

Development of a Diagnostic Test for Monitoring Bladder Cancer Recurrence Using Urinary Matrix Metalloproteinases (MMPs) as Clinical Biomarkers

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Abstract

With increasing cancer therapeutic efficacies and the development of better methodologies for early detection, the cancer survivor population has been growing steadily over the years. Currently 3.5% of the US population is made up of cancer survivors. In addition to the risk of having a recurrence of their primary cancer, these patients also have an increased risk of developing a secondary cancer. To date, due to the increased risk of this patient population, and the need for monitoring methodologies with high performance characteristics, more costly and complex diagnostic procedures generally reserved for diagnosing symptomatic patients have been used for recurrence monitor. However, as this patient population grows, there is a clear need for the development of more cost effective clinical algorithms and assays that would result in more efficient management of cancer survivors.

Matrix metalloproteinases are a family of zinc-dependent endopeptidases that have been shown to be key regulators of tumor growth and metastasis formation. Detection of matrix metalloproteinases (MMPs) in the urine of cancer patients has been shown to correlate with disease status in a variety of cancers, including bladder cancer. Numerous studies have now shown that, in particular, biologically active MMP-2 and MMP-9 are found at higher frequency in the urine of cancer patients than in the urine of normal, age-match, sex-matched controls.

Our group is developing a non-invasive diagnostic test utilizing urinary MMPs, such as MMP-2 and MMP-9, as monitors of disease-free status and cancer recurrence in bladder cancer. Initial studies using urinary levels of MMP-2 and MMP-9 to discriminate disease-free patients from those with bladder cancer resulted in approximately 86% (24/28) sensitivity and 84% (169/202) specificity at a fixed protein level. Further validation of these results and full development of a clinical test to monitor for bladder cancer recurrence are currently underway. In addition, we have incorporated a novel analytical algorithm called Clinical Intervention Determining Diagnostic (CIDD), which results in extremely high (~100%) Negative or Positive Predictive Value, allowing the physician to better manage this patient population.

Study Design

Our lab is developing the use of urinary matrix metalloproteinases to manage the care of cancer survivors by monitoring for recurrence in a non-invasive format and at greater frequency than currently possible. Based on previous studies which have shown that MMP-2 and MMP-9 are found in the urine of cancer patients at greater frequency than in the urine of disease-free individuals, and initial proof of concept study was designed to compare urinary MMP-2 and MMP-9 levels in a set of 230 samples, including 28 samples obtained from bladder cancer patients. "Disease-free" controls were obtained from asymptomatic individuals as well as from cancer-free individuals who suffer from other urological conditions, such as kidney stones, bladder obstruction, incontinence, erectile dysfunction and urinary tract infections. Urine samples were assayed for MMP enzymatic activity by gelatin zymography and total MMP levels were quantitated via ELISA. To determine enzymatic activity, 30ul of unprocessed urine were mixed with 10ul of sample buffer, loaded and separated under non-reducing conditions in SDS-PAGE gels containing gelatin. Gels were incubated overnight in buffer containing calcium and then stained with coomassie blue. Enzymatic activity was visualized as a zone of clearance against a dark blue background where the gelatin was degraded by the MMP.

To determine total MMP levels, 50-100ul of neat urine were run on MMP-2 and MMP-9 specific ELISAs as per the manufacturer's instructions. Data analysis was accomplished via t-tests (assuming unequal variances) and receiver / operator characteristic (ROC) curves for both MMP-2 and MMP-9 values. All studies were conducted in duplicate in a blinded fashion.

Urine Samples from Bladder Cancer Patients Contain Higher Levels of Enzymatically-active MMP-2 and MMP-9 than that from Disease-free Individuals

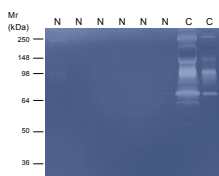


Figure 1 Gelatin zymography was used to assess the MMP enzymatic activity found in the urine of bladder cancer patients as compared to that from disease-free individuals. Both MMP-2 and MMP-9 were detected at significantly higher frequency in the urine of cancer patients (C) compared to urine obtained from disease-free controls (N). A representative zymography gel is shown above.

High Levels of MMP-2 and MMP-9 are Found at Greater Frequency in the Urine of Bladder Cancer Patients than in that from Disease-free Individuals

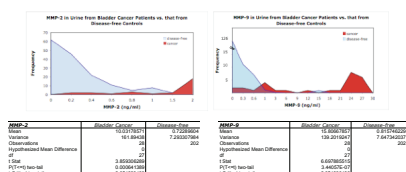


Figure 2 Urine samples obtained from bladder cancer patients (n=28) and disease-free individuals (n=202) were analyzed by ELISA for absolute levels of MMP-2 and MMP-9. Urine obtained from bladder cancer patients contained higher levels of both MMPs as compared to controls and at higher frequency. The graphs above show the number of individuals per range of protein values in each population. Statistical analysis is shown in the table below each graph. In both cases, mean marker concentration was significantly higher in the cancer cohort than in the disease-free samples.

Receiver-Operating Characteristic (ROC) Curves for MMP-2 and MMP-9

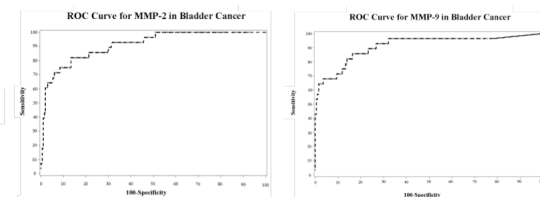


Figure 3 To measure the diagnostic accuracy of the markers, receiver-operating characteristic (ROC) curves were generated. The area under the curve for both markers was approximately 90%.

Biomarker Performance

MMP-9	Cutoff	Sensitivity	[95% CI]	Specificity	[95% CI]
		86% (19/28)	[48% - 84%]	97% (195/202)	[93% - 99%]
MMP-2	Cutoff	82% (23/28)	[67% - 96%]	84% (169/202)	[78% - 88%]
		75% (21/28)	[55% - 89%]	91% (185/202)	[87% - 95%]

Table I Based on the ROC curves generated, two cutoffs values per marker were used to calculate biomarker sensitivity and specificity. Results are shown in the Table above.

Clinical Intervention Determining Diagnostic (CIDD)

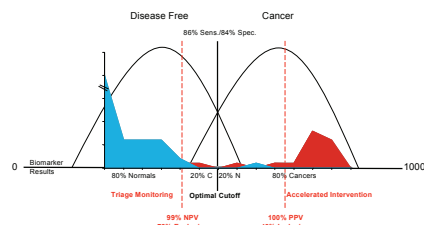


Figure 4 Application of real data to a novel algorithm for determining the need for clinical intervention allows new cutoff points (red dotted line) to be drawn where extremely high negative or positive predictive values (~100%) determine exclusion or inclusion for follow-up intervention. In the example of MMP-9 shown above, at 98% negative predictive value, 84% of disease-free individuals would be excluded from follow-up tests.

Summary

- Initial studies comparing samples obtained from bladder cancer patients and control samples demonstrated that MMP-2 and MMP-9 are found at significantly higher levels and at greater frequency in the urine of bladder cancer patients.
- Receiver-operating characteristic curve (ROC) analysis of each marker, used to determine diagnostic accuracy, resulted in areas under the curve of approximately 90% for each marker.
- Sensitivity and specificity were 86% and 84% for MMP-9 and 82% and 86% for MMP-2.
- Application of CIDD to the data established new cutoffs to be set for each marker. At approximately 100% negative predictive value (NPV) this algorithm allows physicians to exclude patients from unnecessary follow-up tests.