

# MATRIX METALLOPROTEINASE ACTIVITY IN URINE OF PATIENTS WITH RENAL CELL CARCINOMA LEADS TO DEGRADATION OF EXTRACELLULAR MATRIX PROTEINS: POSSIBLE USE AS A SCREENING ASSAY

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## ABSTRACT

**Purpose:** Localized renal cell carcinoma is usually curable by nephrectomy. However, a large fraction of patients already present with metastatic disease, which results in a poor outcome. Currently no clinically relevant screening assay is available to detect early stage renal cell carcinoma. We investigated whether urinary extracellular matrix (ECM) proteins and/or matrix metalloproteinase (MMP) activity may be valuable as a noninvasive indicator of early stage renal cell carcinoma.

**Materials and Methods:** Urine specimens from preoperative patients with renal cell carcinoma and healthy controls were collected. The urinary excretion of the ECM proteins collagen IV, laminin and fibronectin was investigated by immunoblotting. MMP activity was assessed by gelatin zymography and by a fluorescence based microtiter plate activity assay.

**Results:** The full-length forms of all 3 ECM proteins investigated were significantly decreased or absent in renal cell carcinoma urine. Based on criteria established in this study this finding would lead to the correct detection of 95% of patients with renal cell carcinoma (21 of 22) with a false-positive rate of 4.5% (1 of 22 controls). All 11 nonmetastatic cases of the lowest clinical stage (T1N0M0) were correctly identified. The absence of urinary ECM proteins was due to significantly increased urinary MMP activity.

**Conclusions:** Analysis of decreased urinary ECM proteins and analysis of increased MMP activity may have value for the development of a sensitive, high throughput molecular screening assay to detect early stage renal cell carcinoma.

**KEY WORDS:** kidney; carcinoma, renal cell; tumor markers, biological; extracellular matrix proteins; matrix metalloproteinases

Renal cell carcinoma is the third most common malignancy of the urinary tract after prostate and bladder cancer.<sup>1</sup> This condition is usually asymptomatic until a relatively advanced state is reached. Consequently a large proportion of new patients already have metastatic disease at initial diagnosis.<sup>2</sup> In recent years the incidental discovery of asymptomatic renal cell carcinoma has significantly increased due to more widespread use of imaging techniques. A much higher percent of incidentally discovered renal cell carcinomas are localized and of low grade compared with symptomatic tumors.<sup>3</sup> Renal cell carcinoma is usually completely curable by radical or partial nephrectomy provided that the tumor is localized. Mainly due to the increase in incidental discovery the 5-year survival rate has increased to 57.9% from much lower outcomes in the 1970s and 1980s.<sup>4</sup> However, renal cell carcinoma remains a disease that would benefit tremendously from improvements in early detection.

No clinically relevant screening assay is currently available to detect asymptomatic renal cell carcinoma on a larger scale.<sup>3</sup> Existing imaging based methods are not practicable due to the high costs involved. The availability of an effective diagnostic assay may make it possible to screen routinely high and/or low risk populations. The former includes pa-

tients with end stage renal disease and acquired renal cystic disease in whom the risk of renal cell carcinoma is up to 100-fold higher than in the general population.<sup>3</sup> Furthermore, hereditary forms involving renal cell carcinoma, such as von Hippel-Lindau disease and hereditary papillary renal cell carcinoma, are in this category. A sensitive renal cell carcinoma screening test would also have high value for detecting tumor recurrence in patients with renal cell carcinoma after total or partial nephrectomy.<sup>5,6</sup> An ideal screening assay would be noninvasive and could be developed into a high throughput assay relying on commonly available clinical diagnostic equipment and expertise.

We report that urine from patients with renal cell carcinoma even at early stages contains matrix metalloproteinase (MMP) activity, which leads to the degradation of normally excreted extracellular matrix (ECM) proteins. Our data indicate that the presence of MMP activity and absence of ECM proteins in urine could be exploited to develop the first practicable screening assay for renal cell carcinoma detection.

## MATERIALS AND METHODS

**Study population.** A total of 36 patients with renal cell carcinoma who presented for surgery at the urological institute at our institution between July 2001 and January 2002 were enrolled in this study. Patients with other kidney related diseases or who received medications that may interfere with normal kidney functions were excluded from analysis. Also excluded from the study were patients with

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proteinuria or hematuria because this condition would obscure the origin of any potential renal cell carcinoma specific marker. Proteinuria and hematuria were assessed using Labstix Reagent Strips (Bayer Corp., Elkhart, Indiana). Patient age was 25 to 78 years (mean age 54.1). Spot urine samples (30 to 100 ml.) were collected once 2 to 4 weeks before surgery. The diagnosis was confirmed by pathological findings. All patients provided written consent. Control urine samples were also spot voids obtained anonymously from 39 apparently healthy volunteers 23 to 63 years old (mean age 44.5).

**Urine sample collection and processing.** Urine samples were collected in sterile containers and processed immediately. The study populations were divided into 2 groups depending on the intended experimental use. Urine from these groups was concentrated by 2 methods. In group 1 it was dialyzed against 50 mM. ammonium bicarbonate using 12 to 14 kDa. cutoff dialysis tubing (Invitrogen, Carlsbad, California) after adding a protease inhibitor cocktail, 10 mM. ethylenediamine tetraacetic acid and 50 mM. ammonium bicarbonate. Samples were lyophilized and reconstituted in water, resulting in a 30-fold concentration, and then aliquoted and stored at  $-80^{\circ}\text{C}$  for experiments. Urine from group 2 was immediately concentrated 20-fold by ultrafiltration using Centriprep (Millipore, Bedford, Massachusetts) centrifugal filter devices with a 12 to 14 kDa. cutoff and stored at  $-80^{\circ}\text{C}$  for activity based assays. All subsequent assays were standardized by an equal volume of the original urine.

**Immunoblotting.** Urine samples (20  $\mu\text{l}$ .) concentrated 30-fold from patients and controls were separated on 6% polyacrylamide gels. Proteins were electro-transferred onto nitrocellulose membranes and probed with primary antibodies for laminin, fibronectin (Sigma Chemical Co., St. Louis, Missouri) or collagen IV (Rockland Immunochemicals, Gilbertsville, Pennsylvania) and subsequently with secondary antibodies coupled to horseradish peroxidase. Bands were visualized by enhanced chemiluminescence. For semiquantitative analysis Western blots were encoded and 2 researchers not associated with this study were asked to assign to each signal a value of between - and +++++. Discrepancies were resolved by discussion.

**In gel zymography.** Gelatin (1 mg./ml.) was incorporated into 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Urine samples (15  $\mu\text{l}$ .) concentrated 20-fold were mixed with SDS-PAGE sample buffer without dithiothreitol and loaded directly onto gels. After electrophoresis gels were incubated for renaturation at room temperature in a buffer containing 50 mM. tris, pH 7.5, 150 mM. NaCl, 5 mM.  $\text{CaCl}_2$  and 2.5% Triton-X100 overnight. Gels were further incubated in the same buffer lacking Triton-X100 for 16 to 18 hours. Afterward gels were stained with Coomassie blue to visualize white negative bands indicating the presence of protease activity.

**In vitro proteolysis of fibronectin.** For each experiment 0.3  $\mu\text{g}$ . purified human fibronectin (Roche Molecular Biochemical, Indianapolis, Indiana) were incubated with 150  $\mu\text{l}$ . 20-fold concentrated renal cell carcinoma or control urine at  $37^{\circ}\text{C}$ . At different time points (0, 1, 3, 6 or 16 hours) 25  $\mu\text{l}$ . were removed from the reaction and analyzed for the disappearance of full-length fibronectin by SDS-PAGE and Western blot, as described.

**Fluorescence based assay for metalloproteinase activity.** Urine samples (50  $\mu\text{l}$ .) concentrated 20-fold were mixed in duplicate with 2  $\mu\text{g}$ . fluorescein conjugated collagen IV substrate (Molecular Probes, Eugene, Oregon) and  $10\times$  reaction buffer containing 0.5 M. tris-HCl, 1.5 M. NaCl, 50 mM.  $\text{CaCl}_2$  and 2 mM. sodium azide, pH 7.6, into 96-well plates in a total volume of 200  $\mu\text{l}$ . Plates were incubated at room temperature for 20 hours. Proteolytic activity was monitored using a fluorescence microplate reader and background was subtracted. As a control, metalloproteinase activity was inhibited by

including 2.5 mM. 1,10-phenanthroline. Standard curves were measured using collagenase-IV from *Clostridium histolyticum* (Molecular Probes).

## RESULTS

To identify a urinary marker for the detection of renal cell carcinoma we initially investigated the following hypothesis. Normal renal epithelial cells have a polarized morphology and secrete most proteins in strictly polarized fashion apically into the tubule lumen or basolaterally into the interstitial space.<sup>7</sup> In contrast, carcinoma cells (epithelial derived malignant tumors, including renal cell carcinoma) have partially or completely lost cell polarity to a degree that usually correlates with the degree of malignancy. In cell culture models we have previously shown that loss of epithelial polarity results in nonpolarized exocytosis of proteins.<sup>8</sup> Our hypothesis was that proteins normally secreted basolaterally into the interstitial space are secreted by renal cell carcinoma cells into the luminal space, resulting in their excretion with urine. Therefore, detecting such proteins would indicate the presence of renal cell carcinoma, potentially even at early disease stages.

To test this hypothesis we investigated the ECM proteins laminin, collagen IV and fibronectin, which are known to be secreted basolaterally by renal epithelial cells. Urine samples from 22 preoperative patients with renal cell carcinoma and 22 healthy controls were analyzed. Patients with renal cell carcinoma who had hematuria or proteinuria were excluded from analysis. Contrary to our hypothesis, these 3 ECM proteins were significantly decreased or absent in renal cell carcinoma urine samples compared with controls. Figure 1, A shows examples of Western blot analyses indicating that

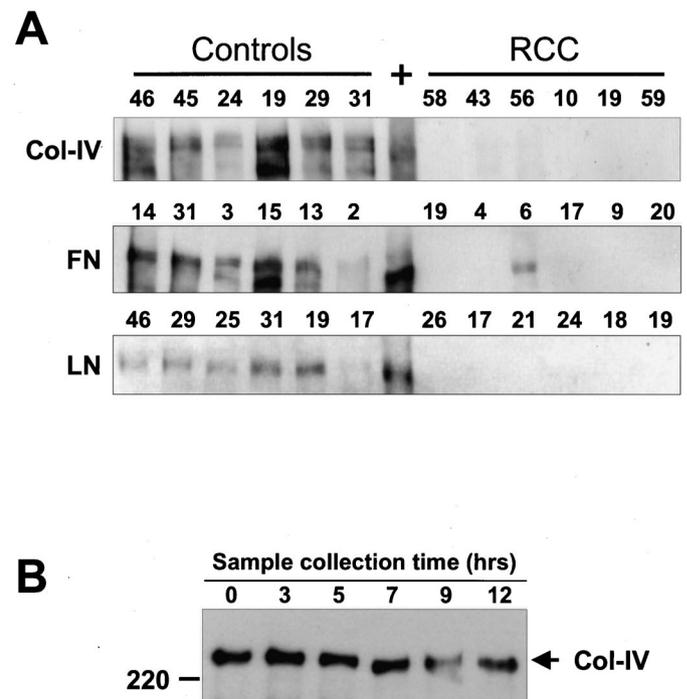


FIG. 1. Analysis of urinary ECM proteins. A, concentrated urine samples corresponding to equal original volumes from patients with renal cell carcinoma or controls were separated by SDS-PAGE, followed by immunoblotting with antibodies against collagen IV (Col-IV), fibronectin (FN) or laminin (LN). Regions of full-length proteins are shown. Values above lanes indicate patient and control numbers. +, positive controls (human serum for fibronectin, and total human kidney lysate for laminin and collagen IV). B, excretion profile of collagen IV in consecutive urine specimens collected at different times from healthy volunteer.

the full-length forms of all 3 ECM proteins were dramatically reduced in renal cell carcinoma urine.

The table lists semiquantitative analysis of these results with pathological findings. If we arbitrarily defined the absence (-) of at least 2 ECM proteins as indicative of renal cell carcinoma, this analysis would detect renal cell carcinoma correctly in 21 of 22 patients with renal cell carcinoma (95%). Only 1 of 22 controls (4.5%) showed 2 undetectable ECM proteins. Interestingly the absence of ECM proteins was detected even in all 11 patients with nonmetastatic disease of the lowest clinical stage (T1N0M0), which was typically asymptomatic and discovered incidentally. This finding indicates that the analysis of urinary ECM proteins may have value for the early detection of renal cell carcinoma.

For this analysis all specimens were collected at random times and standardization was done by using an equal volume of urine. To exclude the possibility that the excretion of ECM proteins may vary depending on the time of specimen collection successive samples from healthy volunteers standardized by equal volume were analyzed for full-length collagen IV (fig. 1, B). Collagen IV excretion varied only modestly between sample collection times. Therefore, the time of specimen collection is unlikely to account for the observed differences in patients with renal cell carcinoma and controls.

We then investigated whether overall patterns of excreted proteins differed in patient and control urine specimens. Figure 2 shows electrophoretic patterns of urinary proteins of 12 patients with renal cell carcinoma and 12 controls. While changes in the band patterns in some patients and controls were detectable, they were not observed consistently. No general protein degradation was apparent in renal cell carcinoma urine. This result indicates that urinary ECM proteins are specifically affected in renal cell carcinoma.

We hypothesized that the absence of ECM proteins was due to increased excretion of MMPs in the urine of patients with renal cell carcinoma. To test this hypothesis we incubated purified human fibronectin with equivalent amounts of urine from controls or patients. Figure 3 shows that patient but not control urine contained proteolytic activity that led to significant degradation of fibronectin. Proteolytic activity could be inhibited by the zinc chelator 1,10-phenanthroline, indicating that the involved enzymes belonged to the metalloproteinase family (fig. 4). To investigate further the excretion of MMPs urine specimens were analyzed by gelatin zymography. Figure 5 shows that renal cell carcinoma urine contained elevated levels of gelatinases compared with controls.

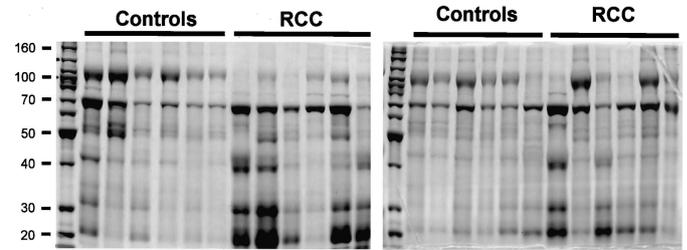


FIG. 2. Total urinary protein patterns. Concentrated urine samples corresponding to equal original volumes from patients with renal cell carcinoma (RCC) or controls were separated by SDS-PAGE, followed by staining with Coomassie blue. Values indicate molecular weight markers in kDa.

As a first step toward a high throughput screening assay for urinary MMP activity that may be suitable for screening large subject populations, we designed the following assay. Urine samples of 18 patients with renal cell carcinoma and 17 controls (different groups from the aforementioned analyses) were incubated with collagen IV heavily labeled with fluorescein, so that fluorescence was quenched. Proteolytic digestion results in fluorescence de-quenching due to the release of highly fluorescent peptides. The increase in fluorescence is proportional to the proteolytic activity and it is measured in a microtiter plate fluorescence reader. Figure 4 shows that the average activity measured by this assay was significantly increased by approximately 4-fold in the renal cell carcinoma population (t test p <0.0001).

DISCUSSION

We report that urine from patients with renal cell carcinoma contains elevated levels of MMP activity, which results in the degradation of normally excreted, full-length ECM proteins. In principle the absence of ECM proteins and presence of MMP activity can be exploited to develop a noninvasive screening assay to indicate the presence of renal cell carcinoma.

Semiquantification of immunoblot analysis of urinary full-length ECM proteins

| Control* |         |             |             | Renal Cell Ca* |                          |                             |                         |         |             |             |
|----------|---------|-------------|-------------|----------------|--------------------------|-----------------------------|-------------------------|---------|-------------|-------------|
| Sex      | Laminin | Fibronectin | Collagen IV | Sex            | Pathological Tumor Type  | Fuhrman Grade <sup>17</sup> | TNM Stage <sup>18</sup> | Laminin | Fibronectin | Collagen IV |
| M        | +++     | ++          | +++         | M              | Clear cell + granular    | 3                           | T3bN2M1                 | -       | -           | -           |
| M        | +++     | ++++        | +++         | M              | Clear cell               | 3                           | T3bN2M1                 | -       | -           | -           |
| M        | ++      | +++         | ++++        | M              | Clear cell + papillary   | 3/4                         | T1N0M0                  | -       | -           | -           |
| M        | +++     | +++         | +++++       | M              | Papillary                | 2                           | T2N0M0                  | +       | -           | -           |
| M        | +++     | +++         | +++         | M              | Clear cell               | 2                           | T2N0M0                  | -       | +           | +           |
| M        | ++++    | +++         | ++++        | M              | Papillary                | 3                           | T1N0M0                  | -       | -           | -           |
| M        | +++     | ++          | ++          | M              | Clear cell + papillary   | 2                           | T2N0M0                  | -       | -           | -           |
| M        | ++      | ++          | ++          | M              | Clear cell               | 2                           | T2N0M0                  | -       | -           | +           |
| M        | +++     | ++++        | ++++        | M              | Clear cell               | 2                           | T1N0M0                  | -       | -           | -           |
| M        | ++      | +           | ++          | M              | Clear cell               | 2                           | T1N0M0                  | -       | -           | -           |
| M        | +       | +           | +           | M              | Clear cell               | 3                           | T3N0M0                  | -       | +           | -           |
| M        | +++     | +++         | +++         | M              | Papillary                | 2                           | T1N0M0                  | -       | -           | -           |
| M        | +++     | ++++        | +++         | M              | Clear cell               | 2                           | T3aN0M0                 | -       | -           | -           |
| M        | ++      | ++          | +++         | F              | Clear cell               | 2                           | T1N0M0                  | -       | -           | -           |
| F        | ++      | +++         | +++         | F              | Clear cell               | 3                           | T1N0M0                  | -       | -           | -           |
| F        | ++      | ++          | +           | F              | Clear cell               | 3                           | T3bN2M1                 | -       | -           | -           |
| F        | ++      | +           | -           | F              | Clear cell               | 3                           | T3bN2M1                 | -       | -           | -           |
| F        | +       | +           | +           | F              | Clear cell               | 2                           | T3BN2M1                 | -       | -           | -           |
| F        | -       | -           | +           | F              | Clear cell               | 1/2                         | T1N0M0                  | -       | -           | -           |
| F        | -       | ++          | +           | F              | Clear cell               | 2                           | T1N0M0                  | -       | -           | -           |
| F        | ++      | +++         | ++++        | F              | Clear cell + sarcomatoid | 4                           | T1N0M0                  | -       | -           | -           |
| F        | +++     | ++++        | +++         | F              | Clear cell               | 2                           | T1N0M0                  | -       | -           | -           |

Concentrated urine specimens from patients with renal cell carcinoma and age matched controls were analyzed for full-length laminin, fibronectin or collagen IV with samples standardized by equal volume.

\* Since signal intensities in patients and controls far exceeded the linear range of film detection, signals were analyzed semiquantitatively by comparing multiple exposures and defining a range from undetectable (-) to maximum signal (+++++).

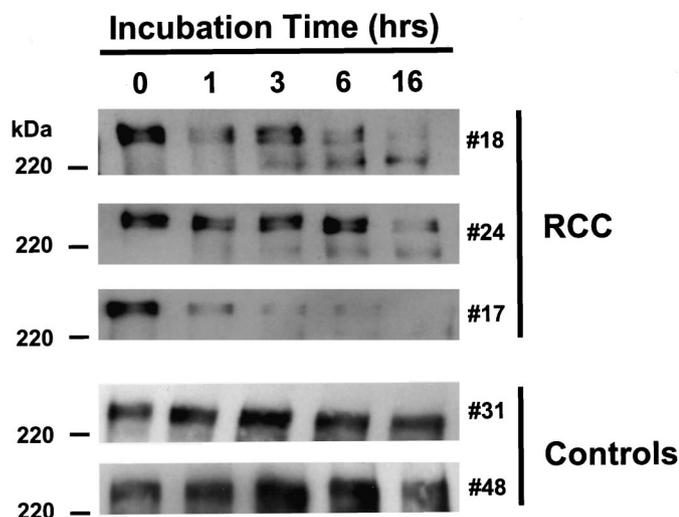


FIG. 3. Renal cell carcinoma (RCC) urine contained proteolytic activity for in vitro degradation of fibronectin. Purified human fibronectin was incubated with concentrated renal cell carcinoma or control urine specimens from 3 patients and 2 controls for indicated times. Disappearance of full-length fibronectin was monitored by Western blot analysis.

A large number of MMPs are known to proteolyse specifically various extracellular matrix proteins under normal and pathological conditions. MMPs have an important role in tumor invasion and metastasis.<sup>9-13</sup> There is evidence for increased MMP expression in renal cell carcinoma. MMP-9 was reported to be significantly elevated in tumor tissue of patients with renal cell carcinoma, whereas MMP-2 was reportedly unaffected.<sup>14</sup> In contrast, Kugler et al found that MMP-2 and MMP-9 are elevated in renal cell carcinoma tumor tissue and their expression level correlates with tumor aggressiveness.<sup>15</sup> Urine was not examined in either study, nor was the expression of MMPs other than MMP-2 and MMP-9.

Since we excluded patients with proteinuria or hematuria from analysis, it is unlikely that the increased level of MMP activity in urine was due to leakage from serum caused by compromised renal barrier function. It is possible that affected MMPs reach the urine by glomerular filtration or direct secretion from the tumor into the urinary space. The latter possibility may be expected to lead to much more

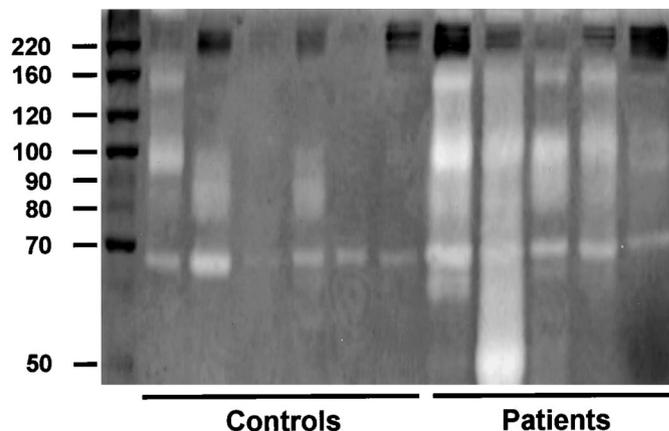


FIG. 5. Gelatinolytic activity in renal cell carcinoma and control urine specimens was analyzed by in-gel gelatin zymography. Molecular weight assignment was only approximate because reduced marker proteins were expected to show different migration behavior than nonreduced proteins in urine. White bands indicate gelatinases. Values indicate molecular weight in kDa.

efficient MMP excretion, although further experiments are required to investigate this question.

Our results indicate that analysis of urinary ECM proteins allows the detection of renal cell carcinoma with high sensitivity (see table). A single false-positive result occurred in 22 healthy controls. The complete absence of urinary ECM proteins in controls was only observed in relatively young and presumably premenopausal females, which suggests that increased urinary MMP activity may be linked to endometrium remodeling. Several MMPs are known to be highly up-regulated under these conditions.<sup>16</sup> Therefore, it may be necessary to exclude premenopausal women from this study or restrict inclusion to certain phases of the menstrual cycle, which must be defined experimentally.

Although MMP activity measured by the fluorescence based assay was an average of 3.4-fold increased in the renal cell carcinoma population compared with controls (fig. 4), there was a greater overlap in the 2 groups compared with the analysis of ECM proteins. Higher sensitivity of the ECM assay may have been due to the fact that the urinary ECM proteins are subjected to proteolytic activity at body temperature in the urinary tract for an extended period before voiding. This scenario would cause "in-body enzymatic

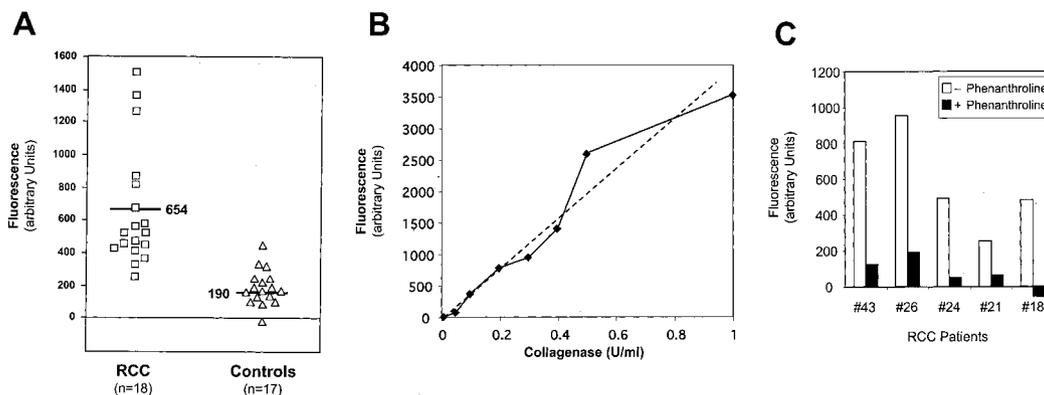


FIG. 4. Detection of MMP activity using fluorescence based microtiter plate assay. A, concentrated urine samples from patients and controls were mixed in 96-well plates in duplicate with reaction buffer containing fluorescent substrate, namely collagen IV modified with fluorescein. Proteolytic activity was indicated by increased fluorescence, which was monitored with fluorescence microplate reader. Background was subtracted and average fluorescence values were plotted for patients and controls. Values of duplicate measurements varied by less than 10%. Investigated specimens were from groups of patients/controls different from those shown in table 1. Average value for renal cell carcinoma was 3.4-fold higher than for controls (t test  $p < 0.0001$ ). Difference was also significant by Wilcoxon and median tests. RCC, renal cell carcinoma. B, standard curve measured using collagenase IV from *Clostridium histolyticum* under otherwise identical assay conditions shows fluorescence values within linear range of assay. C, in 5 randomly selected renal cell carcinoma urine specimens zinc dependency was investigated by including 2.5 mM, 1,10-phenanthroline, which resulted in almost complete inhibition of proteolytic activity.

signal amplification" and, therefore, higher sensitivity. However, the simplicity of the fluorescence based activity assay makes it much more suitable for rapid, high throughput analyses and optimization of this assay may result in improved differentiation of patients from controls.

#### CONCLUSIONS

Even at early stages of disease patients with renal cell carcinoma excrete significantly elevated levels of MMP activity with the urine, causing the degradation of full-length ECM proteins. The analysis of ECM proteins and/or MMP activity in urine has high potential as a marker to allow noninvasive, high throughput screening for the early detection of renal cell carcinoma.

Xin Li and Yuehua Li assisted with data analysis.

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