Development of 3 Multi-PCR Based Microsatellite Instability Analysis (MSA) for Bladder Cancer Detection

Thomas Reynolds

NEXT Bio-Research Services, LLC

Maxie Gordon

HJM Cancer Research Foundation Corporation

David Moon

HJM Cancer Research Foundation Corporation

Chulso Moon (✉ csmoon6@gmail.com)

HJM Cancer Research Foundation Corporation

Research Article

Keywords: Bladder Cancer, Loss of Heterozygosity, Microsatellite Instability, Triplex MSA assay

Posted Date: October 8th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-944157/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Several studies have shown that microsatellite changes can be profiled in urine for the detection of bladder cancer. Use of microsatellite analysis (MSA) for bladder cancer detection requires a comprehensive analysis of up to 15 to 20 markers, which were based on amplification and interpretations of many individual MSA markers and can be technically challenging. Here, in a way to develop fast, more efficient, standardized and less costly MSA for detection of bladder cancer, we have developed 3 multiplex PCR based MSA assay, all of which were analyzed by genetic analyzer. First, we have selected 16 MSA markers based on 9 selected publications. Based on samples from Johns Hopkins University (JHU Sample, first set sample), we have attempted to develop MSA assay based on doublet, 2 tube based multiplex PCR combined with singlet, 1 tube based single plex PCR. While this assay was initially successful, we have encountered a reproducibility issue and we then developed MSA based on triplet, 3 tube based multiplex PCR (Triplet MSA assay). From second set samples (6 cancer patients' and 14 normal individuals' sample), our Triplet Assay with 15 MSA markers correctly predicted all of 6/6 cancer samples to be cancerous sample and 14/14 samples from normal individuals as normal samples. This result suggests that our Triplet MSA Assay combined with genetic analyzer is a potentially time and cost-effective genetic assay for bladder cancer detection and can be used potentially as a dependable assay in patient care.

Introduction

Microsatellite instability (MSI) is a molecular tumor phenotype resulting from genomic hypermutability and is initially discovered as genome-wide variations in the length of microsatellite sequences. As part of familiar colon cancer syndrome, MSI is most common with individuals with Lynch syndrome [1-3]. However, since its initial discovery, MSI has been a generalized phenomenon in a wide spectrum of sporadic cancers [4-8], and the underlying mechanisms for these cases of sporadic cancers seem to be based on epigenetic mechanisms, namely methylation of MLH1. Probably the most deleterious outcome of MSI, either inherited or sporadic solid tumors, the accumulation of frameshift mutations in tumor-associated genes, which will be crucial pathways in human carcinogenesis. the development of cancer Molecular diagnosis of MSI is currently achieved by examining PCR products from a few (typically 5–7) informative microsatellite markers (MSI–PCR) [9,10].

Bladder cancer (here we refer transitional carcinoma of bladder) is a major cause of morbidity and mortality in the western world [11]. Our understandings of its etiology, molecular characteristics associated with disease progression and management guidelines render bladder cancer an ideal candidate for screening [12,13]. Due to the lack of disease-specific symptoms, diagnosis and follow-up of bladder cancer has remained a challenge to medical community, not only to the urologists but also to the primary care physicians [13-15]. Cystoscopy, a gold standard for the detection of bladder cancer, is invasive, sometimes carries unwanted complications, while the cost is relatively high. Urine cytology is of limited value due to low sensitivity particularly to low-grade tumors. Urgent need for new qualitative and quantitative molecular tests designed to identify cellular and subcellular alterations exclusively

associated with bladder cancer have been explored [14-16]. Therefore, over the last two decades, several new “molecular assays” for the diagnosis of urothelial cancer have been developed. Overall, application of several molecular assays in conjunction with traditional screening methods has demonstrated promising results [16-20]. Based on histopathology and clinical presentations, two different types of noninvasive bladder cancer have been classified and this include the frequently recurring papillary tumor (Ta), and the more aggressive carcinoma in situ (CIS). While either type can progress into invasive tumors (T1–T4), the chance that low-grade Ta tumors progress to invasive disease is much less likely than high-grade Ta tumors and CIS. It is commonly accepted that noninvasive tumors have been grouped as ‘superficial bladder cancer’ and that noninvasive (that is, Ta and CIS) tumors is distinguished from invasive tumor, which invade the basement membrane [17-19].

LOH is typically identified by comparing the DNA isolated from tumors to normal DNA, such as that isolated from blood. This LOH can be detected using a method known as microsatellite instability analysis (MSA) [1-3]. Abnormalities involving the p16 (chromosome 9p21) and p53 (17p13) are associated with superficial transitional cell carcinoma (TCC) and these loci are the two most common regions of LOH in bladder cancer [20-27]. LOH at 9p has been shown to have prognostic value in non-muscle –invasive bladder cancer, while other loci implicated in progression of bladder cancer are characterized including 18q, 4p, 16, 20 and 21 [28-35]. LOH is typically identified by comparing the DNA isolated from tumors to normal DNA, such as that isolated from blood. Short tandem repeat (STR) regions also known as microsatellite regions) within chromosomes are unstable in cancerous cells and are deleted, causing a loss of heterozygosity (LOH) in the tumor sample. Microsatellite instability analysis (MSA) targets such tandem repeats in genomic DNA to evaluate loss of heterozygosity (LOH) that occurs with tumor cell transformation [24-26]. These biomarkers, originally developed at Johns Hopkins University represent a panel of fifteen short tandem repeat (STR), or microsatellite regions that have been shown to detect deletions in the DNA isolated from urine sediment of bladder cancer patients before the cystoscopic evidence of tumor [34-36]. Several studies have shown that these microsatellite changes can be profiled in urine for the detection of bladder cancer cells [37–44]. MSA combines 15 to 20 markers from a region with a high percentage of LOH. This method proved overly sensitive for low- and high-grade lesions with sensitivities of 67%, 86% and 93% for recurrent G1, G2 and G3 lesions, respectively, and a specificity of 88% [37-44]. Moreover, MSA could predict a recurrence before cystoscopically detected evidence in all studies with extended follow-up [44]. Although MSA has a high potential, automation of the assay, multi center studies and excluding patients with persistent leukocyturia are essential if this analysis is to become clinically important. The discovery, validation, and translation of biomarkers for the early detection of cancer is the primary focus of the Early Detection Research Network (EDRN), an initiative of the National Cancer Institute (NCI). A prospective study sponsored by the EDRN was undertaken to determine the efficacy of a novel sets of MSA markers for the early detection of bladder cancer [45,46]. This work and data analysis were performed through a collaboration between academia and industry partners. Out of 500 patients which 300 cancer patients and 200 benign controls, a summary analysis of samples, exclusive of various confounding variable and

sample errors, showed >80% sensitivity and specificity in cancer patients as compared to healthy controls (manuscript in prep). [45,45].

Microsatellite analysis for detection of cancer has several significant technical challenges specifically in allele calling and interpretation. Lab to lab variability due to instrumentation and personnel created differences in assay performance. Additionally, both stochastic effects and variations in peak height effects between the DNA derived from urine sediment samples and DNA derived from the blood samples provided more variation in the results which added to interpretation differences. These differences were especially evident in samples which produced results slightly above or below cut off ratios established for LOH. Hence, qualification of STR assay present significant challenges including establishing the parameters to be used for determining LOH from potential tumor cells isolated from urine sediment [34-44].

Recently, in a way to develop standardized and less costly MSA for detection of bladder cancer, we have developed 3 multiplex PCR based MSA assay. In this report, we have presentation several studies. First, we present 16 different markers we selected for our assay development. Then we have presented doublet multiplex PCR based MSA assay and finally, we present our final and viable MSA assay based on triplet multiplex PCR reaction (Triplet MSA assay). We also briefly discuss about use of MSA for early detection of bladder cancer and its role as a tool for surveillance of recurrent bladder cancer.

Results

Selection of 16 markers

Out of selected 9 publications as in Table 1, we have selected 15 different MSA primer sets for 16 markers as in Table 2. These primer sets have shown a repeatedly shown dependable high LOH in bladder cancer samples with exceptionally low number of LOH in normal samples and a total of over 350 patients this combined data has provided over 90% sensitivity and 100% specificity. These 16 different markers have been characterized for separate independent PCR based MSA analysis and desirable cutoff values.

Development of doublet multiplex PCR

Our goal of the study was development of dependable, accurate and less costly test. First, using JHU sample, we have tried to establish doublet, 2 tube multiplex PCR, assay with 1 tube singlet PCR (D13S304 ((AAAG)_n 6-FAM GGCTGCATGAGCCCTAAGTA TGGGTGACACAGTGAGACTCTA) as in Table 2 and 3. We have tried various combinations of primer sets in tube 1 and tube 2. After multiple trial and error, we have finalized our assay as in Table 3. Based on the final assay, we have thus run a 10 cancer and 5 normal sample (JHU sample) as a blind fashion as in Table 4, which has demonstrated 100 % sensitivity and specificity. However, we did realize that after a repeated trial of these markers in table 3, reproducibility

from each different trial has been inconsistent. Therefore, we have then redesigned triplet multiplex PCR as in Table 5 and 6.

Development of triplet multiplex PCR

We have originally designed triplet, three tube multiplex PCR reaction and singlet, one tube single plex PCR primer set, similar to the design of doublet. This primer, D13S304 ((AAAG)_n 6-FAM GGCTGCATGAGCCCTAAGTA TGGGTGACACAGTGAGACTCTA), is not included in the final triplet MSA assay as this marker had resulted in inconsistent PCR amplification. Of note, MP2 marker MBP generates two distinct amplifications which are included in the results as marker MBP and MBPa making a total of 15 amplified markers from 14 primer sets in the multiplex. The cut off values was designed as we did in doublets assay and described in Table 6. From this final triplet assay, we have then repeated multiple times on various samples including original 25 cancer sample and 15 normal samples (JHU samples) and reproducibility has been consistent.

Finally, we have performed, in non-blind fashion, to test 20 QC samples from EDNRN study by our newly established assay. As in table 7, from these QC samples, 6/20 cancer samples are determined to be cancer based on overall evaluation of all 15 markers tested and 14/20 samples from normal individuals are determined to be normal.

Discussion

Many aspects of clinical presentations with expected course of disease progression make bladder cancer as a potential value of screening target and the common consensus at this point is screening high-risk populations [13,14]. Certainly, there are two key aspects because bladder cancer screening is important in upcoming decades. First, the persistently high prevalence of smoking is expected to function as key hazard for long-term urothelial carcinogenic effects for the next several generations. Second, as it is highly unlikely for bladder cancer to have capacity to metastasize before they become invasive.[19-21] Therefore, this makes it valuable opportunity to make an early detection of bladder cancer, in a window of timing between tumor origination and invasion Certainly, management of noninvasive cancers is associated with fewer morbidities and is more effective than that of invasive tumors [13] as in this stage of tumor development, cystectomy, systemic chemotherapy or chemo radiation therapy are not required .

Many independent groups have previously confirmed the superior sensitivity of MSA (75–96%) compared to cytology (13–50%) in various clinical settings [35-44, 48-61] and based on 377 cancer samples from 1997 to 2001 reports, the sensitivity was 90 percent and specificity was 100 percent [34,35,36,42,44, 45,53,54,58]. In contrast to conventional cytology, it appears that microsatellite analysis (MSA) can detect low-grade and low stage disease as accurately as high-grade and high-stage disease [35-44,47-61]. Frigerio et al [37] found that the combined use of cytology and LOH analysis had high sensitivity for identifying primary tumors and had the ability to detect almost all recurrent diseases in voided urine. Likewise, van Rhijn et al. [43] has demonstrated, out of 47 Caucasian patients with confirmed superficial bladder TCC (37 pTa, 10 pT1) at initial diagnosis, three important clinical applications of MSA. First, MSA

correctly identified 94% (44/47) of primary tumors. Second MSA has correctly identified 92% (12/13) of tumor recurrences. Third, MSA predicted chance of future recurrences as 75% (9/12) of tumor recurrences were molecularly detected 1–9 months before cystoscopy evidence of recurrent disease.

In summary, several important studies clearly demonstrated that not only urine microsatellite analysis reliably detected superficial bladder tumors, but also was a reliable test for detecting and predicting tumor recurrences and such detection rate can be improved in combination with urine cytology. In this sense, it is important to notice that meta-analysis done by van Rhijn et al [57], who had done an extensive literature review for use of 18 markers and concluded that MSA, ImmunoCyt, NMP22, CYFRA21-1, LewisX and FISH are the most promising markers for surveillance [57-68]. Nevertheless, so far, there are not enough clinical evidence to warrant the substitution of the cystoscopy follow-up scheme by any of the currently available urine marker tests. Likewise, currently, data so far are not consistent with the sole use of molecular tests in patients with high risk of developing bladder cancer. However, a lot of studies have shown molecular tests to have value in not only improving the diagnostic accuracy of high-risk groups of population in the initial diagnosis of bladder cancer but also for the prediction of recurrence, but only when used in conjunction with cytology and cystoscopy; molecular testing can reduce the need for these procedures.

From this study, we have learned several important lessons, most of which were also recognized in other studies. Several points warrant further discussions to achieve to maximize sensitivity and specificity of MSA. First, by establishing a robust genetic marker profile and determining for each of the analyzed microsatellites individual threshold values for LOH/allelic imbalance can maximize the sensitivity of tumor DNA detection without compromising its specificity. In this sense, we have used rigorous statistical analysis based on reported high quality published studies as in Table 1. Second, a careful set-up of dependable methods is necessary to avoid erroneous LOH-judgements due to PCR artefacts repeatedly described by others [41-44]. In fact, our 2-tube based PCR, doublet multiplex PCR, seemed to be initially successful and then, a repeated tests indicated lack of dependable of marker amplification and therefore we have decided not to pursue this assay. Third, performing MSA on a genetic analyzer, which is a commonly used nowadays, needs to be always a standard way of MSA practice, which brings in two major advantages. First, sample-processing can be largely automatized, and results are provided in form of a numerical data-readout, independent of inter-observer variability associated with the complex interpretation of morphological features. Moreover, the determination of LOH ratios on this platform is highly reproducible and provides reliable results even in situations, when the cell conservation is suboptimal for cytological evaluation and/or FISH. In fact, in our experiences, use of standard genetic analyzer like ABI 3100 machine gives MSA a significant advantage as MSA is little affected by changes in PCR conditions or amounts of genomic DNA applied as long as it meets minimum requirement. For example, when we repeated multiple rounds of our triplex PCR, most of the markers resulted in consistent findings. Third, amount of genomic DNA to be analyzed need to be sufficient and we propose to use at least 20 to 30 ng of urine genomic DNA to be safe side DNA, 20 ng for 10 markers and 30 ng for 15 markers. We have used amount of DNA based on this guideline. In fact, sometimes, DNA from normal

urine sample become less than 30 ng and the result of MSA from both 2 and 3 tube PCR became either inconsistent or non-reproducible.

In conclusion, we have presented the data for the development of “Triplet MSA Assay combined covering 15 markers with genetic analyzer” and we further propose that our assay is a potentially time and cost-effective genetic assay for bladder cancer detection. Along the line, we have discussed about various prior data regarding MSA based bladder cancer detection. As in table 7, from 20 QC samples, our Triple MSA correctly predicted all of 6/6 cancer samples to be cancerous sample and 14/14 samples from normal individuals as normal samples. This result is consistent with multiple other reports we have discussed above in terms of diagnostic accuracy. We hope that our assay can potentially improve management of bladder cancer.

Material And Method

Matched Blood and Urine genomic DNA samples

Blood and urine specimens for the qualification study were obtained from Dr David Sidransky's lab at Johns Hopkins University (JHU sample). These include 15 biopsy proved superficial bladder cancer and 10 normal controls and participants in the assay development for MSA study we report here. All of whom were deemed eligible for the study. These samples were previously used for determination of proper cut off value for a series of 10 MSA markers. The parent trial and the secondary analysis reported in this manuscript were approved by the Institutional Review Board from Johns Hopkins University. Written informed consent was obtained from all participants and/or their legal guardians. All research was performed in accordance with relevant guidelines/regulations from Johns Hopkins University. Research involving human research participants had been performed in accordance with the Declaration of Helsinki.

EDRN QC Samples

The prospective study was designed to determine the efficacy of the panel of MSA markers for the detection of bladder cancer and bladder cancer recurrence using the developed clinical MSA assay [45,46]. As a part of the study, a quality control CLIA/College of American Pathology (CAP) accredited laboratory, (QA Lab) University of Maryland Baltimore Biomarker Reference Laboratory (UMB-BRL) performed quality control analysis on 10% of the samples performed by the testing laboratory (QC Samples). The parent trial and the secondary analysis reported in this manuscript were approved by the Institutional Review Board from EDRN. Written informed consent was obtained from all participants and/or their legal guardians. All research was performed in accordance with relevant guidelines/regulations from EDRN. Research involving human research participants had been performed in accordance with the Declaration of Helsinki.

DNA extraction and quantification

Matched blood and urine samples remained de-identified during the study. Genomic DNA from the buffy coat of blood collected in ACD collection tubes was purified by the testing lab using the QIAGEN QIAMP Blood Mini kit according to manufacturer's instructions. Genomic DNA from Urine sediment was purified using the QIAGEN QIAMP Viral RNA Mini kit. The DNA was quantified against a standard curve of human DNA using TaqMan β -actin Detection Reagents from Applied Biosystems. 30 ml of urine samples and 5 ml of whole blood was used for this study

STR Targets and PCR primers

The panel of STR targets for the MSA assay was originally developed at Johns Hopkins University as a radioactive PCR assay [34]. The testing lab converted the radioactive PCR assay to a high-throughput capillary electrophoresis assay of fluorescent PCR products. The resulting assay was comprised of two or three multiplex reactions, of which primers were selected from report in Table 1 and each primer sequence and size of PCR product are outlined in Table 2. The 5' fluorescent primers were obtained from Applied Biosystems (Table 2B). The 3' primers were obtained from Integrated DNA technologies. Following Round three of the qualification studies, the single plex reaction was dropped due to poor performance in the assay. The primers were initially mixed at an equimolar concentration (2 μ M each primer); following refinement of the assay, the primer concentrations were further adjusted. The designing of five sets of multiplex PCR was previously initiated from assay developed for EDRN study [45,46].

Multiplex PCR: Doublets (Two tube) multiplex PCR

For doublet PCR reaction, a master mix for each PCR reaction such that the final amounts/concentrations of each reagent are as follows: MgCl₂ at 2.5 mM for D13S802, 4mM for PCR 1, 4mM for PCR 2 dCTP and dGTP at 0.2 mM in all reactions; dUTP at 0.2mM; dATP at 0.46 mM AmpliTaq Gold at 2 units per reaction. For D13S802, only one set of primer was performed (single PCR). For all of single tube and 2 multiplex PCR, same PCR condition (95°C for 11 min For 30 cycles: 94 °C for 1 min. 55°C for 1 min. 72°C for 1 min. 60°C for 45 min. 4°C forever) was used. Molecular concentration for each primer and sets of primers used in each of single PCR and doublets multiplex PCR are presented in Table 3A. An example of master mix preparation for 35 test samples (JHU sample) and 2 controls is shown below is presented in Table 3B. Acceptance Criteria for each peak was calculated from DNA obtained from H460 cancer cell lines and is discussed below. Based on the final assay, a total of 15 patients (10 cancer and 5 normal sample (JHU sample)) were blindly tested (Table 4).

Multiplex PCR: Triplet (Three tube) multiplex PCR

We have run five sets of optimization reactions (qualification) to design triplet, 3 tube PCR, multiplex PCR reaction, using primers in Table 5 and 6. For the First three rounds of qualification, the STR regions were amplified using AmpliTaq Gold DNA Polymerase Applied Biosystems). The PCR conditions were 4 mM MgCl₂/0.2 mM dCTP/0.2 mM dGTP/ 0.4mM dUTP, 0.5 mM dATP/ 2 units Amplitaq Gold. MP1, MP2 were amplified 4 ng of total genomic DNA, respectively, for both blood and urine DNA, and MP3 and the D13S804 Singleplex reaction were amplified using 6 ng of blood or urine DNA in a 25 μ l final volume.

PCR Cycling conditions were 95 °C for 11 minutes/32 cycles of 94 °C 1 minute, 55 °C for 1 minute, 72 °C for 1 minute/ 60 °C for 45 minutes/ 4 °C hold. For Rounds 4 and 5 of the qualification, the PCR amplification conditions were modified to utilize 1X FastStart Taqman Probe Master (Roche). The DNA concentrations and the PCR cycling conditions remained the same.

Capillary electrophoresis

Capillary electrophoresis was performed on 1 µl of PCR product using the 3100 Genetic analyzer from Applied Biosystems (Foster city, CA). Matched blood and urine samples were run in the same injection to prevent difference in run conditions between matched specimens. Following electrophoresis, the data was analyzed using Gene mapper v3.1 (Applied Biosystems) and the peak sizes and peak heights were exported as a tab delimited text file.

Calculation of Loss of Heterozygosity

The data exported from the 3100 analyzer was imported in Microsoft Excel for analysis. In order for the data to be acceptable, the positive control DNA (HL-60 genomic DNA (ATCC)) had to pass strict peak height and size criteria before the sample data could be analyzed (Table 6B). If these criteria were acceptable, the ratio of the blood to urine heterozygous alleles using the following formula: Ratio= ((urine 1 allele 1 peak height/ urine 1 allele 2 peak height)/(blood 1 allele 1 peak height/blood 1 allele 2 peak height)). This ratio was then compared to cutoff values previously determined by calculation of ratios of 50 matched normal blood and urine specimens.

Data analysis

Data Analysis was performed by GeneMapperID, “Positive” results indicated a variant or missing allele at the loci indicated. Target Loci are known cancer markers. Loss of Heterozygosity (LOH) at the loci indicate when one of the two alleles is missing in the urine sample at a heterozygous loci in the buccal sample. LOH can only be detected at heterozygous loci hence homozygous loci are uninformative. The overall MSA Genotype is calculated as Negative, LOH-Low (1-2 markers), LOH-Medium (3-4 markers), and LOH-High (>4 markers). Tumors typically acquire more LOH markers as they grow and advance in stage. Unless stated otherwise in the Validation Protocol, the following acceptance criteria were applied to determine the suitability of each assay:

ABI 3100 DNA Analyzer Acceptance Criteria

To be deemed negative (or no LOH detected) a heterozygous locus must have RFU peak heights between 200 RFUs and 100,000 RFUs with a peak height ratio between the cut off values determine for each STR marker (see Table 2). To be deemed LOH/Positive the samples must have at least one heterozygous loci with RFU peak heights that fall between 200 RFUs and 100,000 RFUs with a peak height ratio above or below the cut off values determine for each STR marker (see Table 2). To be deemed Non-Informative the sample has to be homozygous for the locus. Homozygous locus will have only one peak with a sample

RFU peak heights must fall between 200 RFUs and 100,000 RFUs. If the sample does not meet the minimum or maximum RFU for peak height or has no signal at all it will be deemed Non-Evaluable. A minimum of eight (8) loci must be analyzed and meet the Negative, LOH or Non-informative call in order to be deemed passing of sample acceptance criteria. Failed samples will be repeated one time. If a sample fails on the second analysis, then it is deemed to fail sample acceptance criteria and reported as Quantity Not Sufficient (QNS).

Abbreviations

MSI, Microsatellite instability, Polymerase Chain Reaction; CIS, Carcinoma in situ; LOH, Loss of heterozygosity; MSA, Microsatellite instability analysis; TCC, Transitional cell carcinoma; STR, Short tandem repeat; EDRN, Early Detection Research Networks; QC, Quality control; CAP, College of American Pathology; UMB-BRL, University of Maryland Baltimore Biomarker Reference Laboratory; QNS, Quantity Not Sufficient.

Declarations

Ethics approval and consent to participate

The parent trial and the secondary analysis reported in this manuscript were approved by the Institutional Review Board at Johns Hopkins University and EDRN. Written informed consent was obtained from all participants and/or their legal guardians. All research was performed in accordance with relevant guidelines/regulations from Johns Hopkins University and EDRN. Research involving human research participants had been performed in accordance with the Declaration of Helsinki.

Consent for publication

N/A

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available as there are no public repositories for this type of dataset. The data are available from the corresponding author on reasonable request.

Competing interests

CM is founding shareholder of BCD Innovations and TR is founding shareholder of Next Molecular Bio. MG and DM both declare no conflicts of interests.

Funding

This study was supported in part by the Cancer Research Grant from Pyung-Ya Foundation (grant number PY-1; to C.S.M.) and Translational Research Grant from HJM Foundation (grant number HJM-TR1; to C.S.M. and D.M.).

Authors' contributions

CM was the Principal Investigator of the grant that supported this project. CM and TR conceived of and executed the overall study and design, oversaw all molecular data collection, directed the analysis plan. CM and DM wrote the paper. CM, MG, DM and TR together contributed to the analysis plan, contributed to the interpretation of the results, and approved the final manuscript.

Acknowledgements

CM is deeply indebted to Dr. HJ Moon on his encouragement for the completion of this study.

References

1. Aaltonen L, Peltomaki P, Leach F, Sistonen P, Pylkkanen L, Mecklin J, et al. Clues to the pathogenesis of familial colorectal cancer. *Science*. 1993;260:812-6.
2. Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature*. 1993;363:558-61.
3. Thibodeau S, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. *Science*. 1993;260:816-9.
4. Imai K, Yamamoto H. Carcinogenesis and microsatellite instability: the interrelationship between genetics and epigenetics. *Carcinogenesis*. 2008;29:673-80.
5. Yamamoto H. Interrelationship between microsatellite instability and microRNA in gastrointestinal cancer. *World J Gastroenterol*. 2012;18:2745-55.
6. Yamamoto H. An updated review of gastric cancer in the next-generation sequencing era: Insights from bench to bedside and vice versa. *World J Gastroenterol*. 2014;20:3927-37.
7. Yamamoto H, Imai K. Microsatellite instability: An update. *Arch Toxicol*. 2015;89:899-921.
8. Gelsomino F, Barbolini M, Spallanzani A, Pugliese G, Cascinu S. The evolving role of microsatellite instability in colorectal cancer: A review. *Cancer Treat Rev*. 2016;51:19-26.
9. de la Chapelle A, Hampel H. Clinical relevance of microsatellite instability in colorectal cancer. *J Clin Oncol*. 2010;28:3380-7.
10. Bacher JW, Flanagan LA, Smalley RL, Nassif NA, Burgart LJ, Halberg RB, et al. Development of a fluorescent multiplex assay for detection of MSI-high tumors. *Dis Markers*. 2004;20:237-50.
11. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun M. Cancer statistics. *CA Cancer J Clin*. 2009;59:225–49.
12. Botteman MF, Pashos CL, Redaelli A, Laskin B, Hauser R. The health economics of bladder cancer: A comprehensive review of the published literature. *Pharmacoeconomics*. 2003;21:1315-30.

13. Lotan Y, Kamat AM, Porter MP, Robinson VL, Shore N, Jewett M, et al. Key concerns about the current state of bladder cancer: A position paper from the Bladder Cancer Think Tank, the Bladder Cancer Advocacy Network, and the Society of Urologic Oncology. *Cancer*. 2009;115:4096-103.
14. Lotan Y, Svatek RS, Malats N. Screening for bladder cancer: A perspective. *World J Urol*. 2008;26:13-8.
15. Shariat SF, Lotan Y, Vickers A, Karakiewicz PI, Schmitz-Dräger BJ, Goebell PJ, et al. Statistical consideration for clinical biomarker research in bladder cancer. *Urol Oncol Semin Orig Invest*. 2010;28:389-400.
16. Lotan Y, Roehrborn CG. Sensitivity and specificity of commonly available bladder tumor markers versus cytology: results of a comprehensive literature review and meta-analyses. *Urology*. 2003;61:109-18.
17. Bensalah K, Montorsi F, Shariat SF. Challenges of cancer biomarker profiling. *Eur Urol*. 2007;52:1601-9.
18. Babjuk M, Oosterlinck W, Sylvester R, Kaasinen E, Böhle A, Palou-Redorta J, et al. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder, the 2011 update. *Eur Urol*. 2011;59:997-1008.
19. Hall MC, Chang SS, Dalbagni G, Pruthi RS, Seigne JD, Skinner EC, et al. Guideline for the management of nonmuscle invasive bladder cancer (stages Ta, T1, and Tis): 2007 update. *J Urol*. 2007;178:2314-30.
20. Mitra AP, Datar RH, Cote RJ. Molecular pathways in invasive bladder cancer: New insights into mechanisms, progression, and target identification. *J Clin Oncol*. 2006;24:5552-64.
21. Mitra AP, Cote RJ. Molecular pathogenesis and diagnostics of bladder cancer. *Annu Rev Pathol Mech Dis*. 2009;4:251-85.
22. Moon JJ, Lu A, Moon C. Role of genomic instability in human carcinogenesis. *Exp Biol Med*. 2019;244:227-40.
23. Saran KK, Gould D, Godec CJ, Verma RS. Genetics of bladder cancer. *J Mol Med*. 1996;74:441-5.
24. Orntoft TF, Wolf H. Molecular alterations in bladder cancer. *Urol Res*. 1998;26:223-33.
25. Skacel M, Pettay JD, Tsiftsakakis EK, Procop GW, Biscotti CV, Tubbs RR. Validation of a multicolor interphase fluorescence in situ hybridization assay for detection of transitional cell carcinoma on fresh and archival thin-layer, liquid-based cytology slides. *Anal Quant Cytol Histol*. 2001;23:381-7.
26. Lopez-Beltran A, Amin MB, Oliveira PS, Montironi R, Algaba F, McKenney JK, et al. Urothelial carcinoma of the bladder, lipid cell variant: clinicopathologic findings and LOH analysis. *Am J Surg Pathol*. 2010;34:371-6.
27. Ploussard G, Dubosq F, Soliman H, Verine J, Desgrandchamps F, De Thé H, et al. Prognostic value of loss of heterozygosity at chromosome 9p in non-muscle-invasive bladder cancer. *Urology*. 2010;76:513. e13-e18.

28. Cai T, Nesi G, Dal Canto M, Mondaini N, Piazzini M, Bartoletti R. Prognostic role of loss of heterozygosity on chromosome 18 in patients with low-risk nonmuscle-invasive bladder cancer: Results from a prospective study. *J Surg Res.* 2010;161:89-94.
29. Sibley K, Cuthbert-Heavens D, Knowles MA. Loss of heterozygosity at 4p16. 3 and mutation of FGFR3 in transitional cell carcinoma. *Oncogene.* 2001;20:686-91.
30. Yoon DS, Li L, Zhang RD, Kram A, Ro JY, Johnston D, et al. Genetic mapping and DNA sequence-based analysis of deleted regions on chromosome 16 involved in progression of bladder cancer from occult preneoplastic conditions to invasive disease. *Oncogene.* 2001;20:5005-14.
31. Docimo SG, Chow N-H, Steiner G, Silver RI, Rodriguez R, Kinsman S, et al. Detection of adenocarcinoma by urinary microsatellite analysis after augmentation cystoplasty. *Urology.* 1999;54:561.
32. Szarvas T. The diagnostic value of microsatellite LOH analysis and the prognostic relevance of angiogenic gene expression in urinary bladder cancer. *Magy Onkol.* 2009;53:385-9.
33. Bartoletti R, Cai T, Nesi G, Girardi LR, Baroni G, Dal Canto M. Loss of P16 expression and chromosome 9p21 LOH in predicting outcome of patients affected by superficial bladder cancer. *J Surg Res.* 2007;143:422-7.
34. Mao L, Schoenberg MP, Scicchitano M, Erozan YS, Merlo A, Schwab D, et al. Molecular detection of primary bladder cancer by microsatellite analysis. *Science.* 1996;271:659–62.
35. Ellegren H. Microsatellites: Simple sequences with complex evolution. *Nat Rev Genet.* 2004;5:435–45.
36. Seripa D, Parrella P, Gallucci M, Gravina C, Papa S, Fortunato P, et al. Sensitive detection of transitional cell carcinoma of the bladder by microsatellite analysis of cells exfoliated in urine. *Int J Cancer.* 2001;95:364–9.
37. Frigerio S, Padberg BC, Strebel RT, Lenggenhager DM, Messthaler A, Abdou MT, et al. Improved detection of bladder carcinoma cells in voided urine by standardized microsatellite analysis. *Int J Cancer.* 2007;121:329–38.
38. Hoque MO, Lee J, Begum S, Yamashita K, Engles JM, Schoenberg M, et al. High-throughput molecular analysis of urine sediment for the detection of bladder cancer by high-density single-nucleotide polymorphism array. *Cancer Res.* 2003;63:5723–6.
39. de Bekker-Grob EW, van der Aa MNM, Zwarthoff EC, Eijkemans MJC, van Rhijn BW, van der Kwast TH, et al. Non-muscle-invasive bladder cancer surveillance for which cystoscopy is partly replaced by microsatellite analysis of urine: A cost-effective alternative? *BJU Int.* 2009;104:41–7.
40. van der Aa MNM, Zwarthoff EC, Steyerberg EW, Boogaard MW, Nijssen Y, van der Keur KA, et al. Microsatellite analysis of voided-urine samples for surveillance of low-grade non-muscle-invasive urothelial carcinoma: Feasibility and clinical utility in a prospective multicenter study (Cost-Effectiveness of Follow-Up of Urinary Bladder Cancer Trial [CEFUB]). *Eur Urol.* 2009;55:659–68.
41. Wild PJ, Fuchs T, Stoeck R, Zimmermann D, Frigerio S, Padberg B, et al. Detection of urothelial bladder cancer cells in voided urine can be improved by a combination of cytology and standardized

- microsatellite analysis. *Cancer Epidemiol Biomarkers Prev.* 2009;18:1798–806.
42. Steiner G, Schoenberg MP, Linn JF, Mao L, Sidransky D. Detection of bladder cancer recurrence by microsatellite analysis of urine. *Nat Med.* 1997;3:621–4.
43. van Rhijn BWG, Lurkin I, Kirkels WJ, van der Kwast TH, Zwarthoff EC. Microsatellite analysis–DNA test in urine competes with cystoscopy in follow up of superficial bladder carcinoma: A phase II trial. *Cancer.* 2001;92:768–75.
44. Amira N, Mourah S, Rozet F, Teillac P, Fiet J, Aubin P, et al. Non-invasive molecular detection of bladder cancer recurrence. *Int J Cancer.* 2002;101:293–7.
45. ClinicalTrials.gov. Microsatellite Analysis of Urinary Sediment in Detecting Bladder Cancer. 2019. <https://clinicaltrials.gov/ct2/show/NCT00095589>. Accessed 6 Sept 2021.
46. National Cancer Institute. Title. Year. <https://edrn.nci.nih.gov/protocols/108-detection-of-bladder-cancer-by-microsatellite-analysis>. Accessed 6 Sept 2021.
47. Parekh DJ, Bochner BH, Dalbagni G. Superficial and muscle-invasive bladder cancer: principles of management for outcomes assessments. *J Clin Oncol.* 2006;24:5519–27.
48. Linn JF, Lango M, Halachmi S, Schoenberg MP, Sidransky D. Microsatellite analysis and telomerase activity in archived tissue and urine samples of bladder cancer patients. *Int J Cancer.* 1997;74:625–9.
49. Mourah S, Cussenot O, Vimont V, Desgrandchamps F, Teillac P, Cochant-Priollet B, et al. Assessment of microsatellite instability in urine in the detection of transitional-cell carcinoma of the bladder. *Int J Cancer.* 1998;79:629–33.
50. Steiner G, Reinschmidt G, Müller SC. Molecular genetic diagnosis of de novo and recurrent bladder cancer. *Electrophoresis.* 1999;20:280–2.
51. Baron A, Mastroeni F, Moore P, Bonetti F, Orlandini S, Manfrin E, et al. Detection of bladder cancer by semi-automated microsatellite analysis of urine sediment. *Adv Clin Path.* 2000;4:19–24.
52. Christensen M, Wolf H, Orntoft TF. Microsatellite alterations in urinary sediments from patients with cystitis and bladder cancer. *Int J Cancer.* 2000;85:614–7.
53. Schneider A, Borgnat S, Lang H, Régine O, Lindner V, Kassem M, et al. Evaluation of microsatellite analysis in urine sediment for diagnosis of bladder cancer. *Cancer Res.* 2000;60:4617–22.
54. Zhang J, Fan Z, Gao Y, Xiao Z, Li C, An Q, et al. Detecting bladder cancer in the Chinese by microsatellite analysis: Ethnic and etiologic considerations. *J Natl Cancer Inst.* 2001;93:45–50.
55. van Rhijn BW, Lurkin I, Chopin DK, Kirkels WJ, Thiery JP, van der Kwast TH, et al. Combined microsatellite and FGFR3 mutation analysis enables a highly sensitive detection of urothelial cell carcinoma in voided urine. *Clin Cancer Res.* 2003;9:257–63.
56. Zeger SL, Liang KY. An overview of methods for the analysis of longitudinal data. *Stat Med.* 1992;11:1825–39.
57. Van Rhijn BW, Van der Poel HG, van Der Kwast TH. Urine markers for bladder cancer surveillance: A systematic review. *Eur Urol.* 2005;47:736–48.

58. Sourvinos G, Kazanis I, Delakas D, Cranidis A, Spandidos DA. Genetic detection of bladder cancer by microsatellite analysis of p16, rb1 and p53 tumor suppressor genes. *J Urol*. 2001;165:249–52.
59. van Rhijn BW, Smit M, van Geenen D, Wijnmaalen A, Kirkels WJ, van der Kwast TH, et al. Surveillance with microsatellite analysis of urine in bladder cancer patients treated by radiotherapy. *Eur Urol*. 2003;43:369–73.
60. Mian C, Maier K, Comploj E, Lodde M, Berner L, Lusuardi L, et al. uCyt+/ImmunoCyt™ in the detection of recurrent urothelial carcinoma: An update on 1991 analyses. *Cancer Cytopathol*. 2006;108:60–5.
61. Lodde M, Mian C, Comploj E, Palermo S, Longhi E, Marberger M, et al. uCyt+ test: Alternative to cystoscopy for less-invasive follow-up of patients with low risk of urothelial carcinoma. *Urology*. 2006;67:950–4.
62. Kumar A, Kumar R, Gupta NP. Comparison of NMP22 BladderChek test and urine cytology for the detection of recurrent bladder cancer. *Jpn J Clin Oncol*. 2006;36:172–5.
63. Kibar Y, Goktas S, Kilic S, Yaman H, Onguru O, Peker AF. Prognostic value of cytology, nuclear matrix protein 22 (NMP22) test, and urinary bladder cancer II (UBC II) test in early recurrent transitional cell carcinoma of the bladder. *Ann Clin Lab Sci*. 2006;36:31–8.
64. Mian C, Lodde M, Comploj E, Lusuardi L, Palermo S, Mian M, et al. Multiprobe fluorescence in situ hybridisation: Prognostic perspectives in superficial bladder cancer. *J Clin Pathol*. 2006;59:984–7.
65. May M, Hakenberg OW, Gunia S, Pohling P, Helke C, Lübke L, et al. Comparative diagnostic value of urine cytology, UBC-ELISA, and fluorescence in situ hybridization for detection of transitional cell carcinoma of urinary bladder in routine clinical practice. *Urology*. 2007;70:449–53.
66. Bergman J, Reznichuk RC, Rajfer J. Surveillance of patients with bladder carcinoma using fluorescent in-situ hybridization on bladder washings. *BJU Int*. 2008;101:26–9.
67. Raitanen MP. The role of BTA stat test in follow-up of patients with bladder cancer: Results from FinnBladder studies. *World J Urol*. 2008;26:45–50.
68. Babjuk M, Böhle A, Burger M, Capoun O, Cohen D, Compérat EM, et al. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder: Update 2016. *Eur Urol*. 2017;71:447–61.

Tables

Due to technical limitations, tables are only available as a download in the Supplemental Files section.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [EDRNTTable1.pptx](#)
- [EDRNTTable2.pptx](#)
- [EDRNTTable3.pptx](#)

- [EDRNTable4.pptx](#)
- [EDRNTable5.pptx](#)
- [EDRNTable6.pptx](#)
- [EDRNTable7.pptx](#)