Detecting DNA Methylation of the *BCL2, CDKN2A* and *NID2* Genes in Urine Using a Nested Methylation Specific Polymerase Chain Reaction Assay to Predict Bladder Cancer

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Purpose: Detection of methylated DNA has been shown to be a good biomarker for bladder cancer. Bladder cancer has the highest recurrence rate of any cancer and, as such, patients are regularly monitored using invasive diagnostic techniques. As urine is easily attainable, bladder cancer is an optimal cancer to detect using DNA methylation. DNA methylation is highly specific in cancer detection. However, it is difficult to detect because of the limited amount of DNA present in the urine of patients with bladder cancer. Therefore, an improved, sensitive and noninvasive diagnostic test is needed.

Materials and Methods: We developed a highly specific and sensitive nested methylation specific polymerase chain reaction assay to detect the presence of bladder cancer in small volumes of patient urine. The genes assayed for DNA methylation are *BCL2*, *CDKN2A* and *NID2*. The regions surrounding the DNA methylation sites were amplified in a methylation independent first round polymerase chain reaction and the amplification product from the first polymerase chain reaction was used in a real-time methylation specific polymerase chain reaction. Urine samples were collected from patients receiving treatment at Wolfson Medical Center in Holon, Israel.

Results: In a pilot clinical study using patient urine samples we were able to differentiate bladder cancer from other urogenital malignancies and nonmalignant conditions with a sensitivity of 80.9% and a specificity of 86.4%.

Conclusions: We developed a novel methylation specific polymerase chain reaction assay for the detection and monitoring of bladder cancer using DNA extracted from patient urine. The assay may also be combined with other diagnostic tests to improve accuracy.

Key Words: DNA methylation, urinary bladder neoplasms, polymerase chain reaction

Abbreviations and Acronyms

ACTB = β-actin
BCL2 = B-cell lymphoma 2
CDKN2A = cyclin dependent kinase inhibitor 2A
LMP = low malignancy potential
MSP = methylation specific polymerase chain reaction
NID2 = Nidogen-2
PCR = polymerase chain reaction
TCC = transitional cell carcinoma

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For another article on a related topic see page 2377.

Editor's Note: This article is the second of 5 published in this issue for which category 1 CME credits can be earned. Instructions for obtaining credits are given with the questions on pages 2446 and 2447.

BLADDER cancer is the fourth most prevalent cancer in men and the ninth most prevalent cancer in women. In the United States 69,250 new cases of bladder cancer were diagnosed and an estimated 14,990 deaths occurred in 2011.[1] In addition, bladder cancer is the most expensive cancer per patient between diagnosis and death because of its 50% to 80% recurrence rate.[2]

Due to this high rate of recurrence, patients are monitored regularly using

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invasive diagnostic techniques. The current regimen for a patient diagnosed with bladder cancer is cystoscopy every 3 months for the first 2 years after diagnosis, once every 6 months in the following 2 years, then once a year for the life of the patient. However, cystoscopy is an invasive and uncomfortable procedure, and is a possible reason why 60% of patients diagnosed with bladder cancer do not return for the recommended surveillance.[3] In addition, cystoscopy is subjective, thereby resulting in different outcomes depending on the interpretation.[4] Therefore, a sensitive, objective, noninvasive test would be expected to improve patient compliance and result in earlier detection.

Epigenetics controls genetic traits not by varying the DNA sequence comprising genes, but by controlling gene expression through modification of the DNA and the proteins that compact it into chromatin. One such modification is DNA methylation of cytosine in CpG islands. CpG islands are genomic regions, usually present near gene promoters, which have a higher frequency of CpG dinucleotide content than the rest of the genome. Hypermethylation of CpG islands generally correlates with gene silencing.[5] Furthermore, DNA methylation is deregulated in cancer leading to hypermethylation of these CpG islands and the possible silencing of tumor suppressors.[6,7]

Detection of DNA methylation has been shown to be a biomarker for the presence of cancer. DNA methylation has been identified as an indication for the detection, prognosis and stratification of cancer.[8–10] Altered DNA methylation has also been found in all cancers studied to date.[5] Furthermore, such analyses are appealing based on the stability of methylated DNA, as well as the established methods for amplification and detection of DNA methylation such as methylation specific PCR.[8] MSP is the use of primer pairs in a PCR reaction that are complimentary to DNA that is converted by sodium bisulfite and that contains several CpG dinucleotides (ie multiple methylation sites) that can be methylated in vivo. MSP depends on the use of sodium bisulfite to selectively convert nonmethylated cytosine to uracil.[11] This change in sequence allows one to distinguish between methylated and nonmethylated DNA using specific PCR primers.

DNA methylation detection is applicable to bladder cancer due to the shedding of cells into urine. Several studies have analyzed DNA methylation of targets associated with bladder cancer using urine.[12–18] MSP is commonly used to detect DNA methylation because it can be used in a high throughput and quantitative manner.[11,19] However, MSP requires large quantities of urine and yields unreliable results due to the small quantity of cancer cells present. To address this issue we developed a novel nested MSP assay to detect bladder cancer using patient urine. The advantages of this assay are that it can be used to detect small numbers of tumor cells in a urine sample, and that it is highly specific and sensitive. In a pilot case-controlled prospective study we analyzed urine collected from patients with bladder cancer to assess the performance of our assay. We also compared these samples to those of healthy patients and patients with prostate or kidney cancer.

MATERIALS AND METHODS

Patients and Sample Collection

Study participants provided written informed consent and the study protocol received approval from the Israeli Ministry of Health (experimental protocol 9-2008-0588, genetic committee approval 069-2008, local institutional review board approval 1019). Bladder cancer diagnosis was made by the attending physicians and confirmed by histological evaluation of the resected tissue samples. Clinical and pathological data for the population studied are shown in table 1. Urine samples were collected at Wolfson Medical Center (Holon, Israel) during outpatient visits or during the morning before any surgical procedure. Each

Table 1. *Clinicopathological data for patients with bladder cancer and controls*

	Bladder Cancer	
	Pts with TCC	Controls
No. gender:		
F	7	4
M	28	13
Not applicable	7	4
Median age (range)	74 (55–87)	60 (36–81)
No. pathological stage:		
pTa	18	
pT1	13	
pT2	9	
Unknown	2	
No. pathological grade:		
LMP	11	
Low	12	
High	18	
Unknown	1	
No. recurrence:		
Yes	15	
No	17	
Not applicable	10	
No. diagnosis:		
Healthy		5
Cystectomy, no tumor		1
Renal Ca		4
Prostate Ca		5
Kidney stones		2
Benign prostatic hyperplasia		1
Neuroendocrine Ca		1
Breast Ca		1
Inflammation		1
Cystitis		1

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