
CLIN. CHEM. 37/3, 466-471 (1991)

Urinary Fibronectin Fragments (a Potential Tumor Marker) Measured by Immunoenzymometric Assay with Domain-Specific Monoclonal Antibodies

Masahiko Katayama,1 Fumitsugu Hino,1 Kyoko Kamihagi,1 Kiyotoo Sekiguchi,2 Koji Ttani,2 and Ikunoshin Kato1

We found that urinary fibronectin (UFN) in cancer patients was almost all fragmented and consisted mainly of the cell-binding domain. We developed a two-site immunoenzymometric assay for UFN, using two monoclonal antibodies that both recognize this domain of fibronectin. The amount of UFN was expressed as arbitrary units per milligram of creatinine. Some 2% of the 623 healthy subjects had UFN above the clinical cutoff point (200 arb. units/mg creatinine), as did 14% of the 271 patients with nonmalignant diseases. In contrast, concentrations of UFN exceeded the cutoff point in 59% of the 589 patients with cancer. In 37 patients with gastrointestinal cancer tested for UFN and for one or more of three established serum tumor markers, UFN was found in 25, significantly more often than the other markers. These results indicated that UFN is a marker that may be helpful in evaluating many kinds of cancer.

**Additional Keyphrases:** cancer - cutoff value

The adhesive glycoprotein fibronectin (FN),3 Mr 440 000, is widely distributed on cells, in the extracellular matrix, and in plasma. FN plays a role in various cellular processes, including cell-to-substrate adhesion, cell migration, and the regulation of cell morphology (1). Webb and Lin first reported in 1980 (2) that the amount of fragmented FN in the urine of patients with prostatic cancer is higher than in normal subjects. Another group reported that an FN-like protein, with a lower molecular mass than plasma FN, is increased in sera from patients with malignant disease (3). Since then, measurements of the concentration of FN in body fluids and tissues from patients with cancer have usually involved use of a polyclonal antibody to plasma FN. In particular, many examinations have been done to evaluate the clinical usefulness of measurements of plasma FN (4-7). However, FN concentrations in body fluids and tissues are not correlated with malignancy or the metastatic potential of cancer (4-6). Plasma FN does not seem suitable as a tumor marker, because plasma FN is sensitive to clinical events unrelated to the malignancy status (4-6).

We found by immunoblotting that urinary FN (UFN) in cancer patients is almost all present as fragments of different sizes, and that a polyclonal antibody raised against native plasma FN cannot react well with UFN. Monoclonal antibodies can be used to detect the target substance in such situations. We have already developed four monoclonal antibodies against three different domains (the N-terminal, the C-terminal, and the central region) of the FN molecule (9). Here we demonstrate that two monoclonal antibodies,
FN10 and FN30, which are reactive with the central region of the FN molecule, the "cell-binding domain," are suitable for in vitro assays of FN fragments excreted in the urine of cancer patients. The antibodies were used together in a monoclonal immunoenzymatic sandwich for a specific and simple urine test for cancer.

Materials and Methods

Monoclonal antibodies. Two murine monoclonal antibodies, FN10 and FN30, were derived by fusion with the use of splenocytes from a mouse immunized with purified human plasma FN (Collaborative Research Inc., Lexington, MA) and identified as being of the IgG1 subclass. The domain epitopes of these antibodies were characterized as described elsewhere (9, 10). Locations of antibody-binding sites on FN are shown in Figure 1.

Immunoblotting analysis. UFN was purified from 20 mL of a urine sample by use of FN30 monoclonal antibody immobilized on an agarose column. Urine samples for electrophoresis were collected from one healthy subject and three patients with bladder, lung, or stomach cancer. UFN eluted from the column and plasma FN were separated simultaneously by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose sheet by electrophoresis. The bound antibodies were detected with anti-mouse IgG antiserum labeled with horseradish peroxidase (EC 1.11.1.7; Bethesda Research Laboratories, Gaithersburg, MD). All samples were reduced with 2-mercaptoethanol. The nitrocellulose membrane was immunostained with FN10 monoclonal antibody as reported previously (10).

Clinical specimens. For evaluation of the immunoenzymometric assay (IEMA) of UFN, a total of 1483 urine samples from healthy subjects (n = 623), patients with benign disease (271), and patients with cancer (589) were obtained from the National Cancer Center Hospital (Tokyo), Showa University Hospital (Tokyo), Toho University Hospital (Tokyo), Dokkyo University Hospital (Tochigi), and Shiga University Hospital (Shiga, Japan). All of the 589 patients with pathologically and histologically proven cancer, but with no previous treatment by chemotherapy and radiotherapy, were entered in this study. All the urine samples from the cancer patients were collected before surgery. Some of the malignant diseases were staged at the time of diagnosis after the urine samples were collected. To evaluate the correlation between increased UFN and tumor grade, we performed a detailed staging in 58 of the 164 patients with stomach cancer who provided urine samples. The samples were frozen without preservatives at -20 ºC until analysis. Serum samples were also collected from 37 of the patients with gastrointestinal cancer.

Standard material for UFN assay. We used recombinant FN cell-binding fragment as the standards for the assay. Fragments were prepared by the expression of human FN complementary DNAs in Escherichia coli. The construction and expression of the plasmid used were described elsewhere (9).

Assay of creatinine in urine. A commercially available kit ["Creatinine Test Wako," based on the method of Jaffe (11)] was used according to the manufacturer's instructions to assay creatinine in urine.

Cutoff value for UFN. UFN concentrations were expressed in terms of arbitrary units, 1 arb. unit being equivalent to 1 ng of plasma FN. A value ≥200 units/mg of creatinine was considered to be positive for cancer, based on the values found in 623 healthy subjects (200 units/mg is the mean of these observed values + 2 SD).

Sandwich IEMA. Antibody FN10 was purified by Protein A chromatography and labeled with horseradish peroxidase (Boehringer GmbH, Mannheim, F.R.G.). First, 96-well plates coated with antibody FN30 were blocked with bovine serum albumin solution. To each well we added 100 µL of a solution of peroxidase-labeled FN10 antibody plus 20 µL of FN standard (0, 50, 100, 200, 400, or 800 arb. units/mL) or a urine sample. The plate was incubated for 0.5 h at room temperature and washed with de-ionized water. We then added 2.9 mmol/L 3,3’,5,5’-tetramethylbenzidine 2HCl (Sigma Chemical Co., St. Louis, MO) solution as substrate and left the mixture for 15 min at room temperature, after which we stopped the enzyme reaction by adding 100 µL of HCl, 1 mol/L. We measured the absorbance of each well at 450 nm with a Titertek Multiscan densitometer (Flow Laboratories, McLean, VA). The amount of UFN was expressed per milligram of creatinine.

Other clinical markers. Besides UFN, we assayed the concentrations in serum of carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), and α-fetoprotein (AFP) in 37 of the 589 patients with malignant tumors. Serum and urinary markers were collected from these 37 patients before surgery; their cancers were classified as stage III or IV. Serum and urinary markers, except for UFN, were measured with commercially available kits: CEA-EIA kits were purchased from Abbott Laboratories (North Chicago, IL), CA19-9 radioimmunoassay kits from Centocor (Malvern, PA), and AFP radioimmunoassay kits from Pharmacia (Uppsala, Sweden).

Results

Precision of assay and recovery. We evaluated the precision of the assay by assaying two samples of urine
10 times each in a continuous series (intra-assay) or twice each in 10 consecutive assays (interassay). Intra-assay CVs were 7.0% for a UFN concentration of 72.5 arb. units/mL and 6.5% for 141.0 arb. units/mL; interassay CVs were 6.0% for 70.4 arb. units/mL and 7.8% for 143.1 arb. units/mL. Mean analytical recovery of standard UFN (200, 400, or 800 arb. units/mL) added to three urine samples in equivalence ratios was 99.1%.

**Immunoblotting analysis of UFN in cancer patients.**

Three samples of UFN from patients with bladder, lung, or stomach cancer showed a positive reaction, as did plasma FN, in immunoblotting analysis with FN10 (Figure 2). UFN in these three cancer patients had multiple molecular masses in the range of 30,000 to 180,000 Da. The *M*<sub>f</sub> of the antigens detected ranged widely, but were generally in the region of 60,000 or 100,000. The amount of UFN in a healthy subject chosen without conscious bias was so small that no antigen was detected. Nonfragmented FN, *M*<sub>f</sub>, ~220,000, was almost undetectable in the urine samples from the three cancer patients.

**Use of the assay to measure UFN in cancer patients.**

The mean concentration of UFN in the 623 healthy subjects was 68.6 (SD 47.7) arb. units/mg creatinine. The mean concentrations of UFN for different ages and by sex for all 257 subjects tested for suspected disease at Showa University Hospital are shown in Table 1. Among the healthy controls, UFN concentrations in men and women were not significantly different, but were slightly increased in the urine of the healthy subjects ages 60–69 years (Table 1). The mean + 2 SD concentration of UFN in the 623 healthy subjects was 164.0 arb. units/mg creatinine, and the mean + 3 SD was 211.7 arb. units/mg creatinine. Concentrations ≥200 arb. units/mg creatinine, i.e., greater than the mean + 2 SD but less than the mean + 3 SD, were therefore considered to be high. With this as the cutoff, only 13 (2%) of the 623 healthy subjects gave positive results. The distribution of UFN values of the patients and healthy subjects is shown in Figure 3. Concentrations were high in 73 (14%) of the patients with nonmalignant diseases and in 356 (59%) of the patients with malignant diseases.

We assayed UFN in urine from the 58 patients with stomach cancers at different stages. UFN concentrations were high in four (33%) of the 12 patients with stage I stomach cancer, in one (50%) of the two patients with stage III stomach cancer, in 14 (74%) of the 19 patients with stage IV stomach cancer, in 23 (96%) of the 24 patients with recurrent stomach cancer, but not in the one patient with stage II stomach cancer. Metastases to distant sites were detected in two of the 19 patients with stage IV cancer and in nine of the 24 patients with recurrent cancer. UFN concentrations were extremely high in all of these patients with distant metastases.

**Comparison of UFN and serum markers.** We assayed concentrations of UFN and serum markers in urine samples from patients with malignancies (Table 2). UFN was found much more often than the serum markers tested (CEA, CA19-9, and AFP).

**Discussion**

We showed here that UFN excretion was increased in 59% of the patients with various cancers and conclude
that this assay of UFN would be useful for widespread diagnostic screening of many kinds of cancer. We obtained positive rates ranging from 37% to 88% for different kinds of cancer, and a mean of 14% for nonmalignant diseases. However, positive results suggesting the presence of malignancy when only nonmalignant disease was actually present would not be a problem, because false-positive results for healthy subjects were rare (2%). Distribution of UFN concentrations in 58 patients with stomach cancer could be classified into stages. Our results indicate substantial differences between the percentages of patients positive for UFN at stage I and those at stage IV, so we expected a direct correlation between the stage of the tumor and the concentration of UFN in cancer patients. Moreover, the UFN test would indeed be primarily advantageous for patients with resectable stomach cancer (such as stage I).

The amount of UFN is given here per milligram of creatinine. Previous studies suggest that the ratio of urinary protein per unit of creatinine concentration in random (untimed) urines accurately reflects 24-h urinary protein excretion (12, 13). Serial urine samples from the same subject should have consistent values for the UFN concentration when the excretion of UFN is expressed per milligram of creatinine.

The tumor markers CEA, CA19-9, and AFP circulate in increased amounts in sera of patients with gastrointestinal malignant disease. Our study shows that the clinical usefulness of these assays in the detection of cancer is not superior to that of UFN. Increases in UFN seem to be independent of renal function, because the incidence of increased UFN concentrations was not significantly different between patients with renal diseases and those with nonmalignant diseases. In addition, we preliminarily studied the correlation between two urinary markers (albumin and β2-microglobulin) established for early assessment of lesions of glomerulus and UFN in some patients with kidney failure, as well as the correlation between four markers (blood urea nitrogen, serum creatinine, urinary glucose, and urinary protein) for renal function and UFN in some cancer patients. We observed no significant correlation between these six markers and UFN (unpublished). This preliminary examination shows that increases in UFN in the patients are unrelated to renal function. We detected no difference between UFN concentrations in cancer patients pretreated with chemotherapy or radiotherapy and those in untreated cancer patients (unpublished), perhaps because chemotherapy or radiotherapy does not independently cause increased UFN.

Several studies have shown that FNs isolated from normal or oncofetal tissues are similar in many properties but differ slightly in their subunit size; FN of oncofetal origin has some extra domains and glycosylation changes (14–17). However, the actual amount of matrix containing FN produced by tumor cells is often much less than normal. Cultured cells of metastatic carcinomas of rats and humans apparently do not express high concentrations of cellular FN (18, 19); the increased concentrations of UFN in cancer patients may not be the result of an increased expression of cellular FN by the tumor cells.

The increase in UFN concentrations is closely associated with malignant diseases, but the basis for the apparent correlation is not clear. The diversity of molecular masses of UFN suggests that UFN arises from FN fragmented by several FN-degrading proteases. A possible explanation for the increases of UFN in cancer patients might be the relationship between proteases that degrade FN and cell invasion (20, 21). The ability of cells to invade the surrounding connective tissue, an important factor in tumor metastasis, seems to be a multiple-step process requiring degradation of components of the extracellular matrix by proteases on the cell surfaces (22). FN is a large extracellular glycoprotein that interacts in various ways with cell surfaces (I), and
is more sensitive to digestion by various neutral proteases than is laminin or collagen, which are also components of the extracellular matrix (21).

Analysis of the course of tryptic or thermolysin digestion of plasma FN in vitro has shown that the α and β subunits of FN are first rapidly cleaved into 215- and 185-kDa fragments when the Hep-1/Fib-1 and Fib-2 domains are released (10, 23). The 185-kDa fragment is further degraded into the cell-binding domain, a 75-kDa fragment, upon the release of the Gel, Hep-2, and Hep-3 domains. The cell-binding domain of FN, specifically recognized by both the FN10 and FN30 antibodies, seems to resist protease attack, to judge from its being the main component of UFN. The terminal domains released, except for the cell-binding domain, bind to other matrix components: collagen, fibrin, and glycosaminoglycans (1, 10, 23). Thus, the released fragments probably remain in the extracellular matrix in an insoluble state. We could detect the few N- and C-terminal domain fragments of FN in urine samples from both healthy subjects and patients by using monoclonal antibodies specific to the Hep-1/Fib-1, Gel, and Fib-2 domains (unpublished).

Some oncogenically transformed cells have a diminished ability to adhere to FN because of the decreased expression of integrin, the FN receptor (24). Therefore, the cell-binding domain of FN or the degradation products of this domain may tend to be released from the surface of malignant cells into the body fluid. These processes of degradation and release may explain why the UFN detected consists mainly of the cell-binding domain of FN. In our preliminary experiments, we transplanted human tumor tissue into nude mice and observed the gradual increase of UFN excretion from these mice (unpublished data). This study strongly indicates that changes in FN organization in tumor cells may account for the release of FN or its fragments into the blood.

Several low-Mr tumor markers in urine have been investigated (25–28). UFN is different from these markers because it is generated from a large extracellular matrix protein through proteolysis. Perhaps fragmented FN is initially released from tumor sites into the blood, then filtered through the kidney, and thus excreted in the urine. The direct detection of circulating fragments of FN (i.e., the cell-binding domain) in the plasma might be more useful than the assay of UFN in the diagnosis of malignancy. Because there is no method to measure these fragments in plasma separately from intact plasma FN, assay of the concentration of FN fragments in urine, therefore, may be the most practical way to detect degraded FN circulating in body fluids. Several commercial methods have been established for the assay of FN in plasma and other such fluids (29), but the assay sensitivity of these methods is not satisfactory.

Webb and Lin (2) first reported on the amounts of FN in urine from healthy subjects, patients with prostate cancer, and patients with benign urological disease, measuring FN semiquantitatively by gelatin affinity chromatography. We describe here a reliable and reproducible IEMA for measuring the forms of human FN excreted into urine. With this assay we measure fragments made up mainly of the cell-binding domain, fragments that originate from the central portion of one of the subunits of FN and account for about one-fifth of the molecular mass of the native protein. The accuracy of the assay was improved when the monoclonal antibodies FN10 and FN30, specific to the cell-binding domain, were used instead of polyclonal antibodies against intact plasma FN; moreover, a sandwich assay performed with a pair of monoclonal antibodies had better specificity and sensitivity than did assays involving only one monoclonal antibody (unpublished).

The apparent heterogeneity of UFN, together with the possibility that different forms of the antigen have different affinities for polyclonal antibodies, was discovered during immunoblotting. We use the recombinant FN cell-binding domain (9), recognized by FN10 and FN30 monoclonal antibodies, which also recognize plasma FN, in the assay to overcome the problem of multiple molecular forms of native FN (29), which limits the use of the latter as a standard.

We describe here a new urinary tumor marker for many kinds of cancer and find it superior to other markers in the simplicity, speed, and noninvasiveness of its assay. These results suggest that the assay will be useful in the diagnosis of cancer, especially in screening groups undergoing physical checkups and in monitoring cancer patients.

References