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Original Article

Proteomic Analysis of Urinary Fibrinogen Degradation Products in Patients With Urothelial Carcinomas

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Abstract

Despite many years of research efforts and continued progress in the identification of urine markers for detection of bladder cancer, none of the markers described to date has been able to replace cystoscopy and urine cytology, the current gold standards for diagnosis. Here, we present a comprehensive gel-based proteomic study in which we have analyzed the presence and origin of fibrinogen (FG) and its degradation products (FDPs) in the urine of patients with and without urothelial carcinoma (UCs), with the aim of evaluating the potential of two-dimensional (2D) gel FDP profiling for detecting bladder cancer. A total of 151 urine samples collected from patients with UCs of varying degrees of atypia and stages of invasion were compared with a control group consisting of 34 healthy volunteers with no clinical history of bladder cancer. The level and degree of degradation of FG in the urine were determined by 2D gel Western blotting in combination with enhanced chemilumenscence detection. Elevated levels of urine FG/FDPs were found in 99% of patients bearing superficial tumors, in 97% of the cases with early invasive disease, and in 96% of patients with highly invasive tumors. 2D gel profiling of urine FG/FDPs showed that the FG chains exhibited differential susceptibility to *in situ* proteolysis, with the α-chain being the most susceptible and the γ-chain the most resistant. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry identified peptide sequence regions in several of the most representative and common FDPs, which can be of value for producing novel specific antibodies to detect FG/FDPs in the urine. In addition, we present evidence indicating that FG is not produced by normal or malignant urothelium, although it is present both in the stroma of malignant tissue and tumor lesions. Taken together, the data indicate that increased levels of FG/FDPs amounts in the urine are a characteristic feature of bladder cancer, and emphasize the value of 2D gel profiling of urine FG/FDPs for detecting low-grade, noninvasive UCs.

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Key Words: Gel-based proteomics; urothelial carcinoma; urine fibrinogen degradation products.

Introduction

Bladder cancer is the fifth most common malignancy in the world, and represents the second most common type of urological cancer. It includes a wide spectrum of histologically heterogeneous tumor types that arise predominantly in the transitional epithelium (urothelium) lining of the urinary bladder and the ureters. Tumor types of the urothelium include urothelial carcinomas (UCs), squamous cell carcinomas (SCCs), adenocarcinomas, as well as other less frequent lesions (*1–3*). UCs comprise more than 90% of the diagnosed bladder tumors in the Western hemisphere (*4*,*5*) and are broadly divided in two groups—low-grade superficial papillary lesions and high-grade lesions (carcinoma *in situ* [CIS]) (*1*,*2*)—based on histological examination and clinical parameters (*6*,*7*). Superficial papillary tumors (pTa, pT1) represent approx 70% of all bladder tumors at first presentation, whereas 2–5% correspond to CIS or flat lesions (dysplasia, atypia), and the remaining display a metastasized tumor (*8*,*9*). Among the superficial tumors, about 40% are pTa and 30% are pT1 lesions.

Detection and characterization of bladder cancer has relied on classic histopathological techniques for many years. To date, cystoscopy and urine cytology still remain the gold standards in the diagnosis of bladder cancer. Cystoscopy, aided by histological evaluation of the biopsy specimens, is very effective in identifying morphologically visible tumors, but is an invasive procedure that causes a great deal of discomfort to the patient. Voided urine cytology is the oldest diagnostic test being used in the clinic; it is noninvasive, simple, and characterized by high specificity, i.e., yields relatively few false-positives (*10*). Several screening studies, however, have reported low sensitivity for this test, particularly for detecting superficial papillomas and/or low-grade tumors (*11–13*).

A number of noninvasive tests, based on the measurement of tumor-derived molecules that are potentially released into the urine from malignant lesions, have recently become available to clinicians, whereas others are under investigation (*14–22*). These include: the nuclear matrix protein 22 test (NMP-22; Matritech, Inc.), the bladder tumor antigen test (BTA; Bard Diagnostic), and the fibrinogen/fibrinogen degradation product (FG/FDPs) test. Several comparative studies have shown a broad variation in sensitivity and specificity of these tests. For example, the average sensitivity and specificity data published so far are 70.5 and 75.2% for NMP22; 52.3 and 84.6% for the BTA test; and 68.3 and 77.8% for the FDP test (*14*,*18*,*19*). It has been shown repeatedly that cytology provides higher specificity but lacks sensitivity compared to the NMP-22, BTA, and FDP tests (*14–18*), underscoring the need for a better overall test.

In general, the urinary FDP-based tests are simple, rapid, inexpensive, and can be performed in a clinical setting. Although various inflammatory conditions of the urinary tract can result in detectable amounts of FDP in the urine, several studies have shown that the presence of FDPs is far more prevalent in the urine of patients with bladder cancer and that the levels tend to be higher in patients bearing tumors of increasing grade and stage (*23–25*). This correlation may lead to improved sensitivity for detecting more aggressive tumors, although this issue has not been addressed systematically. The commercial Accu-Dx FDP test (the original AuraTek FDP has been replaced with the new label, Accu-Dx, Intracel Corp.) made use of a monoclonal antibody-based immunoassay to qualitatively measure the levels of FG/FDPs in the urine of patients with bladder neoplasias (*24*,*25*). Although the initial data appeared to be very promising, the manufacturer has

since withdrawn the assay because of the lack of stability and the problems associated with the test formulation. Indeed, the sensitivity and specificity of various FDP-based tests reported so far differ widely in different studies ranging from 27 to 100% and from 66 to 86% for sensitivity and specificity, respectively (26–32). Furthermore, as with other urine-based biochemical tests, the ability of the FDP-based tests to detect early-stage and low-grade tumors has not been properly established. It is still unclear whether the low sensitivity of urine FDP-based tests reported in several studies is caused, for example, by low affinity of the antibody used, or by the absence/very low level of FG/FDPs in the urine of some groups of patients with bladder cancer. Additionally, the origin(s) of FG/FDPs in urine of patients with bladder cancer is/are not yet known, a fact that hampers the improvement of urine FG/FDP-based diagnostic.

With these concerns in mind, we present here a comprehensive gel-based proteomic study in which we have assessed the presence of FG/FDPs in the urine and tumors obtained from 151 patients bearing bladder UCs. We show that the occurrence of FG/FDPs in urine samples can be readily assessed by using high-resolution two-dimensional (2D) gel electrophoresis in combination with Western blotting. Using this approach, it is possible to inspect the complexity of FG/FDPs by displaying all fibrinogen subunit chains and their proteolytic derivatives on a single 2D gel immunoblot. The main aims of the study were (1) to determine the occurrence and identity of FG/FDPs in the urine obtained from patients with bladder cancer bearing UCs of various grades of atypia and stages of invasion, (2) to investigate the intratissue localization of fibrinogen in urinary bladder, and (3) to identify peptide sequence regions in the most common urine FDPs, which may be later used as novel epitopes for designing improved FG/FDP-based tests.

Materials and Methods

UC and Nonmalignant Tissue Samples

Tumors and other biopsies removed by transurethral resection or from cystectomies at the Department of Urology at the Skejby Hospital (Aarhus, Denmark) were collected with informed consent from the patient. Small pieces of tumor tissue were dissected by the pathologist and immediately transported to the laboratory. Tumor samples for 2D-polyacrylamide gel electrophoresis (PAGE) analysis, having been cleaned of clots and contaminating tissues, were carefully dissected with the aid of a scalpel (2–3 mm^3), homogenized in a glass homogenizer containing 0.2–0.3 mL of lysis solution (9.8 *M* urea, 2% NP40, 2% ampholytes pH 7.0–9.0, 100 m*M* DTT) for 2D-PAGE analysis and kept at –20°C until use. Tumor pieces for cryostat sections were placed in liquid nitrogen immediately after surgery and were kept at –80°C. The classification and grading of UCs were carried out according World Health Organization 1978 (33) in the Department of Urology at the Skejby Hospital (Aarhus, Denmark). The Scientific and Ethical Committie of Aarhus County (Aarhus, Denmark) approved the project.

Urine Sampling and Processing

Voided urine samples (50–100 mL) were collected preoperatively (within 24 h preceding the operation) from the same patients with UC from whom tissue samples were obtained (*see* previous section). Urines were kept at 4°C overnight and centrifuged at 2000*g* for 10 min. The supernatant was transported to the laboratory for further processing. Samples (20–35 mL) were dialyzed at 4°C against distilled water (four changes) for 24 h using membranes with an 8-kDa cutoff. Control urine samples were obtained from healthy volunteers at the Institute of Cancer Biology and processed as described previously. Dialyzed urine samples were freeze-dried and solubilized in lysis solution (9.8 *M* urea, 2% NP40, 2% ampholytes

pH 7.0–9.0, 100 m*M* DTT) for 2D-PAGE analysis. Twenty microliters of lysis solution were used for 1 mL of freeze-dried urine.

Collection and Analysis of Bladder Tumor Interstitial Fluid

Tumor interstitial fluid (TIF) collection was performed as previously described (*34*). Briefly, fresh tumor samples (0.2–0.3 g) were minced carefully with the aid of a scalpel into small specimens (about 1 mm^3), rinsed in phosphate-buffered saline (PBS), placed in a conical plastic tube containing 0.4 mL of PBS, and incubated for 1 h at 37°C in a humidified CO_2 incubator. Following incubation, the samples were centrifuged at 200*g* for 2 min followed by centrifugation at 2000*g* for 20 min at 4°C. The supernatant was lyophilized and resuspended in 0.3 mL of lysis buffer for 2D PAGE analysis.

2D Gel Electrophoresis and 2D Western Blotting

Freeze-dried samples resuspended in lysis solution were subjected to 2D PAGE as we previously described (*35*). About 30–40 µL of sample were applied to the first dimension, and several gels were run from each sample. Proteins were visualized using a silver staining procedure compatible with mass spectrometry (MS) analysis (*36*). Immunoblotting was performed as previously described (*37*) using a polyclonal antibody (A0080, dilution 1:500) raised against human fibrinogen obtained from DakoCytomation (Denmark). Secondary antibodies conjugated to a peroxidase complex (horseradish peroxidase [HRP]-conjugated goat anti-rabbit antibody; DakoCytomation, Denmark) were used at a dilution of 1:1000. 2D blots were developed using the enhanced chemiluminescent (ECL) assay (Amersham).

The Levels of FG/FDPs in Urine

The presence of FG/FDPs in urine samples obtained from healthy individuals and from patients with UC was judged by semiquantitative analysis of 2D Western ECL blots. The total intensity of the FG/FDP spots displayed on the blot was evaluated using the PDQUEST 7.3 image software. On average, the levels of urine FG/FDPs in patients with UC were found to be at least two orders of magnitude higher than those in the normal controls. Accordingly, immunoblot FG/FDPs patterns were subdivided into two categories: "normal" and "disease phenotype," which corresponded to low/undetectable and high levels of FG/FDPs, respectively. To characterize the level of FG proteolysis, the disease phenotype group was further subdivided into moderate (M) and high degradation patterns (H) based on the visual assessment of a variety of low molecular mass FDPs (less than 30 kDa).

Immunoprecipitation

Rabbit polyclonal anti-human fibrinogen antibodies (A0080, DakoCytomation, Denmark) were chemically crosslinked to protein A/G Sepharose (Amersham) using dimethylpimelimidate as a coupling agent as described elsewhere (*38*). Lyophilized urine samples were solubilized in a buffer containing 150 m*M* NaCl, 50 m*M* Tris-HCl, pH 7.6, 0.1% NP40, and incubated in presence of Ab-fibrinogen-A/G protein sepharose beads for 12 h at 4°C.

Mass Spectrometry

Protein spots were excised from the dry gels followed by rehydration in water for 30 min at room temperature. The gel pieces were detached from the cellophane film, rinsed twice with water, and cut into about 1-mm^2 pieces with subsequent additional washes. Proteins were "in-gel" digested with bovine trypsin (unmodified, sequencing grade; Roche Diagnostics, Mannheim, Germany) for 12 h as described by Shevchenko and colleagues (*39*). MS was performed on a Reflex IV matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer, equipped with a Scout 384 ion source.

All spectra were internally calibrated using the tryptic autodigested ions (805.417/906.505/1153.574/1433.721/2163.057/2273.160) visible in all spectra. Spectra were processed by the Xmass 5.1.1 and BioTools 2.1 softwares (Bruker Daltonik, GmbH). Searching was performed against a comprehensive NCBInr database, compiled from GenBank CDS translations, PIR, SWISS-PROT, PRF, and PDB, using the MASCOT software. No restriction on the protein molecular mass and taxonomy were applied. Because proteins were recovered from gels, a number of fixed modifications (acrylamide modified cystein, i.e., propionamide/carbamidomethylation) as well as variable ones (methionine oxidation and protein N-terminus acetylation) were included in the search parameters.

Immunohistochemistry

Fresh tumor pieces were placed in formalin fixative and were paraffin-embedded for archival use. Five-microliter sections were cut from the paraffin-embedded tissue blocks and mounted on Super Frost Plus slides (Menzel-Gläser, Braunschweig, Germany), baked at 60°C for 60 min, deparaffinized, and rehydrated through graded alcohol rinses. Heat-induced antigen retrieval was performed by immersing slides in 10 m*M* citrate buffer (pH 6.0) and microwaving in a 750-W microwave oven for 10 min. The slides were then cooled at room temperature for 20 min and rinsed abundantly in tap water. Nonspecific staining of slides was blocked (10% normal goat serum in PBS buffer) for 15 min, and endogenous peroxidase activity quenched using 0.3% H_2O_2 in methanol for 30 min. Fibrinogen was detected with a primary antibody (dilution 1:500), followed by a secondary antibody conjugated to a peroxidase complex (HRP-conjugated goat anti-rabbit antibody; DakoCytomation, Denmark, dilution 1:1000). Finally, color development was done with 3,3′-diaminobenzidine (Pierce, IL) as a chromogen to detect bound antibody complex. Slides were counterstained with hematoxylin. Normal goat serum, instead of primary antibody, was used as a negative control. Standardization of the incubation and development times allowed an accurate comparison of expression levels in all cases.

Indirect Immunofluorescence

Eight-microliter cryostat sections from frozen tissue were placed on round cover slips, washed three times with PBS, and treated for 5 min with methanol at –20°C. Cover slips were washed several times with PBS, covered with 20 µL of the primary antibody (A0080, DakoCytomation, Denmark) and incubated for 60 min at 37°C in a humidified box. Next, the formed antibody complexes were detected with tetramethyl rhodamine isothiocyanate-labeled secondary antibody (dilution 1:50). Cover slips were washed extensively with PBS, then once with distilled water, and covered with DAKO mounting medium. Normal goat serum, instead of primary antibody, was used as a negative control. Sections were imaged using either standard epilumenscence fluorescence microscopy (Leica, DMRB, Deerfield, IL) or laser scanning microscopy (Zeiss 510LSM; Oberkochen, Germany).

Results

Specificity of the Fibrinogen Antibody

The specificity of the fibrinogen antibody used in this study (A0080, DakoCytomation) was determined using normal human plasma (source of native fibrinogen; Korda, Life Science), as well as a commercially available purified fibrinogen preparation (Kordia, Life Science). As shown in **Fig. 1**, the antibody reacts with all three fibrinogen chains (α, 67 kDa; β, 58 kDa; γ, 47 kDa) yielding multiple charge trains that correspond to posttranslational modifications. The antibody also recognized a heterogeneous group of proteins spots of apparent

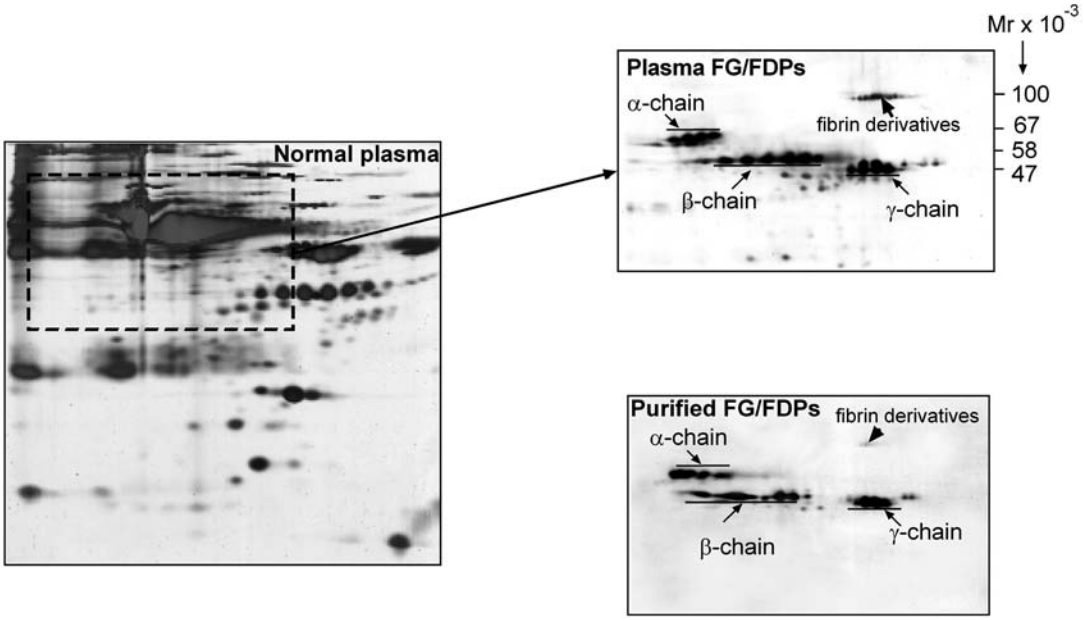

Fig. 1. Specificity determination of the antifibrinogen polyclonal antibody (A0080, DakoCytomation) by two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) Western blotting of human plasma proteins. **Left panel:** human plasma proteins resolved by 2D PAGE and stained by silver nitrate; **upper right panel:** 2D Western blot of plasma proteins; **lower right panel:** 2D Western blot of purified fibrinogen. The immunoblot shown in the upper right panel corresponds to the area of the 2D gel of plasma proteins limited by the dashed rectangle. Putative crosslinked derivatives of soluble fibrin are indicated with arrowheads.

molecular weights of 100–120 kDa that may represent crosslinked derivatives of soluble fibrin polymer (**Fig. 1**, closed arrowheads).

Western Blot Profiling of FG/FDPs in the Urine of Patients in the Control and UC Groups

A total of 151 urine samples collected from patients diagnosed with UC of various degrees of atypia and stages of invasion, and 34 control urine samples collected from healthy volunteers with no known clinical record of bladder malignancy were analyzed by 2D gel immunoblotting and ECL detection using the A0080 fibrinogen antibody (DAKO) (*see* Supplementary Table). The gel immunoblot analysis indicated that with a few exceptions (*see* Supplementary Table and **Table 1**), the urine samples of patients with UC exhibited much higher levels of FG/FDPs as compared to the normal controls (**Fig. 2**). In addition, the analysis revealed that the degree of fibrinogen degradation varied considerably among patients with UC (**Fig. 2**). An overall trend of the data were the increasing proteolytic degradation of fibrinogen, and the upregulation of the amount of urinary FG/FDPs in high-grade and stage UCs (**Fig. 2B–D**). Moreover, the results showed that the fibrinogen chains display differential susceptibility to *in situ* proteolysis, with the α-chain being the most susceptible and the γ-chain the lesser. The intact form of the fibrinogen γ-chain was detected in all the urines from patients diagnosed with UCs, including highly invasive stages of the disease (**Fig. 2B–E**), whereas the α-chain was more susceptible to degradation and as a result could not be detected in most of the superficial lesions (**Fig. 2B**,**C**).

Table 1
Urine FG/FDP Patterns in Normal Individuals and Patients Diagnosed With UC of Different Grade and Stage

Urine type	No. of donors/ patients	FG/FDP 2D phenotype			Disease FG/FDPs phenotype (%)	Normal FG/FDPs phenotype (%)
		Normal phenotype	Disease phenotype			
			Moderate (M) degradation	High (H) degradation		
Normal	34	32	1	1		94%
Superficial (GI-GIII, pTa)	74	1 (1.3%)	48 (65%)	25 (33%)	99%	
Early invasive (GI-GIII, pT1)	29	1 (3.6%)	13 (45%)	15 (52%)	97%	
Highly invasive (GII-GIV, pT2-4)	48	2 (4.1%)	9 (18%)	37 (77%)	96%	
Overall					97%	94%

To assess the presence of FG/FDPs in urine samples obtained from the control group and the UC group, the intensities of the FG/FDP spots were evaluated by quantitative analysis of the 2D gel Western ECL blots (*see* Materials and Methods). We found that the levels of FG/FDPs in the UC group were on average at least two orders of magnitude higher than those in the control group. Based on this observation, we subclassified the immunoblot patterns into two categories, namely "normal" and "disease phenotype" (**Fig. 2**). The disease phenotype was further subdivided in moderate (M) and high degradation patterns (H) based on the visual assessment of a variety of low-molecular-mass FDPs (less than 30 kDa) as illustrated in **Fig. 2B–E**.

Most of the patients diagnosed with high-grade/stage UC lesions exhibited more intense and a higher number of degradation products of fibrinogen in the urine as compared with patients bearing superficial papillary tumors (**Table 1** and **Fig. 2B–E**). As shown in **Table 1**, the proportion of patients with superficial tumors (pTa), early invasive (pT1), and highly invasive (pT2-4) tumors that exhibited the M and H disease phenotypes were 65 and 33%, 43 and 54%, and 18 and 77%, respectively.

The analysis of the 34 normal urine samples revealed very weak or even undetectable levels of FG/FDPs (normal phenotype) (**Table 1** and **Fig. 2A**), although in two individuals we observed elevated levels of FG/FDPs similar to those that are characteristic of the disease phenotype (false-positives). One of them, however, was later found to suffer from chronic cystitis.

Origin of FG/FDPs in the Urine

The presence of traces of fibrinogen in the urine of healthy donors has recently been reported by Pieper and colleagues ***(40)***. The origin of the peptides is unknown at present as, under normal physiological conditions, only small amounts of proteins reach the urinary bladder as a result of the plasma filtration process that take place in the kidney glomeruli. In the case of patients with bladder cancer, however, it is likely that the presence of plasma proteins in the urine may be caused by, in part, bleeding caused by tumor growth. To address this possibility, we analyzed

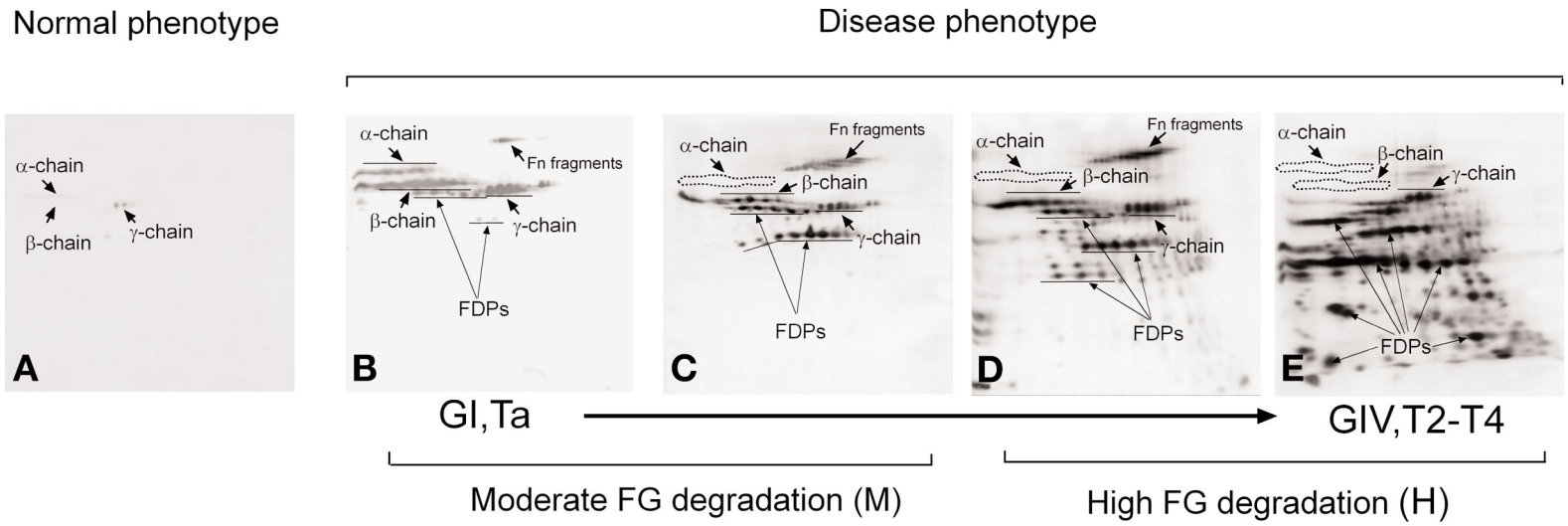

Fig. 2. Two-dimensional gel Western blotting patterns of FG/FDPs in urines from healthy individuals **(A)** and patients bearing urothelial carcinomas of various grades and stages **(B)** GI, pTa; **(C)** GII, pT1; **(D)** GIII, pT1; **(E)** GIV, pT2-4.

(by means of 2D PAGE and silver staining) several diseased urine samples that showed high levels of FG/FDPs. As shown in **Fig. 3**, two urine samples (UC1779-1 and 1756-1) displayed much higher levels of hemoglobin β-chain, indicating the presence of blood in the sample, whereas others (UC 1776-1 and 1774-1) failed to show any detectable levels of this protein (**Fig. 3**), implying that fibrinogen and its degradation derivatives originate mainly from the tumor and/or the tumor microenvironment.

Immunostaining of tumor sections with the fibrinogen antibody showed that fibrinogen, and perhaps FDPs, are present in the surrounding stroma of both nonmalignant (**Fig. 4A**,**C**) and tumor lesions (**Fig. 4E**), but not in the epithelial cells themselves. The latter was confirmed indirectly by in vitro experiments that showed no traces of FG/FDPs in several urothelial cell lines (RT112, T24, HU609, and HU961, data not shown). Clusters of fibrin/fibrinogen were also visible in the cavities of capillaries decorated by the anti-factor VIII-related antigen (AbF8/86, DAKO) (compare **Fig. 4C**,**D**). Immunohistochemical analysis of paraffin-embedded sections of several UC lesions further indicated that the immunoreactivity for FG/FDPs is considerably higher in the stroma of high grade/stage tumors (**Fig. 5A–D**). Moreover, 2D gel blot analysis of whole tumor lysates showed comparable levels of degradation products as observed in the corresponding urine (**Fig. 6**, compare tumor 1291 and urine 1291; **Fig. 6** compare tumor 1789 and urine 1789). In addition, immunoblot and MS analysis of the TIF that bathes the tumor microevironment ***(34**,**41)*** revealed the presence of FG/FDPs in TIF recovered from a few UCs (**Fig. 7**).

Identification of Specific Urinary FDPs by Immunoprecipitation and MALDI-TOF

Although the 2D gel patterns of FG/FDPs in the urine samples of patients with UC varied significantly from patient to patient (**Fig. 2**), some of the FDP spots were detected in most of the disease urines examined. Because these FG peptides may serve as a potential source for producing more specific antibodies for detecting FG/FDPs, as well as for developing more effective and reliable FG/FDP-based diagnostic tests, we characterized several of them using MS. To facilitate the identification, peptides were first enriched by immunoprecipitation using protein A/G Sepharose beads bound to anti-fibrinogen antibodies (A0080, DAKO) (**Fig. 8**). Several prominent FDP peptides indicated with circles in **Fig. 8** were excised from the dry silver-stained gel and analyzed by MALDI-TOF. The identity of these FDP peptides is given in **Table 2**.

Discussion

To gain a better understanding of the origin of FG/FDP in the urine, and to evaluate their potential value for detecting bladder cancer, we performed a detailed gel-based proteomic analysis of FG/FDPs present in the urine of 151 patients diagnosed with UCs of various degrees of atypia and stages of invasion. Our data showed that the repertoire of fibrinogen components in the urine is very complex and highly variable from patient to patient, a fact that may hamper the accurate detection of FG/FDPs in samples using enzyme-linked immunosorbent assay-based immunoassays. 2D PAGE portrays all fibrinogen components with high resolution in a single 2D gel image and provides a convenient and reliable method for monitoring FG/FDPs. Of the 151 patients diagnosed with UCs, 147 individuals (97%) showed increased levels of FG/FDPs in the urine, whereas the rest (3%) either lacked or exhibited very low levels of these components.

Previous studies have shown that FDPs are more accurate for detecting high-grade invasive diseases as compared to early stage

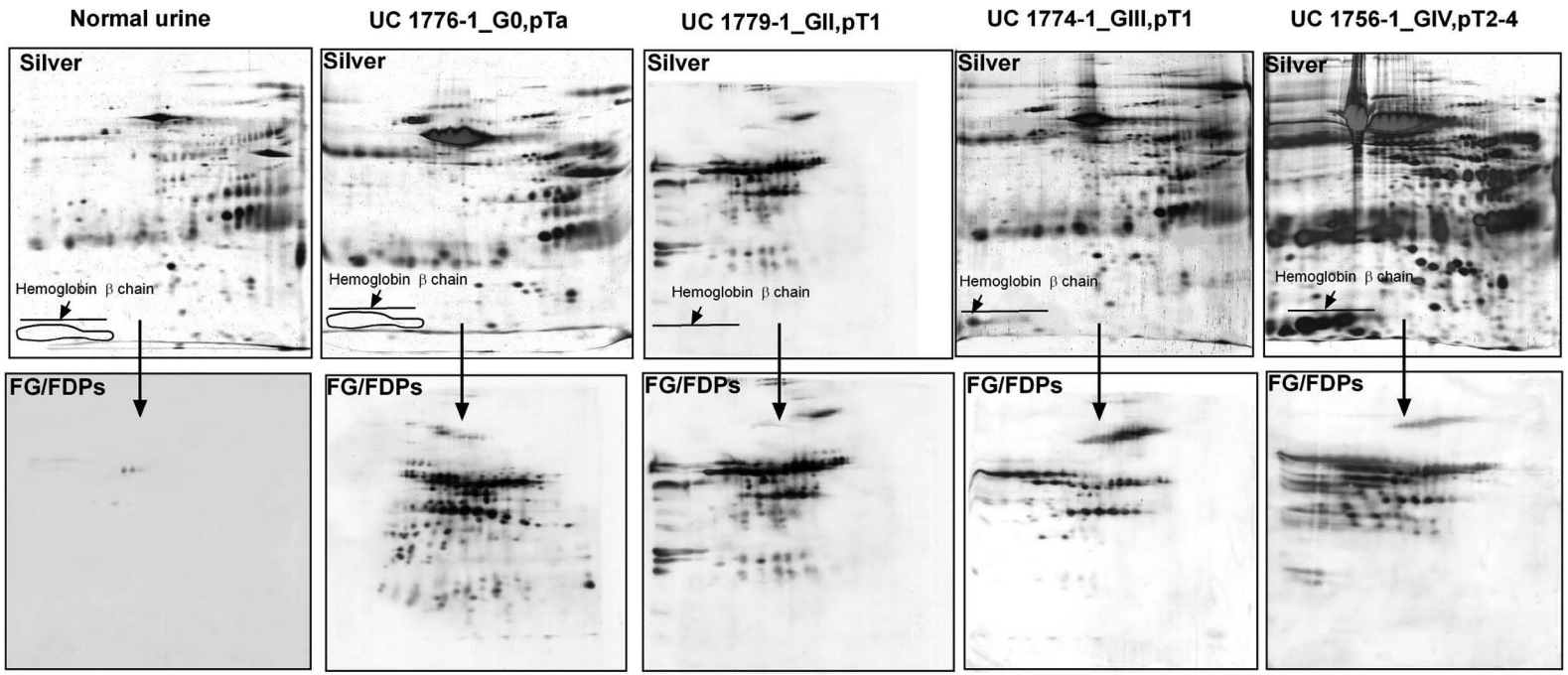

Fig. 3. Urine proteins from several patients with urothelial carcinoma separated by two-dimensional polyacrylamide gel electrophoresis and stained by silver nitrate (**upper panels**) and Western blotted using the fibrinogen antibody (**lower panels**). The position of hemoglobin β-chain (a blood marker) is indicated.

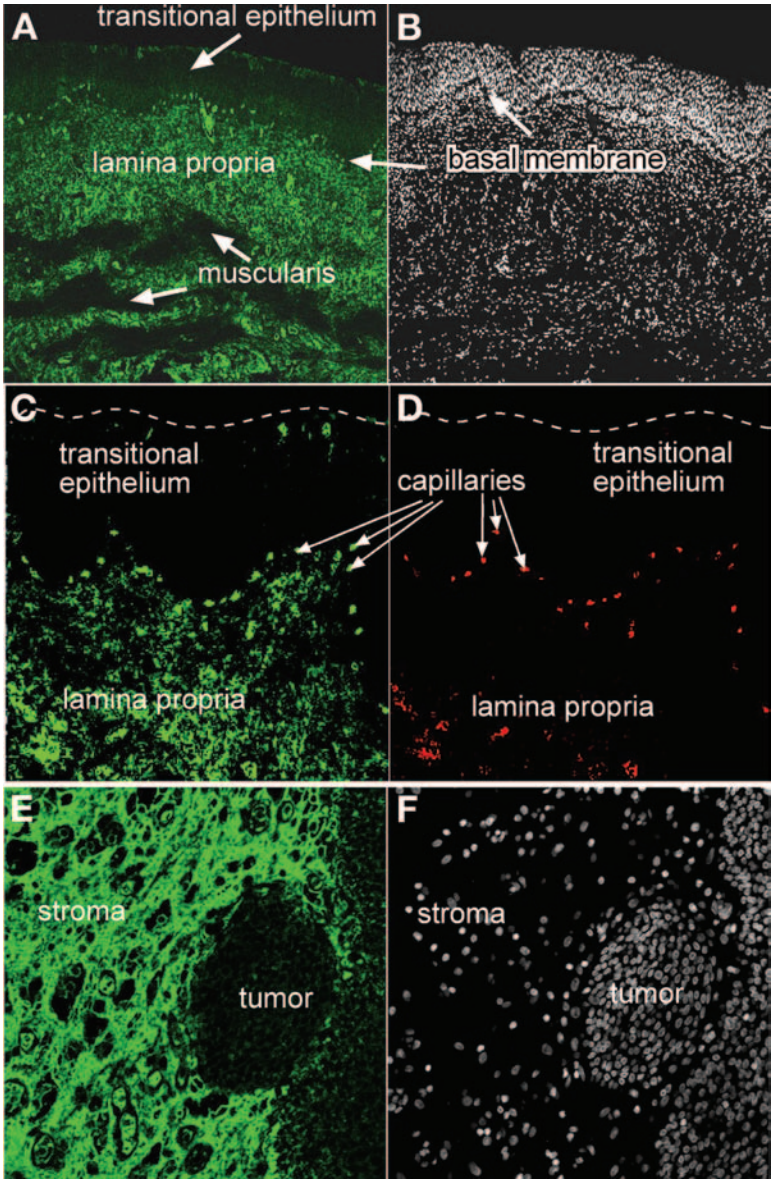

Fig. 4. Indirect immunofluorescence analysis of nonmalignant areas of the bladder and the corresponding urothelial carcinoma specimens obtained from the same patient. Clusters of fibrinogen and its degradation derivatives were detected by staining with antifibrinogen polyclonal antibody (A0080, DAKO) **(A**,**C**,**E)**. Blood capillaries were revealed by staining with an anti-factorVIII-related antigen (F8/86, DAKO) that is specific for endothelial cells **(D)**. Tissue structure in sections was decorated by staining with the monomeric cyanine nucleic acid stain TO-PRO3 **(B**,**F)** specific for nuclei.

tumors (*25*,*27*,*30*,*32*). Our proteomic studies, however, revealed that the percentage of positive detections, when comparing patients with superficial, early invasive, and highly invasive tumors, was comparable (99, 97, and 96%, respectively; **Table 1**) in all three groups. As a whole, these data indicate that elevated levels of FDPs in the urine are a characteristic feature of bladder cancer, and that measuring the levels of urinary FG/FDPs holds great

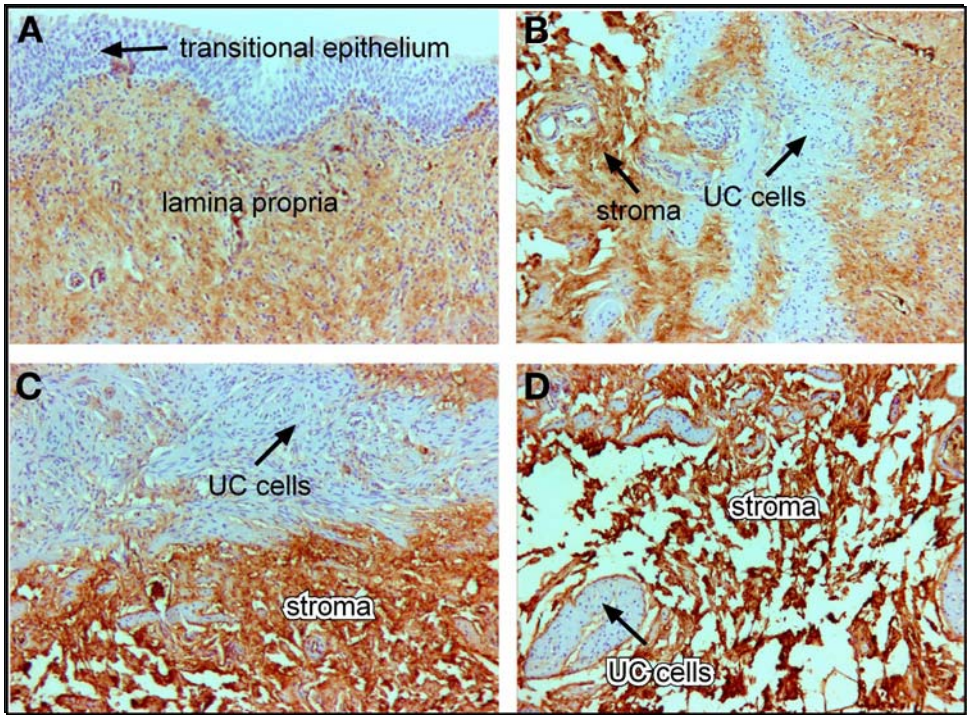

Fig. 5. Immunohistochemistry of cryostat sections from nonmalignant samples and urothelial carcinomas of various stages and grades. Immunohistochemistry was carried out using a fibrinogen polyclonal antibody (A0080, DAKO) followed by counter-staining with Hematoxilin.

promise for detecting low-grade, noninvasive tumors.

Analysis of the urine samples from 34 healthy volunteers showed that FG/FDPs are either absent or present at very low levels. Interestingly, elevated levels of FG/FDPs (false-positives) were detected in two of the control samples; one of which was from a woman that we later found to be suffering from chronic cystitis. These results are not unexpected as it is well known that conditions, such as inflammation, toxicity, renal cancer (affecting the filtration process in the kidney glomeruli and tubuli), and urinary tract stones may lead to increased levels of plasma proteins in the urine (*42*,*43*).

So far, the proposed mechanisms by which FG/FDPs may access the urine of patients with bladder neoplasias are unclear (*30*). Thus, it has been postulated that fibrinogen circulating in the blood may reach the urine as a result of bleeding caused by tumor invasion. Even though the occasional bleeding of invasive tumors may contribute to the accumulation of FG/FDPs into the bladder cavity, our results showed that patients with superficial UCs had a considerable amount of FG/FDPs in the urine, despite the fact that there were no signs of bleeding. Presently, it is widely accepted that fibrin(ogen) is not normally present outside the blood vessels, although it can easily reach the perivascular tissues during wounding and in some malignancies (*44*,*45*).

It has been also suggested that increased permeability of the tumor cell membrane permits the direct transduction of FG/FDPs into the urine (*29*,*30*,*46*). Our results, however, showed that fibrinogen and its derivatives are not produced by normal or by malignant urothelium. More importantly, we found that the stroma of nonmalignant areas contain considerable

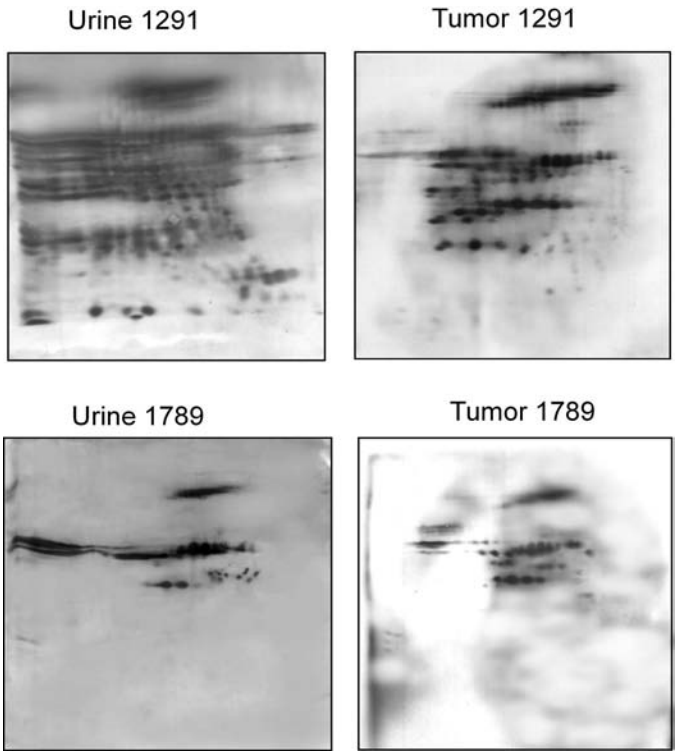

Fig. 6. Two-dimensional gel Western blot profiling of FG/FDPs from urine samples (patients 1291 and 1789) and the corresponding tumor specimens.

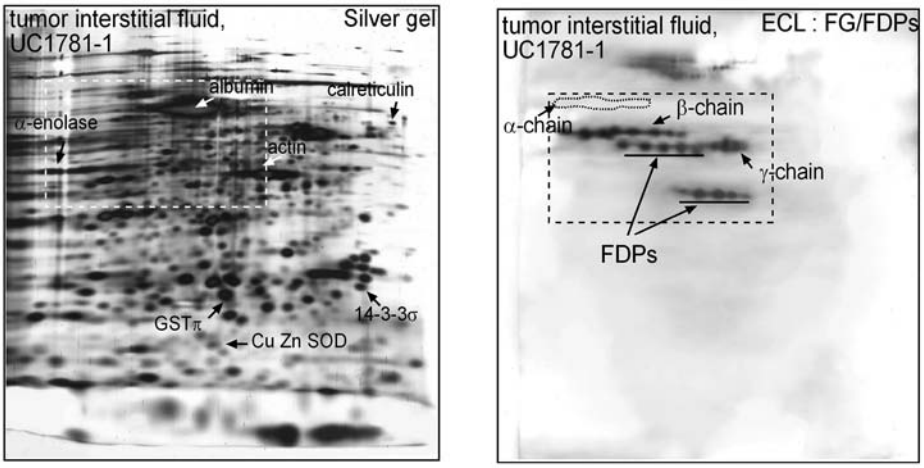

Fig. 7. Tumor interstitial fluid proteins from UC 1748-1 separated by two-dimensional (2D) isoelectrofocusing (IEF) polyacrylamide gel electrophoresis and stained with silver nitrate (**left panel**). The corresponding 2D gel Western FG/FDPs pattern is shown on the **right panel**.

amounts of FG/FDPs, most likely derived from passive or active transport of some plasma proteins through the capillary vessels. Moreover, the deposition of FG/FDPs seems to increase in high-grade and -stage tumors supporting the notion that fibrin/fibrinogen may facilitate

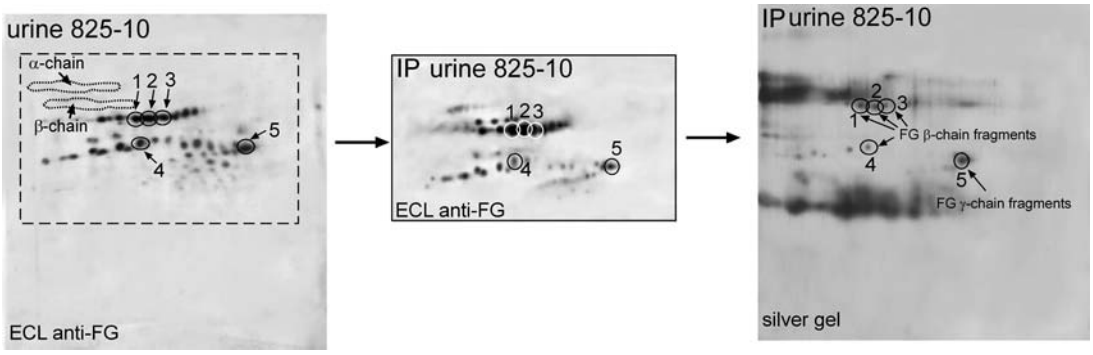

Fig. 8. Mass spectrometry of FDP peptides. The identity of several FDP spots was determined by matrix-assisted laser desorption/ionization time-of-flight after immunoprecipitation of FG/FDPs with the fibrinogen antibody bound to sepharose-protein A.

Table 2
MALDI-TOF Identification of Several FDPs Presented in the Urine of the Most Patients With Developed Bladder UCs

FDP spot[a]	Identification	No. of peptides matched	MASCOT score	Sequence coverage
1	Fibrinogen β-chain, precursor	19	227	152–491 aa
2	Fibrinogen β-chain, precursor	7	98	295–491 aa
3	Fibrinogen β-chain, precursor	9	85	152–491 aa
4	Fibrinogen β-chain, precursor	9	105	161–491 aa
5	Fibrinogen γ-chain, precursor	6	78	1–301 aa

[a]The FDP spots are indicated with their corresponding numbers in **Fig. 8**.

tumor stroma formation by mechanisms that are perhaps analogous to wound repair (*45*,*47*,*48*). Thus, it is known that malignant cells produce the angiogenic factor (vascular endothelial growth factor) that increases vessel wall permeability in the tumor microvasculature resulting in leakage of plasma proteins such as plasminogen, clotting factors, and fibrinogen into the extravascular space (*49–51*). Recently, we described the protein characterization of a near fluid (the TIF) that perfuses breast carcinomas and that is composed of more than 1000 proteins—either secreted, shed by membrane vesicles like exosomes, or leaked as a result of cell death—produced by the complex network of cell types that make up the tumor microenvironment (*34*,*41*). 2D Western blot analysis of the TIF recovered from UCs of various grades and stages of invasion confirmed the presence of FG/FDPs in the tumor microenvironment.

The presence of FG/FDPs in the extracellular matrix of nonmalignant samples may help to interpret the relatively high levels of FG/FDPs

Supplementary Table
Patients With UCs of Various Histopathological Grade of Atypia and Tumor Stage

Count	Tumor ID[a]	IHC classification (grade/stage)[b]	Urine FG/FDPs phenotype[c]	Age	Gender[d]
		Superficial UCs (GI-III,Ta)			
1	62-9	GIII,Ta	Moderate	70	M
2	81-5	GIII,Ta	Moderate	65	M
3	150-7	GII,Ta	Moderate	67	M
4	154-3	GII,Ta	High	76	M
5	192-9	GII,Ta	Moderate	64	M
6	217-9	GII,Ta	High	51	M
7	269-2	GII,Ta	Moderate	62	M
8	335-6	GI,Ta	Moderate	77	F
9	381-1	GII,Ta	Moderate	77	F
10	387-6	GI,Ta	Moderate	72	M
11	450-16	GII,Ta	Moderate	83	M
12	455-1	GII,Ta	Moderate	41	M
13	521-1	GI,Ta	Moderate	27	M
14	532-8	GII,Ta	Moderate	44	M
15	542-13	GII,Ta	Moderate	65	F
16	716-2	GIII,Ta	High	42	M
17	763-13	GII,Ta	High	66	M
18	775-1	GIII,Ta	Moderate	70	M
19	776-1	G0,Ta	Moderate	54	M
20	793-1	GI,Ta	Moderate	67	M
21	807-1	G?,Ta	Moderate	74	M
22	825-10	GIII,Ta	Moderate	75	M
23	829-3	GII,Ta	High	63	F
24	835-1	GII,Ta	High	70	M
25	854-3	GII,Ta	High	71	F
26	854-4	GII,Ta	High	72	F
27	916-1	GI,Ta	High	51	F
28	916-6	GI,Ta	Moderate	53	F
29	925-1	GII,Ta	High	62	M
30	925-3	GII,Ta	Moderate	63	M
31	929-1	GI,Ta	Moderate	70	M
32	956-2	GIII,Ta	High	75	F
33	968-1	GII,Ta	Moderate	71	M
34	969-1	GII,Ta	normal	60	F
35	995-1	GII,Ta	Moderate	53	F
36	997-1	GII,Ta	Moderate	52	M
37	968-1	GII,Ta	Moderate	71	M
38	1009-1	GII,Ta	High	70	M
39	1017-1	GIII,Ta	High	79	M
40	1020-2	GIII,Ta	Moderate	77	M

(Continued)

Supplementary Table *(Continued)*

Count	Tumor ID[a]	IHC classification (grade/stage)[b]	Urine FG/FDPs phenotype[c]	Age	Gender[d]
41	1024-2	GIII,Ta	Moderate	63	M
42	1045-1	GII,Ta	Moderate	69	M
43	1058-3	GII,Ta	Normal	67	M
44	1060-1	GII,Ta	Moderate	59	M
45	1066-1	GIII,Ta	High	80	M
46	1079-1	GII,Ta	Moderate	57	M
47	1084-1	GI,Ta	Moderate	58	M
48	1092-1	GIII,Ta	High	66	M
49	1094-2	GIII,Ta	Moderate	49	M
50	1118-1	GII,Ta	High	59	M
51	1145-1	GII,Ta	High	79	M
52	1146-1	GII,Ta	High	53	M
53	1174-1	G0,Ta	Moderate	54	M
54	1206-1	GI,Ta	Moderate	62	M
55	1216-1	GII,Ta	Moderate	73	M
56	1241-2	GII,Ta	Moderate	70	M
57	1246-1	GI,Ta	High	81	M
58	1250-1	GI,Ta	High	65	F
59	1282-2	GII,Ta	High	77	M
60	1325-1	GII,Ta	Moderate	54	F
61	1587-3	GII,Ta	Moderate	94	M
62	1707-2	G0,Ta	Moderate	69	M
63	1732-3	G0,Ta	Moderate	77	M
64	1762-1	GII,Ta	High	75	F
65	1764-1	GII,Ta	Moderate	68	M
66	1772-1	G?,Ta	Moderate	71	M
67	1776-1	G0,Ta	High	69	M
68	1785-2	GII,Ta	High	72	M
69	1786-1	GII,Ta	Moderate	60	M
70	1787-1	GII,Ta	High	65	M
71	1790-1	GI,Ta	Moderate	38	F
72	1791-2	GI,Ta	High	65	M
73	1802-1	GII,Ta	High	64	F
74	1805-1	GII,Ta	Moderate	80	M
		Early invasive Ucs (GI-III,T1)			
75	195-11	GIII,T1	High	69	M
76	320-2	GIII,T1	Moderate	69	M
77	347-10	GIII,T1	High	79	M
78	351-5	GIII,T1	Moderate	78	M
79	444-5	GIII,T1	Moderate	84	M
80	485-1	G?,T1	High	59	F
81	729-4	GIII,T1	Moderate	89	F

(Continued)

Supplementary Table *(Continued)*

Count	Tumor ID[a]	IHC classification (grade/stage)[b]	Urine FG/FDPs phenotype[c]	Age	Gender[d]
82	744-1	GIII,T1	Moderate	76	M
83	757-1	GII,T1	Moderate	69	M
84	760-1	GIII,T1	Moderate	72	M
85	785-1	GIII,T1	Moderate	71	M
86	847-1	GIII,T1	Normal	82	M
87	936-1	GIII,T1	High	78	F
88	950-1	GIII,T1	High	81	M
89	967-1	GIII,T1	Moderate	67	M
90	1036-1	GIII,T1	Moderate	79	M
91	1049-1	GII,T1	High	82	M
92	1056-1	GIII,T1	High	58	F
93	1075-1	GIII,T1	High	72	F
94	1098-2	GIII,T1	High	83	F
95	1121-1	GIII,T1	High	42	M
96	1191-1	GIV,T1	High	76	M
97	1191-2	GIII,T1	Moderate	76	M
98	1311-1	GIII,T1	High	74	M
99	1270-1	GIII,T1	High	82	M
100	1177-1	GIII,T1	Moderate	56	M
101	1779-1	GIII,T1	High	67	M
102	1783-1	GIII,T1	High	78	M
103	1785-1	GIII,T1	Moderate	72	M
		Highly invasive UCs (GII-IV,T2-4)			
104	116-3	GIII,T2-4	High	80	M
105	163-2	GIV,T2-4	High	71	M
106	195-14	GIII,T2-4	Moderate	74	M
107	376-9	GIII,T2-4	High	64	F
108	389-1	GIV,T2-4	High	76	M
109	475-T1	GIII,T2-4	High	69	M
110	715-4	GIII,T2-4	Moderate	73	M
111	752-5	GIII,T2-4	High	53	M
112	788-1	GIII,T2-4	High	78	M
113	816-5	GIII,T2-4	Moderate	76	M
114	817-1	GIII,T2-4	High	69	M
115	846-3	GIII,T2-4	Moderate	72	F
116	852-1	GIII,T2-4	High	82	M
117	864-1	GIII?,T2-4	High	51	M
118	868-1	GIII,T2-4	High	60	F
119	902-1	GIV,T2-4	High	65	F
120	905-1	GII,T2-4	High	53	M
121	1015-1	GIII,T2-4	High	58	M
122	1016-1	GIII,T2-4	High	70	M

(Continued)

Supplementary Table *(Continued)*

Count	Tumor ID[a]	IHC classification (grade/stage)[b]	Urine FG/FDPs phenotype[c]	Age	Gender[d]
123	1017-3	GIII,T2-4	Low	79	M
124	1026-1	GIV,T2-4	High	64	F
125	1027-2	GIII,T2-4	Normal	69	F
126	1035-1	GIV,T2-4	High	76	M
127	1068-1	GIII,T2-4	Normal	57	F
128	1169-1	GIV,T2-4	High	82	M
129	1170-1	GIV,T2-4	High	65	M
130	1203-1	GIV,T2-4	High	56	M
131	1205-1	GIV,T2-4	High	78	M
132	1218-1	GIII,T2-4	High	53	M
133	1243-1	GIII,T2-4	High	56	M
134	1271-1	GIII,T2-4	Moderate	58	M
135	1271-2	GIII,T2-4	High	?	?
136	1291-1	GIV,T2-4	High	48	M
137	1304-1	GIV?,T2-4	High	84	F
138	1316-1	GIII,T2-4	High	62	M
139	1322-1	GIV,T2-4	High	51	F
140	1664-2	GIII,T2-4	Moderate	79	M
141	1664-3	GIV,T2-4	High	79	M
142	1672-3	GIII,T2-4	High	66	M
143	1707-1	GIII,T2-4	High	69	M
144	1756-1	GIII,T2-4	High	90	M
145	1770-1	GIII,T2-4	High	76	F
146	1774-1	GIII,T2-4	Moderate	48	M
147	1777-1	GIII,T2-4	High	64	M
148	1780-1	GIV,T2-4	High	63	M
149	1789-1	GIII,T2-4	High	62	M
150	1798-1	GIII,T2-4	High	62	F
151	1803-1	GIII,T2-4	Moderate	56	M

[a]Tumor ID indicates a patient number (XXXX-) and a presentation number (-Y) that are separated with a dash.
[b]UCs were classified according WHO 1978 (**33**).
[c]FG/FDPs immunoblot phenotypes were determined and classified as described in Materials and Methods.
[d]M, male; F, female.

that we observed in urine samples obtained from patients with superficial noninvasive UCs. At present, however, it is not clear how FG/FDPs and other TIF components reach the urine, although recent studies have shown that the interstitial fluid pressure is elevated in tumors (**52**). Because of the high permeability of the tumor vessels to fluids and plasma proteins, the microvascular pressure leads to interstitial hypertension in tumors that is associated with the development of the tumor vasculature (**53**). It is then possible that the tumor interstitial hypertension (**52**) may contribute to the release of extravascular proteins into the bladder cavity.

Future attempts to develop more accurate test to measure the levels of FG/FDPs in the urine should focus on the preparation of more specific antibody probes. Our data showed

that α-, β-, and γ-chains of FG exhibited different susceptibilities to proteolysis *in situ* and that the FG 2D gel degradation patterns varied considerably from patient to patient depending on the grade and stage of the tumors (**Fig. 2**). Consequently, for future peptide–antigen design it is a crucial to select FG fragments that are most representative of those present in the urine samples of patients with UC. Toward this goal, we detected several FDP spots that were present in all the urines displaying the disease phenotype (**Figs. 2** and **8**). The sequence coverage obtained from the MALDI-TOF data showed that FDPs corresponding to spots 1, 2, 3, and 4 (**Fig. 8**) were derived from the fibrinogen β-chain and contained C-terminal fragments covering amino acids 152 to 491, 161 to 491, and 295 to 491, respectively (**Table 2**). Spot 5, on the other hand, corresponded to a N-terminal fragment of fibrinogen γ-chain that covered amino acids 1 to 301. Our results also indicated that the γ-chain of fibrinogen was the most resistant to proteolytic degradation *in situ* in line with prior in vitro experiments that showed differential susceptibility of the lysyl and arginyl residues in the α-, β-, and γ-chains of purified FG to plasmin degradation (***54***). Taken together, these data suggest that the fibrinogen γ-chain as well as C-terminal fragment of the β-chain (152–491 aa) may be the most suitable candidates for a peptide-antigen design strategy for developing highly-specific antibodies for a urinary FG/FDP-based test.

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