors and four cases of T₁ tumors that eventually progressed to T₂₋₄ tumors. Importantly, we found that in most of these bladder cancer cases, the level of ADAM12 in the urine decreased following surgery, was minimal during the tumor-free period, but then increased again upon recurrence of tumor. Thus, monitoring ADAM12 in the urine of patients with bladder cancer might be a useful noninvasive diagnostic test, and it is possible that urinary ADAM12 could even be a marker of primary bladder cancer. Compared with cytology, measurement of ADAM12 levels was a more sensitive marker for detecting early-stage and/or low-grade tumors. Cytology is known to be less sensitive in early-stage and low-grade cancers (38, 39); therefore, a combination of cytology and measurements of the ADAM12 level could increase the sensitivity to almost 100%. To further validate the sensitivity, a larger sample size needs to be examined, and a larger study of patients with nonneoplastic bladder disorders should be included to predict the specificity of the assay. This study thus adds to our recent study on breast cancer, in which we reported that increased urinary levels of ADAM12 were found to correlate with breast cancer progression. In fact, we found that the "strength" (i.e., with regard to sensitivity, accuracy, and false-negative ratios) of ADAM12 in differentiating patients with breast cancer from those without was comparable to a number of other tumor markers currently in clinical use. Together, these two studies strongly advocate further studies to determine the efficacy of urinary ADAM12 levels as a routine biomarker for the prediction, diagnosis, and monitoring of progression of disease.

Human ADAM12 is produced in two splice variants, the prototype transmembrane, ADAM12-L, and the shorter secreted, ADAM12-S (14). The bladder cancer microarray we used only allowed us to determine the expression levels of ADAM12-L, whereas for unknown reasons, ADAM12-S was not detected. However, ADAM12-S mRNA could be detected from

bladder cancer tissue by reverse transcription-PCR. We therefore conclude that bladder cancers express both ADAM12-L and ADAM12-S. This is consistent with previous studies which found that levels of RNA for both forms of ADAM12 were increased in cirrhosis, hepatocellular carcinomas, and liver metastases from colorectal cancers compared with normal controls (18).

Consistent with our previous findings (22), we observed low levels of ADAM12 in urine from healthy individuals. In the present study, we were able to detect ADAM12 in the urine from all healthy individuals tested, whereas we previously found that ADAM12 was only detected in ~15% of control samples. The difference in detection rate could be related to differences in sampling and storage of the specimens or the membranes used for electrophoretic transfers in the two studies. We have recently found that Immobilon-P (polyvinylidene difluoride) membranes are more sensitive than nitrocellulose. We investigated which cells in the bladder might produce the ADAM12 that is found in the normal urine. Immunohistochemistry analysis of normal urothelium with an antibody that recognizes both ADAM12-L and ADAM12-S showed that the umbrella cells, the outer layer of specialized cells, exhibited strong immunostaining, whereas the underlying epithelium stained more weakly. We therefore suggest that the normal urothelium represents the most likely source of ADAM12 in normal urine. The identity of ADAM12 in the urine was validated using a number of different domain-specific antibodies. ADAM12 appears as a 68 kDa protein band representing the mature form and a 27 kDa band representing the prodomain that remains associated with the rest of the molecule following secretion (29, 35). The 68 kDa band could represent the mature form of ADAM12-S, a shed or otherwise truncated form of ADAM12-L, or a mixture of the two. To examine whether ADAM12-S is present in the urine of patients with bladder cancer, we examined urine using a polyclonal antibody that specifically recognizes a carboxyl-terminus ADAM12-S peptide. The 68 kDa band was detected in urine from patients with bladder cancer, confirming the presence of ADAM12-S. In contrast, polyclonal antibodies against the carboxyl-terminus of ADAM12-L did not detect a band in bladder cancer urine, suggesting that full-length ADAM12-L or a fragment truncated at the NH₂-terminal part is not present in significant amounts (data not shown). It is still possible,

however, that ADAM12-L could be shed from cell membranes and appear in the urine as a "tail-less fragment." Both previous studies (22) and the data obtained in the present study show that the level of ADAM12 is increased in the urine of patients with cancer. We hypothesize that ADAM12 is produced by the tumor cells and escapes into the urine—and may be designated "tumor ADAM12." Because the production of ADAM12 can be increased by cytokines, including transforming growth factor β (18, 40), we also suggest that the normal urothelium produces

more ADAM12 in the presence of a neighboring tumor—and may be designated "cytokine-induced ADAM12." Further studies are needed to test these hypotheses.

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