

Multi-Analyte Diagnostic Readout (MADR): Combining Protein and DNA Markers to Maximize Clinical Performance

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Abstract

We have recently reported the development of a non-invasive diagnostic assay utilizing urinary Matrix Metalloproteinases (MMPs) as monitors of disease-free status and cancer in high risk bladder cancer populations. Using a novel approach called Clinical Intervention Determining Diagnostic (CIDD), in a study comprised of 86 patients with bladder cancer and 341 cancer-free individuals, we were able to identify with high confidence, ~94% Negative Predictive Value (NPV), those patients who do not have bladder cancer (~38%).

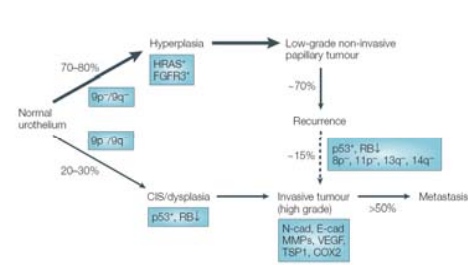
In order to further increase NPV, we have now developed a Real Time PCR based assay for detecting FGFR3 mutations in urine samples. FGFR3 mutations have been identified in 30-50% of bladder cancer patients, and are particularly associated with low-stage non-invasive tumors (Ta) where sensitivity reaches ~70%. In addition, FGFR3 mutations have been previously detected in the urine of bladder cancer patients, making this an attractive non-invasive DNA marker. We are currently analyzing the same sample cohort for the presence of the eight most prevalent FGFR3 mutations, and have modeled results to ultimately combine both the protein and DNA analyses into one assay.

Given the inherent specificity of DNA mutations to cancer cells, the presence of an FGFR3 mutation in the urine of patients undergoing bladder recurrence monitoring would be limited to samples containing mutant DNA and not those from cancer-free individuals. This would effectively increase sensitivity and NPV, while retaining or potentially increasing the number of patients who could be excluded (i.e. Specificity) from receiving invasive procedures. Based on the percent of mutant FGFR3 detected in urine samples from bladder cancer patients in previous studies, a simulation was run on the sample cohort above resulting in 97% NPV while retaining 38% exclusion of patients who do not have bladder cancer.

In addition, using this Multi-Analyte approach, patients below a set MMP cutoff, would be excluded from unnecessary intervention while those patients who are positive for an FGFR3 mutation would receive accelerated intervention. All remaining patients would continue to receive the already existing standard of care procedure.

Introduction

To complement our existing MMP assay, we have developed a non-invasive assay for the detection of FGFR3 mutations in urine. As shown below, FGFR3 mutations occur primarily in low grade, non-invasive tumors, while MMPs are associated with higher grade, invasive tumors. Our FGFR3 assay utilizes a novel sample prep method and a two-step PCR amplification process. As with our previous studies, urinary MMP-9 levels were determined by ELISA and cutoffs were determined to maximize Negative Predictive Value in a population of patients undergoing recurrence monitoring and patients with hematuria undergoing screening for bladder cancer. Using these cutoffs and our proprietary Clinical Intervention Determining Diagnostic concept, patients with MMP-9 levels below the cutoff could be excluded from further intervention. To further maximize NPV, our new assay would combine MMP-9 and FGFR3 mutation detection in the same non-invasive test. Here, we demonstrate detection of FGFR3 mutations in representative urine samples and model the performance of a combined MMP-9/FGFR3 assay using estimated detection rates for FGFR3 from current literature to determine the impact of combining DNA and protein markers into one assay.



Taken from Xue-Ru. Nature Reviews Cancer, vol. 5: 713-725, Sept. 2005.

FGFR3 Mutation Detection Assay

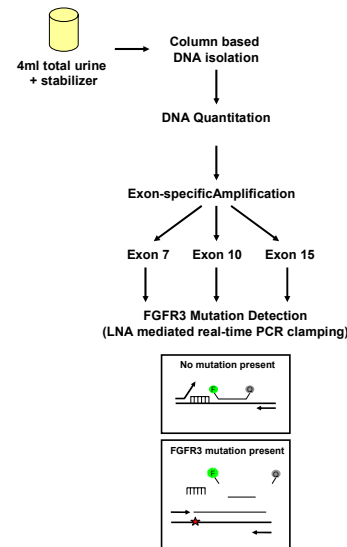


Figure 1. Assay Design. DNA is extracted from 4ml of urine using a modified Qiagen Virus Vacuum kit. The extracted DNA is then subjected to 50 cycles of PCR amplification for three FGFR3 exons (7, 10, and 15). These PCR products are then used as templates for our real-time mutation detection assay. The real-time assay utilizes blocking oligonucleotides containing locked-nucleic acid bases, which suppress the amplification of wild-type DNA.

Detection of 1% FGFR Mutant DNA in 50ng Total DNA

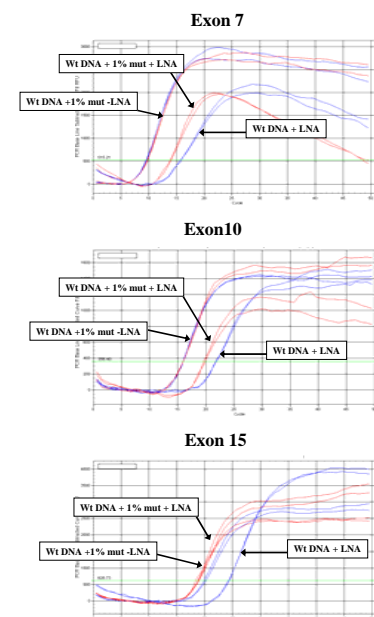


Figure 2. To demonstrate that mutant DNA can be detected in the presence of LNA blocking oligonucleotides, plasmids containing the mutations of interest were added to a final concentration of 1% in a background of known normal urine DNA.

Primary Exon Amplification from 4ml of Urine

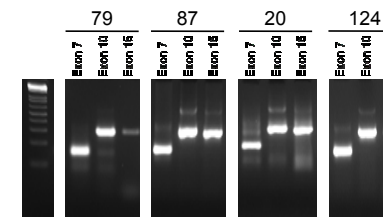


Figure 3. Representative gels of the PCR amplification of exons 7, 10 and 15 of the FGFR3 gene from 4ml urine samples from 4 individual patients. Patients 79, 87 and 20 were diagnosed with bladder cancer of stage Ta, T2 and Ta, respectively. Sample 124 was obtained from a patient who had hematuria but had a negative cystoscopy.

Real Time FGFR3 Mutation Detection in Patient Urine Samples

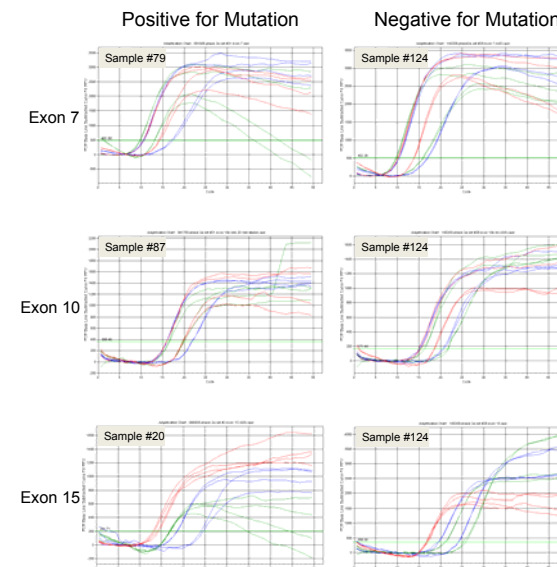
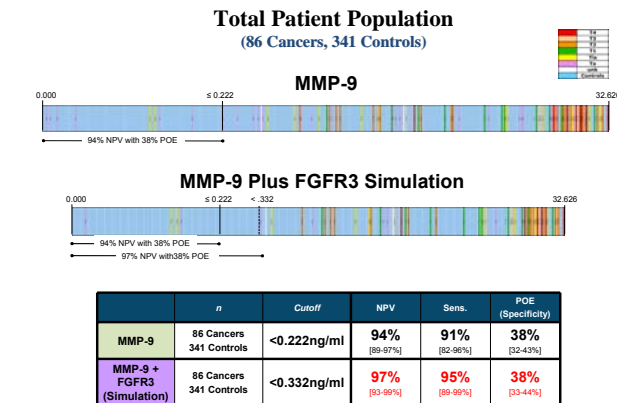
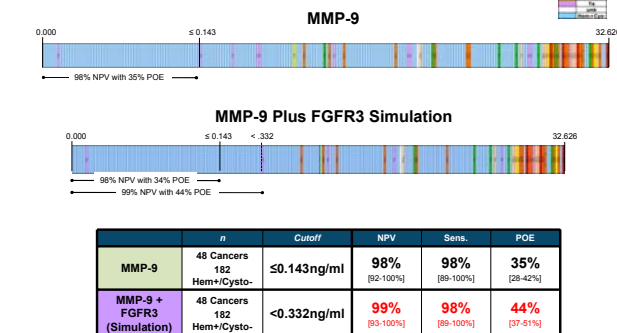


Figure 4. Detection of FGFR3 mutations by Real-time PCR. Real-time PCR amplifications were carried out using the primary PCR products from samples shown above in figure 3. Each sample was amplified in the presence and absence of LNA blocking oligonucleotide. The panels on the left depict real-time PCR amplifications of patient samples positive for FGFR3 mutations in exons 7, 10, or 15. The panels on the right depict a single patient sample that is negative for FGFR3 mutations. Each sample was amplified in the presence or absence of LNA blocking oligonucleotide (green lines). In addition, control urine DNA (blue lines), and control DNA with 1% mutant plasmid (red lines) were also amplified with and without LNA blocking oligonucleotide. In the left hand panels, amplifications of patient samples (green) display a shift to the left, similar to that seen with the 1% mutant plasmid controls (red), demonstrating the presence of a mutation. In the right hand panels, the patient sample (green) amplifies similarly to the control urine DNA (blue) for all exons, suggesting that no mutation is present in this sample.

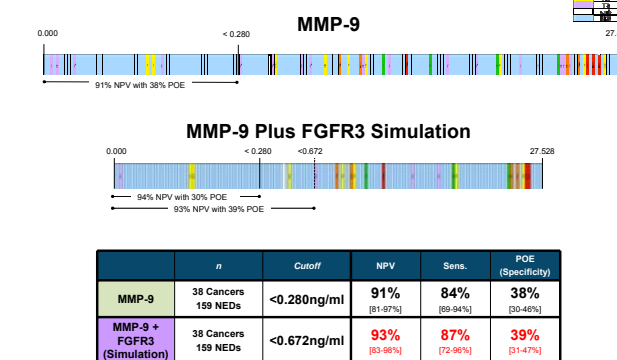
Negative Predictive Value Results



Hematuria Triage Subset (48 Cancers, 182 Hematuria+/Cystoscopy-)



Recurrence Triage Subset (38 Cancers, 159 No Evidence of Disease)



Summary

- FGFR3 mutations are associated with non-invasive bladder cancer while MMPs are associated with invasive tumors.
- We have developed a sensitive, non-invasive assay to detect FGFR3 mutations from as little as 4ml of urine.
- Using the inherent specificity of DNA and the increased sensitivity of proteins, MADR provides one test that delivers extremely high Negative and Positive Predictive Value.