Sample Collection Impacts the Performance of Urinary Matrix Metalloproteinases (MMPs) as Diagnostic BioMarkers for Bladder Cancer Recurrence

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Abstract

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 assay utilizing urinary MMPs, such as MMP-2 and MMP-9, as monitors of disease-free status and cancer recurrence in bladder cancer. In the process of developing the assay, we established the background frequency and levels of both MMP-2 and MMP-9 in the urine of normal controls. Background levels of MMPs were found in the urine of approximately 10% of disease-free males and approximately 30% of disease-free females, with MMP-9 being the most commonly detected endogenous MMP. Little or no MMP-2 was detected in disease-free individuals. We investigated whether addition of a "clean catch" sample collection procedure would decrease the endogenous background levels of MMPs found in a disease-free population. We found that the addition of "clean catch" to our standard operating procedure for sample collection reduced both the frequency and levels of MMPs.

In addition, we tested the potential impact of the " clean catch" sample collection methodology on clinical sensitivity and specificity by performing a small pilot study using urinary levels of MMP-2 and MMP-9 to discriminate disease-free patients from those with bladder cancer. At a fixed protein level resulting in 90% (9/10) sensitivity, addition of "clean catch" procedure to the urine sample collection protocol increased MMP-9 marker specificity from approximately 74% (11/42) to approximately 91% (5/54). Further validation of these results and full development of a clinical test to monitor for bladder cancer recurrence are currently underway.

Introduction

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.

We believe this is a very important emerging issue that could be addressed with the discovery and development of new biomarker associated diagnostic tests. 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. In the course of developing these assays, we observed a slightly higher background of endogenous MMPs in the urine of disease-free females than in that of disease-free males and set out to determine whether this difference might be attributable to contamination of the urine with vaginal secretions and whether the addition of a "clean catch" urine collection procedure would reduce the levels of MMP observed.

Urine Samples from Disease-free Females Contain Higher Endogenous MMP-9 Levels than that from Disease-free Males

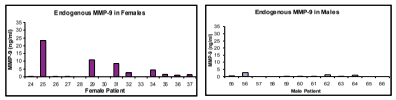

Figure 1 To determine the baseline levels of MMPs found in the urine of disease-free individuals, MMP-2 and MMP-9 ELISA assays were run on urine samples obtained from asymptomatic males and females. Endogenous MMP-9 was detected in a number of urine samples obtained from females but was not found at significant levels in the urine of male controls.

Blood as a Possible Source of MMP-9 in Urine Samples

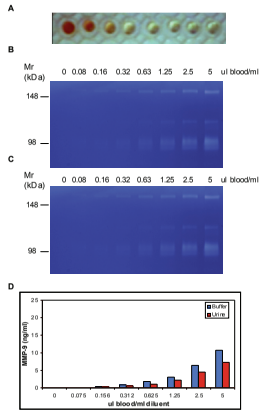

Figure 2 To determine whether blood may contribute to background MMP-9 levels found in some urine samples, increasing amounts of blood (A) were titrated into buffer (B) and urine (C) and MMP-9 levels were monitored by gelatin zymography and ELISA (D). Although large amounts of blood (>1.25ul blood/ml urine) can contribute to MMP-9 contamination in urine, the amount of blood required for MMP-9 detection exceeds the amount of blood typically found in the urine of disease-free individuals and can easily be seen by eye.

Clean Catch Urine Collection Method Reduces Background MMP-9 Levels in Females

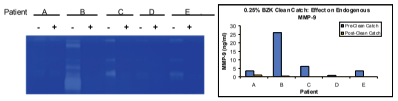

Figure 3 To determine the efficacy of clean catch on reducing the presence of endogenous MMP-9 in the urine of women, 5 females were asked to urinate into a specimen cup, stop stream, use 3 benzalkonium chloride (BZK) towelettes, and continue urination into a separate specimen cup. The resulting 2 urine specimens (pre- and post-wipe) for each female were analyzed via zymography and ELISA. In all five females tested, "clean catch" significantly decreases the amounts of MMP-9 present, suggesting that these are not native to urine itself. Figure 3 shows the ELISA results for each female (A-E) before and after clean catch. This reduction in MMP-9 concentration post clean catch was consistent with a decrease in MMP-9 activity as observed by zymography (left panel).

Use of Clean Catch Towelettes Does Not Interfere with MMP-9 Activity or Detection

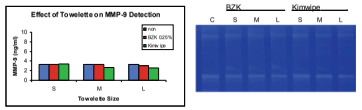

Figure 4 To test whether the benzalkonium chloride (BZK) could affect MMP-9 activity or detection, pieces of the towelette were used to simulate a small (s), medium (m) and large (l) contamination of bezalkonium chloride to the urine sample collected after clean catch. A known amount of pure MMP-9 was added to urine and aliquots of this were exposed to 3 different sizes of towelettes. The urine was then analyzed for levels of MMP-9 via zymography (right panel) and ELISA (left panel). Although a modest decrease was observed when BZK contamination was "large", the decrease was not significant and was equivalent to that observed when a kimwipe was used as a control instead.

Clean Catch Urine Collection Method Decreases the Levels and Frequency of Endogenous MMP-9 Found in the Urine of Disease-free Females

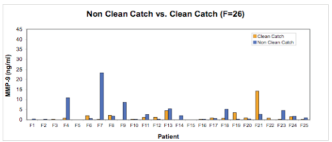

Figure 5 To determine whether addition of the clean catch method to urine sample collection could lower both the levels and frequency of endogenous MMP-9 found in the urine of asymptomatic females, urine samples from 26 females were obtained with and without the clean catch collection method and MMP-9 levels were determined both by zymography and ELISA. A comparison of the MMP-9 concentrations found in the urine of each female with and without clean catch are shown above. Without clean catch, 9 out of 30 women (30%) had 2ng/ml MMP-9 or more, whereas with clean catch, 4 out of 26 women (15%) had MMP-9 concentrations of 2ng/ml or greater. Without clean catch, the mean concentration was 3.573ng/ml (std. dev=6.465), while with clean catch, the mean concentration was 1.361ng/ml (std. dev=2.839).

Clean Catch Urine Collection Method Increases Clinical Specificity

	True Positive (Sensitivity)	True Negative (Specificity)
Bladder Cancer versus Non - Clean Catch Disease -free Controls	9/10 (90%) (55-99 @95% CI)	31/42 (73.8%) 58-86 @95% CI
Bladder Cancer versus Clean Catch Disease -free Controls	9/10 (90%) (55-99 @95%CI)	49/54 (90.75%) (80-97 @95% CI)

Table I To determine the efficacy of clean catch on reducing background MMP-9 levels and thereby improving clinical Specificity, the MMP-9 levels of 10 urine samples obtained from bladder cancer patients were compared to urine samples obtained from disease-free individuals collected with (n=54) and without (n=42) clean catch. In this pilot study, at 90% Sensitivity, addition of the clean catch method improved Specificity from 73.8% to 90.75%.

Summary

- **Urine samples obtained from disease-free females contain higher background levels of MMP-9 than that obtained from males.**
- **Although blood contamination can lead to increased levels of MMP-9 in urine, the amount of blood required exceeds that most commonly seen in the urine of disease-free individuals, suggesting that non-blood vaginal secretions may account for the higher background seen in women.**
- **Addition of a clean catch method to routine urine sample collection reduced MMP-9 levels. In a cohort of 26 disease-free women, the clean catch collection method effectively reduced the frequency (by ~50%) and levels (by ~60%) of MMP-9 detected.**
- **A pilot study comparing samples obtained from bladder cancer patients and control samples with and without clean catch demonstrated that clean catch can improve Specificity from ~75% to ~90%.**