The future of Biomarkers in Prostate and Bladder Cancer

Prof Wim Van Criekinge, CSO
9th August 2015, Colorado Springs
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Overview

Epigenetics
- Introduction
- DNA Methylation & Oncology

MDxHealth
- NEXT-GENeration (Epi)genetic biomarkers
- Prostate Epigenetic Biomarkers
  - confirmMDx & Beyond
- Bladder Epigenetic Biomarkers
Overview

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Defining Epigenetics

- Reversible changes in gene expression/function without changes in DNA sequence
- Can be inherited from precursor cells
- Allows to (re)use one genomes for different purposes
- Allows to integrate intrinsic with environmental signals
Chromatin, a Key Component of Epigenetic Regulation

Cellular DNA is packaged into a structure called chromatin.

The unit of chromatin is the nucleosome, a complex of a histone tetramer with approx. 147 bp of DNA wound around it.
Evolutionary Perspective
epigenetic (meta)information = stem cells

Waddington's Epigenetic Landscape
Evolutionary Perspective

epigenetic (meta)information = stem cells
Epigenetics driving etiology of many human diseases
Cancer is impairing key pathways/modules/networks.
Historically, Cancer Was Considered to be Driven Mostly by Genetic Changes

- Mutations in p53
- Activating mutations in RAS
- Mutations or amplifications of the HER-2 gene
- Chromosomal translocations in myeloid cells and the generation of the BCR-ABL fusion protein
Past decade has shown that Epigenetic Changes are Important in Causing Cancer.

**GENETIC**
- Example: Replication errors
- Altered DNA sequence
- Altered DNA/mRNA/proteins

**EPIGENETIC**
- Example: Chromatin modification errors
- Altered chromatin structure
- Altered levels of mRNA/proteins

Oncogenesis leads to Tumor
Example of Methylation vs Mutation: Colon & Breast Cancer

Source: Schuebel et al 2007
Example of Methylation vs Mutation: Colon & Breast Cancer

Source: Schuebel et al 2007
Example of Methylation vs Mutation: Colon & Breast Cancer

Source: Schuebel et al 2007
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Step I: Bisulfite Treatment

Methylated

\[
\text{CGA CGCGCGCGC}
\]

Na BiSulfite Treatment

\[
\text{CGACGCGCGCGC}
\]

Un Methylated

\[
\text{CGACGCGCGCGC}
\]

\[
\text{UGAGUGUGUGUGU}
\]
Step II: Amplification and Detection

Methylation Specific PCR (MSP)

Using methylation specific primers

PCR Product

Methylated

C G A C G C G C G U C G U
G C T G C G C G C A G C A

Un Methylated

U G A U G U G U G U U G U
G C T G C G C G C A G C A

No PCR Product
DNA Methylation compared to competing technologies

- Frequency of methylation in different cancer tissues is attractive
- Methylation is biologically the most efficient way to shutdown gene
- A small number of biomarkers provides clinically relevant information
- Methylation is highly stable especially relative to mRNA and proteins
- Tumor cell specific methylation patterns detectable in a background of normal cells (higher sensitivity)
Next Generation Epigenetic Profiling

# markers

- **MethylCap_Seq**
- **EpiHealth**
- **Methylation Specific Seq**
- **Deep_Seq**

**Enrichment Sequencing (RUO)**

- Discovery: <50 only models and fresh frozen
- Verification: > 50 All sample types Incl. FFPE
- Validation: # samples

**Targeted Sequencing (IVD)**
Next Generation (Epi)genetic Profiling

Full genome

10^9 10^8 10^7 10^6 10^5 10^4 10^3 10^2 10^1 1
### Next Generation (Epi)genetic Profiling

<table>
<thead>
<tr>
<th>EPI</th>
<th>Whole-genome Bisulphite seq</th>
<th>Enrichment seq (MBD, RRBS)</th>
<th>Probes (450-27K)</th>
<th>Enrichment Targeted Panels</th>
<th>MSP</th>
</tr>
</thead>
<tbody>
<tr>
<td>GENETIC</td>
<td>Whole-genome sequencing</td>
<td>Enrichment seq (Exome)</td>
<td>Enrichment Targeted Panels</td>
<td>PCR</td>
<td></td>
</tr>
</tbody>
</table>

- **Full genome**
- **Enrichment seq**
- **Probes**
- **Targeted Panels**
- **MSP**

The scale for bp ranges from $10^9$ to 1.
Next Generation (Epi)genetic Profiling

Whole-genome sequencing

- Enrichment seq (MBD, RRBS)
- Probes (450-27K)
- Enrichment Targeted Panels
- MSP
- PCR
- Targeted Panels
- Enrichment seq (Exome)

Full genome

- Whole-genome Bisulphite seq

bp

Clinical PCR

RUO sequencing
MDxHealth

Next Generation (Epi)genetic Profiling

Whole-genome Bisulphite seq
Whole-genome sequencing

Enrichment seq (MBD, RRBS)
Enrichment seq (Exome)

Probes (450-27K)
Enrichment Targeted Panels

Enrichment Targeted Panels

MSP
PCR

Full genome

10^9 10^8 10^7 10^6 10^5 10^4 10^3 10^2 10^1 1

 RUO sequencing

Clinical PCR
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Performance of ConfirmMDx genes and methylation technology:

- Reported in over 45+ peer-reviewed scientific publications
- Over 5,000 subjects in studies
- Multinational, academic and community settings
- Prospective and retrospective multicenter, blinded studies

The Prostate 2011
Meta-Analysis Reports Clinical Utility of Epigenetic Assay

BJUI 2011
Assay shows 20% improvement in NPV over histopathology

American Health & Drug Benefits 2013
Reduces healthcare spending by >$588 per patient

American Health & Drug Benefits 2014
10-fold reduction in repeat biopsies in clinical utility study

Journal of Urology 2013
Multi-center clinical validation study demonstrates 90% NPV

Journal of Urology 2014
Multi-center, confirmatory validation study
NPV ~96% Significant PCa
NPV ~90% All PCa

DNA Methylation

Negative Biopsy
Persistent Risk Factors

Avoid Repeat Biopsy

Residual Tissue
Prostate Mapping

confirmMDx
NPV ~96% Significant PCa
NPV ~90% All PCa

Negative Biopsy
Persistent Risk Factors

DNA Methylation

Clinically Significant
or Indolent PCa?

Develop Algorithm for
Significant PCa Risk

Avoid Repeat Biopsy

... and beyond
**confirmMDx Risk Score**

<table>
<thead>
<tr>
<th>Core</th>
<th>Weighed Methylation Intensity</th>
<th>sum</th>
<th>average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>APC</em></td>
<td><em>GSTP 1</em></td>
<td><em>RASSF 1</em></td>
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<tr>
<td>1</td>
<td>0.73</td>
<td>46.95</td>
<td>1.15</td>
</tr>
<tr>
<td>2</td>
<td>0.00</td>
<td>16.05</td>
<td>0.00</td>
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<tr>
<td>3</td>
<td>0.00</td>
<td>0.00</td>
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<td>0.00</td>
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<tr>
<td>9</td>
<td>0.00</td>
<td>0.00</td>
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</tr>
</tbody>
</table>

- Age: 71
- Caucasian
- 1st Biopsy
- HGPIN

3/9 cores positives
confirmMDx Risk Score

Methylation Intensity

0 2 4 6 8 10

Bx- GS 6 GS ≥ 7

p = .457

p < .001

p < .001
Epigenetic Health Index (EHI)  
Risk Score to Further Stratify DNA-Methylation Positive Patients

Algorithm for Risk Stratification Using:
- Epigenetic Risk: Cores Positive and Methylation Intensity
- Clinical Risk Factors: Age, PSA, DRE, Histopathology
## Risk Score Comparison

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NCCN</th>
<th>PCPTRC2</th>
<th>EHI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographic</strong></td>
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<tr>
<td>Age</td>
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<td></td>
<td></td>
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<tr>
<td>Race</td>
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<tr>
<td><strong>Clinical</strong></td>
<td></td>
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<tr>
<td>Family history</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Prior biopsy</td>
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<td></td>
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<tr>
<td>DRE</td>
<td></td>
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<tr>
<td>Pathology of - index Bx</td>
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<tr>
<td>GS</td>
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<td>PSA density</td>
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<td><strong>Molecular</strong></td>
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</tr>
<tr>
<td>PSA</td>
<td></td>
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<tr>
<td>ConfirmMDx+ Bx Cores</td>
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<td></td>
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</tr>
<tr>
<td>Methylation Intensity</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

[http://deb.uthscsa.edu/URORiskCalc/](http://deb.uthscsa.edu/URORiskCalc/)
Epigenetic Health Index (EHI) Multivariate Logistic Regression

Odds Ratio for GS >= 7

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>HGPIN</th>
<th>Age</th>
<th>% + positive cores</th>
<th>PSA</th>
<th>Atypia</th>
<th>EpiScore Methylation intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Odds Ratio</td>
<td>0.1</td>
<td>0.1</td>
<td>1</td>
<td>4.5</td>
<td>2.5</td>
<td>16</td>
</tr>
</tbody>
</table>

Risk Factor
Epigenetic Health Index (EHI)

Methylation Intensity (weighed in logistic regression model)

PSA (8.2 ng/ml)

3/9 Positive Cores

Age-specific baseline for methylation positive men with HGPIN

EHI on 1st Biopsy 8.33
Epigenetic Health Index (EHI)

Methylation Intensity (weighed in logistic regression model)

PSA (8.2 ng/ml)

3/9 Positive Cores

Age-specific baseline for methylation positive men with HGPIN

EHI on 1st Biopsy 8.33

Pathology on 2nd Biopsy GS 8
ConfirmMDx and Epigenetic Health Index (EHI)

ConfirmMDx Flow Chart

- Negative Prostate Biopsy
- DNA Methylation
- EHI
- Avoid RBx

- High Risk Score
  - Repeat Biopsy
- Low Risk Score
  - Active Follow Up

ConfirmMDx:

- Improves stratification on decision for repeat Biopsies
- Helps reduce unnecessary repeat Biopsies
- EHI identifies men with clinically significant PCa outperforming traditional risk score methods like PSA and PCPTRC2

AUC

- PSA: AUC 0.57
- PCPTRC2: AUC 0.62
- EHI: AUC 0.74

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Identification and Validation of the Methylated TWIST1 and NID2 Genes through Real-Time Methylation-Specific Polymerase Chain Reaction Assays for the Noninvasive Detection of Primary Bladder Cancer in Urine Samples

Isabelle Renard a, Steven Joniau b, Ben van Cleynenbreugel b, Catherine Collette a, Christophe Naomé a, Ilse Vlasssenbroeck a, Hubert Nicolas c, Jean de Leval d, Josef Straub a, Wim Van Criekinge a, Wissem Hamida d, Majed Helbel d, Alexandre Thomas d, Laurence de Leval e,f, Katja Bierau a, David Waltregny d,f,*
Bladder Confirm MDx

**Biomaterial tested**

First step: 
BCa cell lines

Second step: 
Bladder tissues

**Experimental strategy**

Pharmacologic unmasking strategy for re-expression analysis of epigenetically targeted genes in four BCa cell lines

MSP analysis of the candidate methylated genes in formalin-fixed paraffin-embedded tissue samples from non-tumoural and cancerous bladder tissue specimens

**Results obtained**

Generation of a list of candidate methylated genes

Identification of methylation markers enabling us to discriminate, with a 100% specificity, UC from non-tumoural bladder tissues
**Bladder Confirm MDx**

**Third step:**
Urine samples from patients with or without BCa

MSP testing of the ten best performing tissue methylation markers in urine samples from patients with or without evidence of BCa (urine marker selection set)

Identification of the five most specific and sensitive urine methylation markers for identifying patients with BCa

MSP testing of the five most specific and sensitive urine methylation gene markers for identifying patients with BCa (urine training set)

Identification of a 2-methylated gene panel (TWIST1 and NID2) showing the highest performance for identifying patients with BCa

Validation of the two methylation gene markers in an independent set of urine samples (urine validation set)
Biomarkers validated by independent studies

Hypermethylation of *TWIST1* and *NID2*

in Tumor Tissues and Voided Urine in Urinary Bladder Cancer Patients

Zeynep Yegin, Sezgin Gunes, and Recep Buyukalpelli

Bladder cancer like other cancers arises from the accumulation of many genetic and epigenetic changes that lead to the activation of proto-oncogenes or inactivation of tumor suppressor genes. We aimed to investigate the methylation patterns of *Twist homolog 1 (TWIST1)* and *nidogen-2 (NID2)* genes in bladder cancer. Fifty-six histologically confirmed bladder tumor samples and paired 24 urine samples constituted the study group and was compared with 15 age- and gender-matched noncancerous individuals. DNA was purified from both tumor and urine samples. The methylation status of the two genes was analyzed by methylation-specific polymerase chain reaction (MSP) in both urinary bladder cell carcinoma samples and urine samples. Sensitivity and specificity values of the method were assessed and compared with the results of the cytology test. Methylation of the urine samples, respectively. The sensitivity of *TWIST1* and *NID2* genes (87.5% and 95.8% in urine samples, respectively), was higher compared with urine cytology (62.5%) for cancer detection. The sensitivity of any of the two genes was 88.8% (8/9) for low-grade cases. The sensitivity of urine cytology was 33.3% for the same low-grade cases. To be used in the early noninvasive diagnosis of bladder cancer, the combined methylation analysis of *TWIST1* and *NID2* genes may be a simple, noninvasive, sensitive, and specific method for detecting cancer cells in urine.
Diagnosis of Bladder Cancer Recurrence Based on Urinary Levels of **EOMES, HOXA9, POU4F2, TWIST1, VIM, and ZNF154** Hypermethylation

Thomas Reinert¹, Michael Borre², Anders Christiansen¹, Gregers G. Hermann³, Torben F. Ørntoft¹, Lars Dyrskjøt¹*

¹ Department of Molecular Medicine, Aarhus University Hospital, Aarhus, Denmark, ² Department of Urology, Aarhus University Hospital, Aarhus, Denmark, ³ Department of Urology, Frederiksberg Hospital, Copenhagen University, Frederiksberg, Denmark.

**Abstract**

**Background:** Non muscle invasive bladder cancer (NMIBC) has the highest recurrence rate of any malignancy and as many as 70% of patients experience relapse. Aberrant DNA methylation is present in all bladder tumors and can be detected in urine specimens. Previous studies have identified DNA methylation markers that showed significant diagnostic value. We evaluated the significance of the biomarkers for early detection of tumor recurrence in urine.

**Methodology/Principal Findings:** The methylation levels of **EOMES, HOXA9, POU4F2, TWIST1, VIM, and ZNF154** in urine specimens were measured by real-time PCR (Methylight). We analyzed 390 urine sediments from 184 patients diagnosed with NMIBC. Urine from 35 age-matched control individuals was used to determine the methylation baseline levels. Recurrence was diagnosed by cystoscopy and verified by histology. Initially, we compared urine from bladder cancer patients and healthy individuals and detected significant hypermethylation of all six markers (P<0.0001) achieving sensitivity in the range 82--89% and specificity in the range 94--100%. Following, we validated the urinary hypermethylation for use in recurrence surveillance and found sensitivities of 88--94% and specificities of 43--67%. **EOMES, POU4F2, VIM and ZNF154** were more frequently methylated in urine from patients with higher grade tumors (P≤0.08). Univariate Cox regression analysis showed that five markers were significantly associated with disease recurrence: **HOXA9** (HR=7.8, P=0.006), **POU4F2** (HR=8.5, P=0.001), **TWIST1** (HR=12.0, P=0.015), **VIM** (HR=8.0, P=0.001), and **ZNF154** (HR=13.9, P<0.001).

Interestingly, for one group of patients (n=15) we found that hypermethylation was consistently present in the urine samples despite the lack of tumor recurrences, indicating the presence of a field defect.

**Conclusion/Significance:** Methylation levels of **EOMES, HOXA9, POU4F2, TWIST1, VIM, and ZNF154** in urine specimens are promising diagnostic biomarkers for bladder cancer recurrence surveillance.
Biomarkers validated by independent studies

A Noninvasive Multianalyte Urine-Based Diagnostic Assay for Urothelial Cancer of the Bladder in the Evaluation of Hematuria

R. Jeffrey Kanes, MD; Cecilia A. Fernandez, PhD; and Anthony P. Shuber, MS

Abstract:

Objective: To test whether a noninvasive urine-based multianalyte diagnostic readout assay that uses protein and DNA biomarkers can risk stratify patients with hematuria into those who are or are not likely to have bladder cancer and those who should receive standard care.

Patients and Methods: This prospective, observational, multicenter, single-assessment study was conducted between June 12, 2009, and April 15, 2011. Eligible patients presented with hematuria and as part of their evaluation underwent cystoscopy. Urine samples were analyzed for the presence of mutant FGFR3 and quantified matrix metalloproteinase 2 and the hypermethylation of TWIST1 and NID2. A patient’s chance of having (positive predictive value [PPV]) or not having (negative predictive value [NPV]) cancer was determined by FGFR3 alone or by all 4 biomarkers, respectively.

Results: Cystoscopy/biopsy diagnosed 690 of 748 patients as negative and 58 as positive for bladder cancer. Of 21 patients identified by FGFR3 as highly likely to have cancer, 20 were also positive by cystoscopy/biopsy, resulting in a PPV of 95.2% (20 of 21), with specificity of 99.9% (689 of 690). The 4-marker combination identified 395 patients as having a low likelihood of cancer. Of these, 56.2% (388 of 690) also had negative biopsy/cystoscopy findings, resulting in an NPV of 98.2% (388 of 395). In total, 416 of the 748 patients with hematuria (55.6%) were identified with extremely high NPV and PPV to have or not have bladder cancer.

Conclusion: This multianalyte assay accurately stratified patients with high confidence into those who likely do or do not have bladder cancer. This test was developed to enhance and not to eliminate referrals for urologic evaluation.
Biomarkers validated by independent studies

MP-06.01
A 3-Gene DNA-Methylation Biomarker Panel Sensitive Detects Bladder Cancer and Discriminates Between High-grade and Low-grade Disease in Voided Urine
Hermanns, Thomas¹; Oltchov, Mislav²; Ekaterina; Savio, Andrea³; Zdravc, Darko⁴; Bhindi, Bimal⁵; Kuk, Cynthia⁶; Noon, Aidan⁷; Rendon, Ricardo⁸; Waltregny, David⁹; Lo, Kirk C.; van der Kwast, Theodorus; Finelli, Antonio⁴; Fliesner, Neil⁴; Bapat, Bharat³; Zloti, Alexandre³
¹Department of Surgical Oncology, Division of Urology, Princess Margaret Cancer Centre, University of Toronto, Toronto, ON, Canada; ²Department of Urology, Mount Sinai Hospital, Toronto, ON, Canada; ³Department of Urology, University Health Network, Toronto, ON, Canada; ⁴Department of Urology, Mount Sinai Hospital, Toronto, ON, Canada; ⁵Department of Urology, Dalhousie University, Halifax, NS, Canada; ⁶Department of Urology, University of Liege, Liege, Belgium; ⁷Department of Pathology, University Health Network, University of Toronto, Toronto, ON, Canada

Introduction and Objectives: Voided urine provides an excellent source of exfoliated bladder cells and is ideal to detect bladder cancer (BC) biomarkers. Using two different genome-wide methylation-array profiling platforms in Toronto, CA and Liege, BE, several differentially relevant genes (incl. TWIST1, NID2 and RUNX3) versus high-grade (HG) BCs were commonly in methylation of the 3 genes to identify BC in urine samples.

Methods: Voided urine from patients with LG (n=34) and HG (n=34) cancers as well as from BC-free controls (n=34) were studied. Status of the 3 genes was analyzed in the urine using the Infinium Epigraph assay. Methylation levels (percent methylated) were obtained for each sample. Association between LG versus HG status was analyzed using Mann-Whitney U-test. Univariate regression models were used to create ROC curves and combined biomarker discrimination, respectively.

Results: Median PMRs for HG, LG and no BC were significantly different for each gene (TWIST1: HG: 22, LG: 1, no BC: 0; NID2: HG: 27.4, LG: 7.5, no BC: 4.7; RUNX3: HG: 3.5, LG: 0.01, no BC: 0; all p<0.001). The PMRs for all genes were significantly higher in BC than in no BC cases (all p<0.001) and in HG BC compared to LG/no BC cases (all p<0.001). The AUC to predict BC was 0.83 (95% CI: 0.76-0.90) for TWIST1, 0.81 (95% CI: 0.72-0.89) for NID2 and 0.73 (95% CI: 0.64-0.82) for RUNX3. The AUC to predict HG BC was 0.86 (95% CI: 0.78-0.94) for TWIST1, 0.8 (95% CI: 0.71-0.79) for NID2 and 0.77 (95% CI: 0.67-0.86) for RUNX3. When combining all genes, the AUC was 0.87 (95% CI: 0.80-0.94) to predict BC and 0.87 (95% CI: 0.79-0.95) to predict HG BC.

Conclusions: Combination of the 3 epigenetic markers is a very promising tool for sensitive and specific detection of BC and discrimination between HG and LG BC in voided urine.
Overview DNA based bladder cancer markers

detection

prognosis

TWIST1
NID2

epigenetic
Overview DNA based bladder cancer markers

detection

epigenetic
- TWIST1
- NID2
- VIM
- OSR1
- ONECUT
- 2
- OTX1

genetic
- p16
- CHH1
- MLL2
- ARID1A
- KDM6A
- FGFR3
- P53
- hTERT

prognosis

- GSTP1
- RASSF1
- APC
- FHT
- LAMC2
- SFRP1
- PMF1
- ISL1
- ALDH1A3
- PCDH7
- ITIH5
- KLF4

EP300, MLL3, MLL2, ARIDNA1, KDMA6A, ATM, ATRX, BRCA2, ATR, STAG2, ERCC2, PDGFRA, CDKNA2A, RB1, CDKN1A, NF1, PIK3CA, FBXW7, CDH1, NFE2L2, MALAT1, NCOR1, PRX
Select best biomarker panel for detecting BC in urine of patients with Hematuria

Hematuria

Bladder tumor 3-28%
Validation Study / case-control / 160 Patients

MSP

TWIST1
NID2
(TWIST1 neutral)

SNaPshot

ONECUT2
OTX1

Mutation

FGFR3
TERT
PIK3CA
KRAS
NRAS
HRAS
Optimal model: NPV > 99%, 97% sensitivity
Conclusions

• A limited number of bladder cancer specific methylation markers can be measured in urine to accurately detect the presence of bladder cancer in hematuria patients.
Conclusions

- A limited number of bladder cancer specific methylation markers can be measured in urine to accurately detect the presence of bladder cancer in hematuria patients.

- ConfirmMDx for Bladder can be used as a rule-in for cystoscopy (in case of hematuria) with a very high NPV and very high sensitivity thereby resulting in a significant reduction in the number of cystoscopies.
Conclusions

- A limited number of bladder cancer specific methylation markers can be measured in urine to accurately detect the presence of bladder cancer in hematuria patients.

- ConfirmMDx for Bladder can be used as a rule-in for cystoscopy (in case of hematuria) with a very high NPV and very high sensitivity thereby resulting in a significant reduction in the number of cystoscopies.

- It represents a significant improvement in PPV as compared to standard of care. Potential in the recurrence setting is actively investigated.
Q&A

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