High-Throughput Molecular Analysis of Urine Sediment for the Detection of Bladder Cancer by High-Density Single-Nucleotide Polymorphism Array

Mohammad Obaidul Hoque, Juna Lee, Shahnaz Begum, Keishi Yamashita, James M. Engles, Mark Schoenberg, William H. Westra, and David Sidransky

Abstract

The detection of urothelial malignancies remains challenging. The majority of patients diagnosed with bladder cancer require life-long surveillance for disease recurrence. Monitoring strategies rely predominantly on invasive endoscopic techniques, which are inconvenient and uncomfortable. Multiple in vitro diagnostic technologies have been developed to supplant the contemporary standard of care. The U.S. Food and Drug Administration has approved several assays, but because of inferior performance characteristics (low sensitivity and specificity) these tests have not made a significant impact on practice, to date. We sought to develop a test for bladder cancer with better performance characterization detection based on a novel molecular approach.

Matched urine and peripheral blood lymphocyte samples were obtained before surgery from 31 patients with bladder cancer (10 pTa, 4 pT1, and 17 pT2≥). DNA from these samples was subjected to allelic imbalance analysis using HuSNP chips and was validated in parallel with microsatellite analysis for loss of heterozygosity and microsatellite instability. Peripheral blood lymphocyte and urine DNA obtained from 14 individuals without clinical evidence of genitourinary malignancy served as controls.

Thirty-one of 31 (100%) urine DNA samples from patients with bladder tumors were found to have 24 or more single-nucleotide polymorphism (SNP) DNA alterations. In general, SNP alterations were more common in tumors than pTa or pT1 tumors. SNP alterations were not identified in nine normal control subjects and in four of five patients with hematuria.

These data support the noninvasive HuSNP chip assay in urine DNA as a valuable tool for the detection of bladder cancer (on a high-throughput-automated platform).

Introduction

Bladder cancer is the fourth most common cancer in men and eighth in women in both incidences and mortality (1–3). Worldwide, an estimated 243,000 cases of bladder cancer occur each year; incidence rates are highest in industrialized countries, in which over 90% of bladder cancers are of transitional cell origin (4). Seventy percent of patients with a de novo diagnosis of TCC have superficial tumors (Tumor-Node-Metastasis stages Ta, Tis, or T1) and can be treated by transurethral resection of the tumor alone. Even after complete transurethral resection of all visible lesions, 50–70% of superficial bladder tumors recur and 10–20% progress in stage and grade (5). Relatively few reliable diagnostic tools are available for the detection of TCC. The development of such tools would permit the evaluation of intriguing clinical issues such as the value of early detection and the role of molecular markers in prognostic evaluation of bladder cancer patients at the time of initial diagnosis.

Cystoscopy is the gold standard for detection of bladder cancer, but it is an invasive and uncomfortable procedure. Urine cytology remains the only accepted noninvasive method for detection of bladder cancer and tumor recurrence, yet the sensitivity of urine cytology in low-grade tumors is not satisfactory (6). Several potential diagnostic markers for bladder cancer were identified, including nuclear matrix protein-22, bladder tumor antigen, and telomerase (7, 8). Although these markers were more sensitive than urine cytology for detecting bladder cancer, their use was limited by low specificity (7). Other methods for the noninvasive diagnosis of bladder cancer, including hyaluronic acid (9), Lewis X antigen (10), fibrinogen degradation product (11), and MCM5 (12), are still in development. All these methods are based on the detection of elevated or altered proteins from cancer cells.

Alternative approaches for noninvasive cancer detection rely on the identification of aberrant nuclear acids from neoplastic cells. Aberrations of the ratio between the copy numbers of two alleles of heterozygotes (microsatellite analyses; Refs. 13–17) or between the copy numbers of two different genes (quantitative PCR; Refs. 18–20) can be used to detect genomic alterations in cancer cells. Adequate sensitivity for bladder cancer detection using microsatellite analysis can be achieved by testing 20 highly informative loci (15). However, it may be difficult to obtain adequate amounts of DNA from urine for the amplification of 20 loci individually or in multiplex PCR reactions.

Recently, we performed a genome-wide comparison of standard microsatellite analysis and the HuSNP chip assay to detect LOH in primary bladder tumors (21). Our results were consistent with those reported by other investigators (22–24), supporting a similar ability to detect LOH for the two methods. In this blinded comparative study, we aimed to determine whether a urine DNA assay based on the HuSNP chip could detect lower urinary tract cancers. We found this high-throughput-automated technique to be accurate in detecting AI in urine sediment from bladder cancer patients.

Materials and Methods

Sample Collection. After we obtained written informed consent from 31 patients with TCC (pTa and pT1, n = 10; pT1, n = 4; pT2≥ n = 17), 50 ml of voided urine and 10 ml of whole blood were collected before surgical intervention. The Institutional Review Board of the Johns Hopkins Hospital approved the study. Five control samples were obtained from patients with...
hematuria but no evidence of malignancy and nine from individuals without a history of genitourinary disease (total, 14). For preparation of buffy coats, blood in citrate-coated tubes was centrifuged at low speed and the liquid phase was decanted. Urine samples were spun at 3000 × g for 10 min and washed twice with PBS. All samples were stored at −80°C.

**DNA Extraction.** To obtain a leukocyte pellet, buffy coats were rinsed with TM solution (5 mM MgCl₂ and 20 mM Tris buffer) and spun at 12,000 × g to lyse remaining erythrocytes. Frozen urine cell pellets and leukocyte pellets were digested with 1% SDS and 50 µg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) at 48°C overnight, followed by phenol/chloroform extraction and ethanol precipitation of DNA as described previously (25).

**HuSNP Protocol.** The Affymetrix HuSNP protocol was performed according to the manufacturer’s instructions with little modification, unless otherwise described (21). The genetic map used in the analysis was obtained from the latest Affymetrix release listing relevant SNP loci and their position.

**Statistical Analysis.** Tumor stage (pTa, pT1, and pT2≥) and grade (G1, G2, and G3) were recorded when reported. Mean fractional allelic loss was compared across stages using simple linear regression models. All statistical computations were performed using the SAS system, and two-sided P values were reported.

**Results**

We tested 1494 polymorphic loci by HuSNP chip analysis in paired urine and lymphocyte DNA from 31 patients with bladder cancer and 14 control subjects. Representative images generated by the software manufacturers are shown in Fig. 1. The median number of informative markers per patient was 319 (range, 87–376). Thirty-one of 31 (100%) urine DNAs from bladder cancer patients harbored AI in at least 24 SNP markers. AI was detected frequently in known area of chromosomal loss: 9p [23 (74.19%) of 31], 9q [21 (67.74%) of 31], 17p [16 (51.61%) of 31], 20q [16 (51.61%) of 31], 14q [14 (45.16%) of 31], 4q [13 (41.43%) of 31], 5q [13 (41.43%) of 31], 13q [12 (38.70%) of 31], 10q [12 (38.70%) of 31], and 8q [11 (35.48%) of 31]. Overall, the median number of SNPs displaying AI in urine DNA samples from cancer patients was 39 (range, 24–119). The median number of SNPs with AI was 34 (range, 24–93) for pTa, pT1, and pT2 tumors and 60 (range, 33–119) for pT2≥ tumors. The General Linear Model (GLM) procedure revealed a trend toward significant differences in AI between these two groups of bladder cancers (P = 0.08; Fig. 2; see Table 2). Urine DNA from none of the nine control volunteers displayed AI at any marker. However, one of five control patients with hematuria and non-neoplastic disease harbored AI at 10 SNP markers in the urine DNA.

The cystoscopy, cytology, and radiology results in relation to AI and tumor characteristics at the time of urine sample collection for the 31 bladder cancer patients are shown in Table 1. The number of SNPs displaying AI was less in pTa and pT1 tumors than in pT2≥ tumors (Fig. 2 and Table 2). We detected urine AI in two pTa tumors (8041 and 8045) and one pT1 tumor (8051) with an indeterminate cytology result (eventually positive by final pathology on biopsy). For the two pTa tumors, cytology results were also negative but suspicious for malignancy, whereas the pT1 tumor cytology was positive (Table 1). For this pT1 tumor, the imaging results were also consistent with the positive cytology and HuSNP analysis findings. In a previous study (26), one patient with a negative cytology had a tumor recurrence within 6 months after a positive LOH test in the urine DNA by microsatellite analysis. The HuSNP assay performed on the same urine DNA was also positive 6 months before diagnosis for this noninvasive superficial bladder tumor. However, the HuSNP chip assay remains to be validated in a prospective monitored setting to detect recurrent tumor with serial urine sampling.

In 28 of the 31 bladder cancer patients, data on conventional cytology and AI were available for a comparison. Urine cytology was negative in six cases in which the HuSNP assay was positive, and in one of these cases (8041), the cytoscopy result was indeterminate. In this case, both cytoscopy and cytology failed to detect cancer, but the HuSNP assay successfully displayed AI on several chromosomal arms. The sensitivity for conventional cytology in bladder cancer detection was 14 of 28 (50%), similar to studies reported previously (6). Cytology was negative in all 14 control cases with non-neoplastic disease.

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**Fig. 1.** Representative images of fluorescence intensities for SNP array hybridization to paired lymphocyte and urine DNA samples. For each sample pair, a call of AI was made. The chromosomal region for each marker is indicated in parentheses below each image. Patient 4045 is a control with nonmalignant urinary disease. All other patients harbor bladder cancers of various stages.

**Fig. 2.** Fractional allelic loss (FAL) index for all TCC stages by the SNP analysis. Bars, the mean for each group of subjects [mean FAL are 0.15 and 0.25 for stage pTa plus pT1 (stage I and stage II) and pT2≥ (stage III), respectively]. Loss of all informative markers showed a trend toward significance between pTa plus pT1 versus pT2≥ (P = 0.08).
reasonable that this assay picked up AIs from all of the cancer cases, information on 312 loci across all chromosomes. It is, therefore, the majority of bladder cancers. Using the HuSNP chip, we obtained in an effort to identify several genetic changes potentially present in we and others used 13

27), based on microsatellite analysis. In most of the previous studies, samples from bladder cancer patients by the HuSNP assay compared through urine DNA analysis.

TCCs (8046, 8047, 8061, and 8063 in Table 1) reinforce the potential found that this noninvasive molecular method detected all cases of detected genomic deletions and/or amplifications in cancer cells. We –

crosatellite or SNP analyses; Refs. 13

Discussion

Aberrations of the normal ratio between heterozygote alleles (microsatellite or SNP analyses; Refs. 13–17 and 23) can be used to detect genomic deletions and/or amplifications in cancer cells. We previously validated the ability of the HuSNP assay to detect AIs in primary human bladder cancer tissue DNA (21). In this study, we sought to determine whether this genome-wide approach could be used for the detection of bladder cancer by testing urine DNA. We found that this noninvasive molecular method detected all cases of cancer, even those negative by standard cystoscopy and cytology. Our findings that four of the bladder tumors detected in this study were not TCCs (8046, 8047, 8061, and 8063 in Table 1) reinforce the potential use of this diagnostic strategy for detecting cancer of different origins through urine DNA analysis.

Urine DNA AIs were present in 100% of the examined urine samples from bladder cancer patients by the HuSNP assay compared with a reported sensitivity of 85% or greater in previous studies (15, 27), based on microsatellite analysis. In most of the previous studies, we and others used 13–20 highly informative microsatellite markers in an effort to identify several genetic changes potentially present in the majority of bladder cancers. Using the HuSNP chip, we obtained information on 312 loci across all chromosomes. It is, therefore, reasonable that this assay picked up AIs from all of the cancer cases examined. Thus, array-based technology may be more thorough and more informative in detecting bladder cancer. A more limited number of SNPs can also be theoretically incorporated into a bladder chip that targets commonly LOH chromosomal regions. Unlike microsatellite analysis, however, the HuSNP chips cannot detect microsatellite instability.

AIs were not detected in the urine from nine healthy subjects and four of five patients with symptoms of urinary tract disease but without evidence of neoplasia. In one patient without cancer, we found AIs on several chromosomal arms by the HuSNP assay, still consistent with occasional “false positive” findings by others (28) using microsatellite analysis. Studies detecting LOH or microsatellite instability in chronic inflammatory diseases that may predispose to cancer [e.g., pancreatitis (29) and colitis (30), pulmonary sarcoidosis (6)] suggest that chronic inflammatory conditions are occasionally accompanied by microsatellite alterations similar to those found in tumors. Chronic inflammation may also intensify oxidative stress, producing enough DNA damage to exceed the capacity of DNA-repair mechanisms (30) or may itself produce microsatellite changes, as suggested by Jackson et al. (31). Whether these clonal changes are present in activated lymphocytes or other cellular components is not known. It is also possible that a small fraction of false positive urine samples may contain a small tumor clone that was not yet detected by cystoscopy or cytology. To improve the assay, SNPs prone to documented false positive readings could also be eliminated.

We and others have shown that LOH/microsatellite instability analysis at urine DNA is superior to urinary cytology, and in some patients, this approach can predict recurrences earlier than urethro-
cystoscopy, the clinical gold standard of bladder cancer detection (15, 26). HuSNP analysis may be more sensitive than microsatellites because a small proportion of cells harboring molecular changes may fall below the threshold of detection by microsatellite analysis but

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*American Joint Committee on Cancer Staging: p, pathologic stage; T, tumor size; N, node status; M, metastatic status.
†ND, not done; SM, suspicious for malignancy; ID, indeterminate.
‡Imaging, radiologic evaluation.
§INS, in situ.

Table 2 AI in relationship with disease stages

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within the range of the HuSNP assay. There may be more variation in determining peak ratios by microsatellites than the more linear analysis based on oligomer hybridization for the chips. Moreover, molecular assays, in general, may detect tumor not visible at cystoscopy because flat lesions, such as carcinoma in situ, exfoliate easily, yet remain undetected by the urologist (32).

We have shown that the array-based HuSNP chip assay on urine sediment provides a simple, noninvasive method to diagnose primary bladder cancers with high sensitivity and specificity. Because the total number of bladder cancer samples and controls tested by all molecular methods is still small, additional prospective studies using a large number of patients and more representative control subjects will be required to determine the true use of this assay. We believe that additional improvement of this methodology can lead to a significant increase in the sensitivity and reliability of DNA testing using urine.

Several potential applications of this technology are similar to those expected from the analysis of plasma DNA (33, 34), including the detection and monitoring of tumor growth and the evaluation of the effectiveness of tumor chemotherapy or radiation therapy. Botezatu et al. (35) and Serydьuk et al. (36) detected tumor DNA from noninvasive malignancies in the urine of cancer patients (pancreatic and colorectal cancer), thus demonstrating that at least small amounts of short tumor DNA sequences might bypass the kidney barrier and be secreted into urine. Although present in a much smaller copy number per cell than mRNA, DNA has better stability than mRNA (37). If confirmed in larger patient groups, urinalysis for tumor DNA might become a valuable tool for virtually every cancer entity with known DNA alterations.

We have, thus, demonstrated that the HuSNP assay on urinary DNA is a promising diagnostic test for bladder cancer. The test detected bladder cancers of all stages and grades, including those often missed by urine cytology. This simple and noninvasive method for detecting genito-urinary tract malignancy is readily automated and has many potential clinical applications, including primary diagnosis, monitoring for relapse, and measurement of therapeutic response.

Acknowledgments

We thank Mary Ellen Haisfeld-Wolfe for collecting the necessary samples.

References

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