

Identification and validation of the methylated TWIST1 and NID2 genes through real-time Methylation-Specific PCR (MSP) assays for the non-invasive detection of bladder cancer in urine samples.

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Introduction

Each year in the U.S. and EU, bladder cancer is diagnosed in >160,000 men and women and results in >48,000 deaths. While the five-year survival rate for early-stage bladder cancer is high, there are significant challenges for early detection as well as monitoring for disease recurrence or progression, which can occur in up to 70% and 25% of cases respectively. Urine cytology and cystoscopy are the current standard-of-care for bladder cancer detection and surveillance. Cytology is highly specific (~90-95%) but poorly sensitive (~35%), especially for early-stage disease. Cystoscopy is highly sensitive but is invasive, costly and not without risk. As a result, there is great interest in a non-invasive approach that can more reliably identify bladder cancer in symptomatic or high-risk patients. OncoMethylome Sciences (ONCO) has developed a urine-based DNA methylation assay with high sensitivity, specificity, and reproducibility. Such an assay could be especially useful for detecting early-stage bladder cancer, while minimizing the need for invasive cystoscopies in negative patients.

Material and Methods

Marker identification: Candidate genes were identified using re-expression profiles of bladder cancer cell lines. Promoter sequences were linked with gene expression to identify valid, epigenetically-silenced genes. An established pharmacologic unmasking strategy (5-aza-2'-deoxycytidine (DAC) and trichostatin A (TSA)) for re-expression analysis of epigenetically-targeted genes was combined with proprietary advanced bioinformatics tools to identify genes prone to promoter methylation [1].

Marker selection in bladder tissue: Differentially-methylated genes were validated in tissue using real-time methylation-specific PCR (real-time MSP). Best performing tissue markers were selected for testing DNA in urine samples.

Urine sample collection and preparation: Prospective, randomly collected urine samples from 3 centers in Belgium were used. All study participants provided written informed consent for the trial and the study protocol received appropriate approval from the relevant ethical committee. Symptomatic patients, attending a urology clinic and ultimately diagnosed with bladder cancer or other non-malignant urological disorders, provided a urine sample for use in real-time MSP and cytology analysis. Bladder carcinoma was diagnosed by cystoscopy, upper tract imaging, ultrasound and cytology. Cases were later confirmed by histology assessment of the resected tissue samples. Patients with other urological cancers were excluded. From the ongoing trial, 496 urine samples were tested (Marker Selection, Training and Validation sets). Those included 339 samples from patients with no evidence of cancer and 157 samples from patients covering all stages of bladder cancers with 92% representing early-stage disease (non muscle invasive bladder cancer). The collected urine samples were immediately stabilized with Stabilur® tablets (1 tablet per 10 ml of urine - Cargille Laboratories). These stabilized urine samples were conserved at room temperature for up to 48h before centrifugation (3000g for 10 min). The sediments were stored at -20°C up to 6 months before DNA isolation and further processing. An aliquot of all urine samples was also used for cytology analysis.

DNA preparation and processing: DNA was isolated from urine sediments using a standard method (Qiagen #158908 and Qiagen #158912) and quantified using the Picogreen® dsDNA quantitation kit (Molecular Probes, #P7589) following the manufacturer's directions. Up to 1.5µg DNA was used for the bisulphite modification using a commercially available kit (Zymo, #D5002). This reaction selectively deaminates unmethylated cytosine residues resulting in a conversion to uracil, while 5-methyl cytosine residues are not modified. The modified DNA was eluted into 20µl Tris-HCl (1mM, pH 8.0) and then stored at -80°C up to 6 months before further processing.

Real-Time MSP: Analyte quantifications were performed by real-time MSP assays. These consisted of parallel amplification/quantification processes using specific primers and probes for each Analyte and Molecular Beacon® assay formats on an ABI Prism® 7900HT instrument (Applied Biosystems). Two analytes defined in the real-time MSP were used. ACTB was used as a reference gene, using primers which were outside any CpG islands.

The thermal profile: The following was used for all genes: 95°C for 5min, 95°C for 30sec, 57°C for 30sec, and 72°C for 30sec for 45 cycles. The total PCR volume was 12 µl (including 2.4µl DNA template) in a 384-well PCR plate.

Quantification: The results were generated using the SDS 2.2 software (Applied Biosystems), exported as Ct values (cycle number at which the amplification curves cross the threshold value, set automatically by the software), and then used to calculate copy numbers based on a linear regression of the values plotted on a standard curve of 20 - 2 x 10⁶ gene copy equivalents, using plasmid DNA containing the bisulfite modified sequence of interest. Cell lines were included in each run as positive and negative controls, and entered into the procedure at the DNA extraction step. A run was considered valid when the following five criteria were met: a) slopes of both standard curves above -4 (PCR efficiency > 77.8%); b) R² of at least 4 relevant data points above 0.990; c) routinely included NTC not amplified; d) 10% of a 1µg conversion reaction of the positive cell line assay control was detectable; and e) 10% of a 1µg conversion reaction of the negative cell line assay control was not detected within the standard curve.

Table 1: Sequence details and targets

Gene	Primer/Target	Sequence	Target Location
TWIST1	Forward primer	5'-GTTAGGTTCTGGGGGCGTGTGT-3'	Chromosome 7, between positions 1912410 and 1912443 (RefSeq NM_00474)
	Reverse primer	5'-CGGTGCGCTTCTCGAGGAA-3'	
	Molecular beacon	5'-CGACATGCGCGGGGGAAGAAATGTTTGGTCAATGTC-3'	
	Amplicon size	77 bp	
NID2	Forward primer	5'-GGGTTTAAAGAGTTTATTTT-3'	Chromosome 14, between positions 5160916 and 5160915 (RefSeq NM_00781)
	Reverse primer	5'-CTAAGAAATCCCTTAAGCT-3'	
	Molecular beacon	5'-CGACATGGTGTGTAAGGTTGGGGTGAAGCGGCATGTCTG-3'	
	Amplicon size	99 bp	
ACTB	Forward primer	5'-TAGGAGTATAGTGTGGGAAGTT-3'	Chromosome 7, between positions 553828 and 553832 (RefSeq NM_00101)
	Reverse primer	5'-AACACCAATCAACAAACAAATCAC-3'	
	Molecular beacon	5'-CGACTGCGTGGGGTGGTGTGAGGAGGTTAAGGAGTCTG-3'	
	Amplicon size	103 bp	

Results

Marker selection in bladder tissue: Based on re-expression, the 74 most differentially methylated gene sequences were validated on retrospectively collected tumors from 91 bladder cancer patients and 39 histopathologically normal tissue samples using real-time MSP assays. Several markers reliably detected bladder cancer in those tissue samples (data not shown). The individual performance of the best performing markers in tissue has been reported previously [2].

Assay validity in urine samples: 496 urine samples were processed using real-time MSP. The real-time MSP assays produced valid results in 94% of the urine samples (Table 1). Validity was assessed by measuring ACTB.

Marker testing in urine samples: The top 10 methylated markers found in bladder tissues were tested in a urine marker selection set (see [2] for details) consisting of 218 urine samples (cancers and controls). The 5 best performing markers were selected for further validation.

Urine Training set: 2-gene methylation markers, TWIST1 and NID2, demonstrated the best performance in this set of samples consisting of 48 valid cancer samples and 121 valid control samples. A sensitivity of 88% with a specificity of 94% was obtained using this 2 gene panel (Table 2 - interpretation model: if any marker gave a copy number above the individual cut-off, then the sample was scored as "positive").

Urine Validation set: The 2-gene panel was then validated on an independent validation set including valid samples from 35 bladder cancers and 57 control cases. Sensitivity and specificity rates of 94% and 91% respectively were reached in this set of samples (Table 2).

Notably, sensitivity rates of 80-89% were found for early stage Ta and low-grade bladder cancers in each set of samples (Table 2).

A Receiver Operating Characteristics (ROC) curve for a logistic regression model using the 2 markers was created by combining the results obtained in the training and validation sets. The area under the curve (AUC) was 0.93 with a 95% CI of 0.90-0.96 at a significance of P<0.0001 (Figure 1).

The sensitivity and specificity of the 2-gene panel was similar for samples collected at the 3 clinical sites (Table 3).

Table 3: Performance of the methylation assays for the NID2/TWIST1 gene panel based on their site of collection (urine training and validation sets combined).

Sample collection site	Sensitivity rate, in % (# methylated / # total)	Specificity rate, in % (# non methylated / # total)
Site 1	92 (11/12)	90 (19/21)
Site 2	89 (42/47)	94 (75/80)
Site 3	92 (22/24)	94 (72/77)

Combination of methylation testing and cytology

results: Sensitivity and specificity of the 2-gene panel and of cytology are detailed in Table 4. Sensitivity is much higher for the methylation markers (88-94%) than for cytology (48-49%) while both tests were associated with high specificity rates of 91-94% and 95-97% respectively. Combination of both tests lead to a slight increase in sensitivity (96-97%) and a slight decrease in specificity (86-93%) for detecting bladder cancer. The Negative and Positive Predictive Values (NPV and PPV) of MSP results for both urine sets was 95% and 86% respectively. When methylation and cytology results are combined, NPV reaches 98% whereas PPV is 83%.

Conclusion

Detection of the methylated TWIST1 and NID2 genes in voided urine samples using Methylation Specific PCR provides a highly (> 90%) sensitive and specific, non-invasive approach for detecting bladder cancer in high-risk, symptomatic patients.

References: 1) Straub, J. et al., Abstract: A64-AACR Molecular Diagnostics in Cancer Therapeutic Development (2007): Base5, a versatile, highly integrated high-throughput methylation profiling platform for Methylation-Specific PCR based marker identification applied to CRC. 2) Renard, I. et al., Abstract: A62-AACR Molecular Diagnostics in Cancer Therapeutic Development (2007): Feasibility of a urine-based DNA methylation assay for early detection of Bladder Cancer. 3) Renard, I. et al., Abstract: A64-Cancer Epigenetics (2008): A real-time Methylation specific PCR (MSP) Assay for the Early Detection of Bladder Cancer.

Table 1: Percentage of valid methylation assays in the various urine sample groups.

Urine set	Patient group	Number of samples	Number of valid methylation assays (%)
Selection set	Cancer	68	62/68 (91)
	Control	150	143/150 (95)
	Total	218	205/218 (94)
Training set	Cancer	54	48/54 (89)
	Control	130	121/130 (93)
	Total	184	169/184 (92)
Validation set	Cancer	35	35/35 (100)
	Control	59	57/59 (97)
	Total	94	92/94 (98)
3 sets	Cancer	157	145/157 (92)
	Control	339	321/339 (95)
	Total	496	466/496 (94)

Table 2: Sensitivity and specificity rates of the methylated NID2/TWIST1 gene panel in the urine training and validation sets.

	Urine training set	Urine validation set
	Sensitivity rate, in % (# methylated / # total) [95% CI]	Sensitivity rate, in % (# methylated / # total) [95% CI]
All Cancers	88 (42/48) [78-97]	94 (33/35) [87-102]
Ta	80 (16/20)	88 (14/16)
Tis	100 (4/4)	100 (2/2)
T1	100 (12/12)	100 (11/11)
T2	83 (10/12)	100 (6/6)
Low grade	80 (12/15) [60-100]	89 (17/19) [76-103]
High grade	91 (30/33) [81-101]	100 (16/16)
	Specificity rate, in % (# not methylated / # total) [95% CI]	Specificity rate, in % (# not methylated / # total) [95% CI]
Controls	94 (114/121) [90-98]	91 (52/57) [84-99]

Figure 1: Receiver Operating Characteristics (ROC) curve for the 2-methylated gene panel (training and validation sets combined).

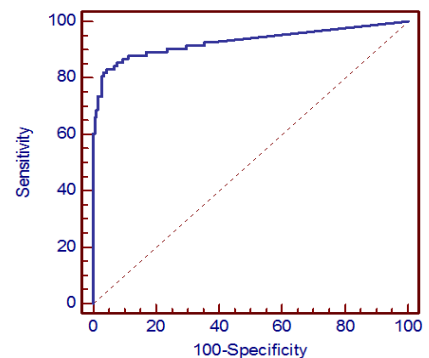


Table 4: Performance overview of NID2/TWIST1 MSP, cytology and their combination for the training and validation urine sets.

		Sensitivity, in % (# positive / # total) [95% CI]	Specificity, in % (# negative / # total) [95% CI]
Training set	Methylation	88 (42/48) [78-97]	94 (114/121) [90-98]
	Cytology	48 (23/48) [34-62]	97 (117/121) [94-100]
	Combination	96 (46/48) [90-101]	93 (112/121) [88-97]
Validation set	Methylation	94 (33/35) [87-102]	91 (52/57) [84-99]
	Cytology	49 (17/35) [33-65]	95 (54/57) [89-101]
	Combination	97 (34/35) [92-103]	86 (49/57) [77-95]

