An Inexpensive, Point-of-Care Urine Test for Bladder Cancer in Patients Undergoing Hematuria Evaluation

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Although hematuria (blood in urine) is the most common symptom of bladder cancer, 70–98% of hematuria cases are benign. These hematuria patients unnecessarily undergo costly, invasive, and expensive evaluation for bladder cancer. Therefore, there remains a need for noninvasive office-based tests that can rapidly and reliably rule out bladder cancer in patients undergoing hematuria evaluation. Herein, a clinical assay for matrix metalloproteinases (“Ammps”) is presented, which generates a visual signal based on the collagenase activity (in urine of patients) on the Ammps substrates. Ammps substrates are generated by crosslinking gelatin with Fe(II) chelated alginate nanoparticles, which precipitate in urine samples. The cleavage of gelatin-conjugated alginate (Fe(II)) nanoparticles by collagenases generates free-floating alginate (Fe(II)) nanoparticles that participate in Fenton’s reaction to generate a visual signal. In a pilot study of 88 patients, Ammps had 100% sensitivity, 85% specificity, and a negative predictive value (NPV) of 100% for diagnosing bladder cancer. This high NPV can be useful in ruling out bladder cancer in patients referred for hematuria evaluation.

1. Introduction

≈79,000 new cases and 16,870 deaths will be attributed to bladder cancer in 2017.[1] The most common symptom of bladder cancer is hematuria with ≈85% of the bladder cancer patients experiencing gross or microscopic hematuria.[2] The American Urological Association recommends cystoscopy, urine cytology, and upper urinary tract imaging for all patients that present with hematuria after ruling out other benign etiologies. The dilemma, however, is that only 2–5% of patients with microscopic hematuria and 10–30% with gross hematuria are diagnosed with bladder cancer after full evaluation.[3] Therefore, a large number of patients undergo a costly and invasive workup unnecessarily. Among the patients who are ultimately diagnosed with bladder cancer, ≈50% of these patients will experience tumor recurrences in their lifetime[4] and thus many are destined to lifelong follow-up with serial cystoscopies and treatment of recurrent tumors. As a result, bladder cancer has the highest lifelong treatment costs of all cancers.[5]

While cystoscopy is the gold standard for both diagnosis and surveillance of bladder cancer, its high associated cost and invasive nature lead to low referrals and poor compliance with surveillance protocols.[6] As a result, few noninvasive tests have been developed with the aim of replacing or decreasing the frequency of cystoscopies. Unfortunately, these tests have low sensitivity (50–70%) or specificity (30–87%).[7,8] are expensive to perform, and are not easily implemented in a clinic setting. Thus, there remains a need for the development of novel tests that allow for cost-effective, noninvasive detection of bladder cancer.

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Matrix metalloproteinases (MMP) are a potential target for such tests. MMPs are a family of zinc-dependent endogenous proteolytic enzymes that show collagenase activity to degrade the extracellular matrix (ECM). [9] Statistically significant increased activity of collagenases (MMP2, MMP9, and sometimes MMP7 and MMP13) has been found in the urine of patients with bladder cancer, [10] making these attractive biomarkers for bladder cancer diagnosis, surveillance, and possibly screening. Moreover, MMPs are an excellent prognostic tool for a poor survival rate, suggesting that MMPs are directly related to survival rates. [11] In addition to the survival rates, it has been reported that abnormal expression of MMP2 and MMP9 was associated with a high risk of tumor recurrence in patients with stage T1 bladder cancer. [12] In this paper, we have generated novel assays called Ammps that can detect MMPs in a highly sensitive and cost-effective manner and therefore may be utilized to sensitively detect patients that do not have bladder cancer.

### 2. Results and Discussion

Ammps is an assay that is highly sensitive to collagenase activity (observed in urine of bladder cancer patients) because it is able to (a) temporarily turn OFF (hide) the catalytic activity of Fenton's reaction catalyst and (b) turn ON (make available) large quantities of the catalyst in the presence of active collagenase. The technology behind Ammps for the detection of bladder cancer is shown in Figure 1. Detection of bladder cancer is based on the collagenase activity of active MMPs 2/9 on gelatin-crosslinked-alginate nanoparticles. The Fe(II) catalyst is “hidden” in these alginate nanoparticles through chelation with alginate (steps 1–3). When patient urine is added that contains active MMPs 2/9, alginate nanoparticles are released and resuspended from the gelatin-crosslinked-alginate aggregates (step 4). The amount of released individual nanoparticles is proportional to the amount of active MMPs 2/9. Step 5: Next, the resuspended nanoparticle solution is separated and is added to the chromophore. Step 6: The previously “hidden” Fe (II) catalyst is activated by adding acid to remove the alginate chelate, and the assay very effectively generates visually observable color output proportional to the amount of active MMPs 2/9. Steps 4–6 are utilized to identify bladder cancer by detecting collagenase in patient’s urine.
MMP1, MMP8, and MMP13) as a linker[33] The resultant intercrosslinked particles now agglomerated as compared to the non-conjugated particles as observed using SEM (Figure 2C). This agglomeration was further confirmed by sizing the particles using coulter counter (average size = 6.8 ± 0.3 µm) (Figure 2D). Although some limited aggregation is seen in SEM images with individual particles, this is a result of SEM preparation. Importantly, these particles can become easily and finely dispersed in solution. In contrast, the particles in Figure 2C are physically crosslinked and precipitate in solution (confirmed visually).

We hypothesized that when collagenase cleaves gelatin-crosslinked-alginate particles, individual alginate particles are regenerated. Therefore, we wished to confirm that the size distribution of gelatin-crosslinked-alginate particles is reduced in the presence of collagenase. Collagenase type IV (a type of collagenase, 1 mg mL⁻¹) in 500 µL PBS was incubated with 50 µL of gelatin-crosslinked-alginate particles (3 mg mL⁻¹) for 25 min and the size of the particles was measured using DLS at 25 min postincubation. It was observed that the size of the particles decreased from 6.8 ± 0.3 µm to 630 nm (Figure 2D), suggesting that incubation with collagenase led to cleavage of gelatin crosslinks, and regeneration of individual particles from aggregates in solution.

In order to determine if the Ammps can produce a visually observable color change, 50 µL of gelatin-crosslinked-alginate particles (3 mg mL⁻¹) in DIH₂O was incubated in the presence of substrate and the change in color was recorded using a camera (Figure S1, Supporting Information). Moreover, the change in absorbance at 560 nm was also recorded using a plate reader to quantitatively determine the progression of the reaction. The addition of HCl to unoxidized IR783 changes its color from green to purple. We determined the range of color change generated in the presence of gelatin-crosslinked-alginate particles, HCl, and IR783. It was observed that in the presence of Ammps the color of the substrate changes from purple to yellow (560 to 400 nm) with time (Figure 3A(i), Figure S2, Supporting Information). This change in color is depicted in the form of a gradient for reference (Figure 3A(ii)). Moreover, we performed this test at different temperatures (4, 30, 40, and 50 °C) and humidity levels (ambient 40% and high 85%) to study the effect of temperature and humidity on the ability of Ammps to detect collagenase activity. It was observed that Ammps could detect collagenase activity at 30 and 40 °C temperatures, and also at ambient 40% and high 85% humidity levels. However, Ammps could not detect collagenase activity at 4 and 50 °C (Figure S3A,B, Supporting Information).

In order to identify the detection limits of Ammps for collagenase, we tested the ability of Ammps to detect different levels of collagenase in PBS and urine samples. PBS solution at 1× was utilized to test the ability of Ammps to detect collagenase activity, whereas for testing the ability of Ammps to detect collagenase activity in urine 70% of 10× PBS + 30% of urine was utilized. In case of urine samples, 70% of 10× PBS solution was utilized because it was determined that the pH can be normalized with this solution in order to achieve the optimal activity of collagenase. PBS (1 mL) or nondiseased urine samples (0.3 mL urine + 0.7 mL 10× PBS) were spiked with collagenase type IV to generate 1 µg mL⁻¹, 1 ng mL⁻¹, 100 pg mL⁻¹, 10 pg mL⁻¹, 1 pg mL⁻¹, and 0.1 pg mL⁻¹ concentrations. A 50 µL solution of gelatin-crosslinked-alginate particles was added to the collagenase spiked PBS or urine samples and incubated at room temperature for 25 min. Next, 300 µL of the solution was retrieved and 6 N HCl, IR783, and H₂O₂ were added to this solution. The color generated at the end of 3 min was recorded using a camera. Moreover, the change in absorbance at 560 nm was also recorded by performing the assay in a 48-well plate and utilizing a plate reader to quantitatively determine the detection limit (Figure S7A, Supporting Information). It was observed that Ammps was able to change the color of the solution from purple to yellow (purple = negative (λ = 560 nm); yellow = positive (λ = 400 nm)) so as to differentiate between 1 and 0 pg mL⁻¹ of collagenase type IV in PBS and urine samples.
In order to confirm if the collagenase retained its activity in the urine samples, collagenase activity was also confirmed using a fluorescent substrate (Figure S4, Supporting Information). This datum shows that the collagenase retained its activity in the normal urine samples that were spiked with different levels of collagenase.

After IRB approval, we collected urine samples from patients seen in the urology clinic for hematuria evaluation, and these samples were deidentified before use. A total of 88 patients (number determined using power analysis for a test of proportions) provided urine samples for Ammps testing. All urine samples were tested on the day of collection. The color of the solution generated by the Ammps was recorded using a camera at the end of 3 min (Figure 3D). To determine the error in interpretation of positive results (color identification), three independent investigators scored the data obtained from Ammps. Moreover, the threshold color for demarcating positive and negative results from Ammps was optimized to obtain the highest sensitivity and specificity toward bladder cancer (Figure S5, Supporting Information). Of the 88 patients who provided urine samples, 23 had a prior history of bladder cancer and these patients were not included in our analysis since we wanted to determine the utility of Ammps in the detection of bladder cancer rather than for bladder cancer surveillance (Figure 3E).

Demographic data for the remaining 65 patients can be found in Figure 3F (for more detailed information see Table S2, Supporting Information). The median age of patients was 64.5 years, 75% were male, median creatinine was 1.0, and only 34 patients (52%) had gross or microscopic hematuria at the time of Ammps testing. Etiology for hematuria was variable with many patients (29%) having prostatic bleeding and 26% having no obvious etiology. Five of 65 patients (8%) received a new diagnosis of bladder cancer. All five patients (100%) had a positive Ammps test. Pathology included two patients with T3 disease, one with T2 disease, one high grade T1, and one patient with low grade Ta. The remaining 60 patients had no evidence of bladder cancer after full evaluation. Of these, 51 patients (85%) had a negative Ammps test and 9 (15%) had false positive tests. Of these nine patients with false positive results, three were diagnosed with prostate cancer, one had a bladder mass that was resected revealing chronic polyploid cystitis, another had recurrent renal cell carcinoma at the left proximal ureteral stump following prior nephrectomy, and only four had completely negative workups (Figure 3E). It is worth noting that

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**Figure 3.** Ammps have 100% negative predictive value for patients without bladder cancer in hematuria patients. A) Oxidation of IR783 dyes (i) in the presence or absence of alginate (Fe/Ca) particles (representative of n = 3). (ii) Color key for increasing oxidation of IR783 in the presence of Ammps with time is shown. B) PBS was spiked with collagenase type IV ranging from 1 µg mL⁻¹ to 0.1 pg mL⁻¹ and Ammps was performed to detect the limit of detection in PBS (representative of n = 3). C) Nondiseased urine was spiked with collagenase type IV ranging from 1 µg mL⁻¹ to 0.1 pg mL⁻¹ and Ammps was performed to detect the limit of detection in urine (representative of n = 3). D) Ammps identifies patients with bladder cancer. Ammps was performed in a blinded fashion on patients’ urine samples and divided into positive (orange-yellow color) and negative (green-purple color) for bladder cancer. Representative images of Ammps performed on patients’ urine samples are shown. Code for patients is shown on top and the results are shown on the bottom. E) After performing cystoscopy, it was observed that the false positive case (negative via cystoscopy and positive via Ammps) had a benign tumor mass but not bladder cancer. E) In the general population of patients, the Ammps had a negative predictive value of 100%, with it being false negative for prostate cancer (three patients), renal cell carcinoma (one patient), chronic polyploid cystitis (one patient), BPH (one patient), UTI (one patient), and complete negative workup (two patients). F) Patient demographics with grade and stage of bladder cancer.
four patients with a false positive test had a different urologic malignancy and one had an inflammatory bladder mass that was resected. Moreover, MMP9 ELISA and collagenase activity assay (DQ-gelatin) performed on the urine of bladder cancer patient had a high correlation (0.54 and 0.82, respectively) with the results obtained from Ammps test, suggesting the presence of and the activity of MMPs in patients’ urine (Figure S4, Supporting Information). Finally, a camera phone application or an absorbance spectrophotometer (such as a plate reader) can be utilized to generate a quantitative output of Ammps for more consistent interpretation of the data (Figures S6 and S7, Supporting Information). Notably, it was observed that a cutoff value of 5 (reciprocal of the absorbance at 560 nm obtained via plate reader) differentiated between the patients with bladder cancer and without bladder cancer with high sensitivity. Furthermore, the varying levels of the reciprocal of absorbance values for bladder cancer positive patients had a low-to-moderate correlation (0.33) with the stage of the bladder cancer.

3. Conclusion

In our pilot study, the sensitivity of Ammps for diagnosing bladder cancer was 100%, while the specificity and negative predictive value (NPV) were 85% and 100%, respectively, which is much more sensitive compared to other commercially available point-of-care urine tests (i.e., BTA-STAT and NMP-22 BladderChek) which have reported sensitivities of 50–70%. Finally, Ammps has the potential to be used as a screening test in resource-poor settings because it follows the ASSURED guidelines (Affordable, Sensitive, Specific, User-friendly, Rapid, Equipment free, and Deliverable) set forth by the World Health Organization (WHO) (Table S1, Supporting Information). On the other hand, current point-of-care tests for bladder cancer such as cystoscopy and BTA-STAT are expensive and require specialized instruments and personnel.

In conclusion, this report introduces Ammps, a point-of-care urine test that may be utilized for the detection and surveillance of bladder cancer. This test can be cost-effective, is instrument free, and can be performed in a clinical setting. Ammps has a high NPV and sensitivity for detection of bladder cancer, and thus also has the potential to serve as a screening test in high-risk populations. With its capacity for bladder cancer diagnostics at the bedside and ability to satisfy the WHO’s ASSURED criteria, Ammps can also have an impact on monitoring of, and possibly screening for, bladder cancer in resource-poor settings. A larger prospective trial is underway to confirm these results.

4. Experimental Section

Fenton’s Reaction Substrate: Fenton’s reaction substrate consisted of 100 µL 6 n HCl (Fisher Scientific, Pittsburgh PA), 100 µL H₂O₂ (R&D Systems, Minneapolis, MN), and 10 µL of 5 mg mL⁻¹ IR783 in deionized (DI) H₂O (Sigma Aldrich, St. Louis, MO). First, the 0.3 mL of sample that had been incubated with Ammps for 25 min was added to the 100 µL 6 n HCl in an appendage tube. Next, 10 µL of IR783 and 100 µL H₂O₂ were added in this sequence. The reaction was carried out for 3 min and a camera was utilized to image the color generated.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.
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