Identification and validation of methylated PENK gene for early detection of bladder cancer using urine DNA

Tae Jeong Oh¹, Eunkyung Lim¹, Bo-Ram Bang², Justin Junguek Lee², Yong Gil Na³, Ju Hyun Shin³, Jae Sung Lim³, Ki Hak Song³ and Sungwhan An¹,²*

Abstract

Background: Early detection of bladder cancer (BCa) offers patients a favorable outcome and avoids the need for cystectomy. Development of an accurate and sensitive noninvasive BCa diagnostic test is imperative. DNA methylation is an early epigenetic event in the development of BCa. Certain specific aberrant methylations could serve as useful biomarkers. The aim of this study was to identify methylation biomarkers for early detection of BCa.

Methods: CpG methylation microarray analysis was conducted on primary tumors with varying stages (T1—T4) and paired nontumor tissues from nine BCa patients. Bisulfite-pyrosequencing was performed to confirm the methylation status of candidate genes in tissues and urine sediments (n = 51). Among them, PENK was selected as a potential candidate and validated using an independent set of 169 urine sediments (55 BCa, 25 benign urologic diseases, 8 other urologic cancers, and 81 healthy controls) with a quantitative methylation-specific real time PCR (mePENK-qMSP). All statistical analyses were performed using MedCalc software version 9.3.2.0.

Results: CpG methylation microarray analysis and stepwise validation by bisulfite-pyrosequencing for tissues and urine sediments supported aberrant methylation sites of the PENK gene as potential biomarkers for early detection of BCa. Clinical validation of the mePENK-qMSP test using urine sediment-DNA showed a sensitivity of 86.5% (95% CI: 71.2 – 95.5%), a specificity of 92.5% (95% CI: 85.7 – 96.7%), and an area under ROC of 0.920 (95% CI: 0.863 – 0.959) in detecting Ta high-grade and advanced tumor stages (T1-T4) of BCa patients. Sensitivities for Ta low-grade, Ta high-grade, T1 and T2-T4 were 55.6, 83.3, 88.5, and 100%, respectively. Methylation status of PENK was not correlated with sex, age or stage, while it was associated with the tumor grade of BCa.

Conclusions: In this study, we analyzed the comprehensive patterns of DNA methylation identified that PENK methylation possesses a high potential as a biomarker for urine-based early detection of BCa. Validation of PENK methylation confirms that it could significantly improve the noninvasive detection of BCa.

Keywords: Bladder cancer, Methylation, Noninvasive, PENK, Urine sediment

Background

Bladder cancer (BCa) is the 5th most commonly occurring cancer, with approximately 550,000 new cases and 200,000 deaths globally in 2018 [1, 2]. It is one of the cancers with the highest lifetime cost because it shows a high rate of recurrence and hence requires continuous invasive monitoring such as cystoscopy [3].

© The Author(s) 2022. Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.
Cystoscopy is a gold standard for the diagnosis of primary tumor or recurrent urothelial carcinoma of the bladder. However, it is an invasive and costly procedure with low compliance [4, 5]. In addition, cystoscopy requires an experienced operator for thorough inspection and accurate diagnosis [6]. Although urine cytology is noninvasive and has high specificity for detection of BCa, its sensitivity is known to be as low as 20 to 50%, especially for low-grade tumors [7].

Numerous potential markers for detecting BCa have been described or are under investigation, and yet only a few have been validated to be clinically useful. The FDA has approved BTA STAT, BTA TRAK, NMP22/BladderChek, and UroVysion for diagnosis and follow-up while Immunocyt/uCyt is approved by the FDA only for follow-up [8, 9]. Among these, only UroVysion is a frequently used tool because it has been shown to be more sensitive than cytology. However, it is notable that the advantage of UroVysion over cytology in terms of sensitivity was largely lost when Ta samples from non-muscle invasive BCa were not included in the analysis [10, 11]. Individual markers have been shown to have insufficient diagnostic power presumably due to false-positive results, thereby decreasing the specificity of these markers. Therefore, the development of highly accurate and noninvasive methods using molecular biomarkers is crucial for early detection of BCa.

Epigenetic alterations are major mechanisms that can inactivate tumor suppressor genes and other cancer-associated genes in various cancers [12, 13]. Aberrant DNA methylation has been recognized as one of the most common molecular alterations in BCa [6, 14]. Detecting DNA hypermethylation in specific genomic regions of urine DNA has been shown to have high potential as a noninvasive diagnostic tool for early detection and surveillance of BCa [2, 15].

Various genome-wide strategies have been used to identify genes that undergo hypermethylation in BCa. Several urine-based epigenetic DNA markers have been shown to have potential for detecting BCa [14, 16–20]. In the present study, we conducted CpG microarray analysis to investigate differentially methylated sites of the genes in primary tumors and paired adjacent nontumor tissues of BCa. Step-wise validation procedures identified the methylation sites of the PENK gene as promising methylation biomarkers for the detection of BCa. Here, we report that PENK methylation assessment by methylation-specific real time PCR [21] can be used as a useful diagnostic tool for detection of BCa.

### Methods

#### Clinical specimens

Fresh-frozen primary tumors and paired adjacent non-tumor tissues from nine BCa patients (Stage T1, n = 5; T2, n = 1; T3, n = 1; T4, n = 2) were collected at the time of surgery. All frozen tissue specimens were obtained from the Chungnam National University Hospital. Each tumor specimen was histologically verified by a board-certified pathologist and archived for further DNA study. A total of 51 voided urine samples, used for bisulfite-pyrosequencing verification, were freshly obtained from BCa patients with varying stages (n = 16), patients with benign urologic diseases (BUD) such as trigonitis, urinary stone, and benign prostate hyperplasia (n = 23), and healthy individuals (n = 12). In the clinical validation for the urine DNA-based methylation test, an independent set of fresh voided urine samples were obtained from patients with BCa (n = 55) at various stages (Ta – T4), patients with BUD (n = 25), and normal healthy subjects (n = 81) as shown in Table 1. Additionally, urine samples

### Table 1  Clinicopathological features of subjects enrolled in this study

| Characteristics | Tissues | Urine samples |
|-----------------|---------|---------------|
| **Healthy control** | -       | 93            |
| Sex – no. (%)   | Male    | 60 (64.5)     |
|                 | Female  | 33 (35.5)     |
| Age, mean (range) | -       | 53.8 (26–85)  |
| **BUD** | -       | 48*           |
| Sex – no. (%)   | Male    | 29 (60.4)     |
|                 | Female  | 19 (39.6)     |
| Age, mean (range) | -       | 52.5 (34 – 83) |
| **Bladder cancer (BCa)** | 9       | 71            |
| Sex – no. (%)   | Male    | 7 (77.8)      |
|                 | Female  | 2 (22.2)      |
| Age, mean (range) | 74.3 (62—81) | 68.8 (33 – 85) |
| **Pathological stage – no. (%)** | -       | 28 (39.4)     |
| Ta              | 5 (55.6) | 35 (49.3)     |
| T1              | 1 (11.1) | 4 (5.6)       |
| T2              | 1 (11.1) | 2 (2.8)       |
| T3              | 2 (22.2) | 2 (2.8)       |
| T4              | 4 (44.4) | 35 (49.3)     |
| **Differentiation grade – no. (%)** | -       | 3 (4.2)       |
| Low             | 5 (55.6) | 33 (46.5)     |
| High            | -       |               |
| Unknown         | -       |               |

*Benign urologic diseases (BUD) included trigonitis, urinary stone, and benign prostate hyperplasia
from patients with kidney ($n=6$) or prostate cancers ($n=2$) were also included. All voided urine samples from BCa patients were collected before definitive surgery. Normal healthy control samples were obtained from individuals without any history of genitourinary malignancy. Voided urine samples ($40$ mL each) were collected into $50$ mL tubes containing preservative buffer (Genomictree, Inc. Daejeon, South Korea), and were then centrifuged at $3000 \times g$ for $10$ min. The pelleted urine sediment was stored at $-20 \, ^\circ C$ until DNA extraction. This study was approved by the Institutional Review Board of ChungNam National University Hospital, Daejeon, South Korea. Written informed consent was obtained from all study participants. This study adhered to local ethics guidelines.

**CpG methylation microarray analysis**

CpG methylation microarray analyses were performed using genomic DNA isolated from primary tumors and paired adjacent nontumor tissues from nine BCa patients with different stages ($T1, n=5; T2, n=1; T3, n=1$; and $T4, n=2$). CpG methylation microarray analysis was conducted as described previously [22] using human $244 \, k$ CpG island microarrays containing $237,000$ oligonucleotide probes covering $27,800$ annotated CpG islands (Agilent Technologies, CA, USA) according to the manufacturer’s instructions. Raw methylation microarray data were submitted to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) with accession number GSE171369.

Methylation microarray data were analyzed using the Agilent Feature Extraction software version 9.3.2.1 and a Genespring software version 7.3.1 (Agilent, CA, USA). To determine differentially methylated targets between primary tumor and paired adjacent nontumor tissue samples, statistical analysis was performed using a parametric analysis of variance test with Benjamini and Hochberg multiple testing correction ($P<0.01$), followed by fold change analysis. Next, multiple-probe enriched genes were further selected as methylation candidate genes if their probes yielded a positive call for methylation in the bladder primary tumor compared to non-cancerous tissues with at least two probes.

**DNA extraction and bisulfite treatment**

Genomic DNA from tissue specimens were extracted using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany). Genomic DNA from urine sediments were isolated using a GT NUCLEIC ACID PREP kit (Genomictree, Inc., Daejeon, South Korea) according to the manufacturer’s instructions.

For methylation assessment, purified genomic DNA were first bisulfite-treated to convert unmethylated cytosine nucleotides into thymidine without changing methylated cytosines using an EZ DNA Methylation-Gold kit (Zymo Research, CA, USA) according to the manufacturer’s instructions. Briefly, DNA was chemically modified with sodium bisulfite at $64 \, ^\circ C$ in the dark for $2.5$ h and then, the bisulfite-modified DNA was purified and eluted in $10 \, \mu L$ of distilled water. Eluted DNA was either immediately used for methylation analysis or stored at $-20 \, ^\circ C$ until the analysis.

**Methylation assessment by bisulfite-pyrosequencing**

To assess methylation status of candidate genes, bisulfite-pyrosequencing was performed as previously described [23]. Bisulfite PCR and pyrosequencing primers were designed to amplify $3$ to $5$ CpG dinucleotides sites in target regions of genes using a PSQ Assay Design software (Qiagen, Hilden, Germany). Sequences of primers used in pyrosequencing are listed in Table 2. These primers were synthesized by Bioneer (Daejeon, South Korea). Genomic DNA was modified by sodium bisulfite using an EZ DNA Methylation-Gold kit (Zymo Research, CA, USA) according to the manufacturer’s instructions. Briefly, $20$ ng of bisulfite-treated DNA was amplified in a $25 \, \mu L$ reaction with primer set and Taq polymerase (Enzymomics, Daejeon, South Korea). PCR amplification was run for $40$ cycles with an optimal annealing temperature.

Pyrosequencing was performed using a PyroGold kit and a PyroMark ID Q96 instrument (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Methylation index ($MtI$) of each gene in each sample was calculated as the average value of $mC/(mC+C)$ for all examined CpGs in target regions. All pyrosequencing reactions included samples without any DNA template as negative controls. Methylation-positive was considered if MtI of primary tumor was greater than that of the corresponding nontumor tissue.

**PENK methylation assessment in urine DNA by real time PCR**

To measure PENK methylation quantitatively in DNA of urine sediment, total genomic DNA obtained from the sediments of voided urine was used for bisulfite treatment. All purified bisulfite-treated DNA was subsequently subjected to real time PCR-based methylation assessment for PENK (named as mePENK-qMSP). Primers and probes were designed to amplify the target region of PENK covering CpG targets ($72$ bp; $+524$ to $+595$ bp) and were synthesized by Integrated DNA Technologies (IDT) (IA, USA). mePENK-qMSP assay have been established with a modified protocol based on the report described by Eads et al. [21] in which the fluorescence-based qMSP quantitated the original methylation level of the interested gene locus using the bisulfite-converted sequences-specific primers and probes. Region of
COL2A1 DNA having no CpG sites was used for methylation-independent amplification as a control to determine the presence of bisulfite-treated DNA [24].

PCR reaction mixture contained 5× AptaTaq PCR master mix (Roche Diagnostics, Mannheim, Germany), PENK methylation-specific primers and probes, and COL2A1-specific primers and probes (Table 2). mePENK-qMSP assay was performed on a 7500 Fast System Real-Time PCR (Thermo Fisher Scientific, MA, USA). Real time PCR was performed with the following thermal cycling conditions: activation at 95 °C for 5 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Heating and cooling rates were set to ≥4 °C per sec and ≥3.5 °C per sec, respectively. For each experiment, BCa cell (RT4) genomic DNA containing fully methylated PENK gene and whole genome amplified genomic DNA of human lymphocyte containing unmethylated PENK gene [25] were used as controls to validate mePENK-qMSP adequacy of each sample batch. Non-template controls were also included for each experiment to detect cross-contamination. Cycle threshold (Ct) values were analyzed using the 7500 software (Thermo Fisher Scientific, MA, USA). For urine DNA testing, Ct values for each experimental set were determined using a manually configured cutoff value. This cutoff value was established using the unmethylated DNA fluorescence level as a baseline. mePENK-qMSP was performed at one time for each sample. The relative level of methylated PENK gene in each sample was determined as 40-△Ct [Ct of amplified PENK gene – Ct of COL2A1 (reference gene)] [26]. Higher values of 40-△Ct represented higher levels of PENK methylation. If Ct of PENK was undetected, the value was considered to be 25, the nearest value to the lowest of 40-△Ct for test results of all samples.

### Table 2 Primer sequences for pyrosequencing and mePENK-qMSP

| Pyrosequencing | Sequences (5’ → 3’)* | Amplicon Size (bp) |
|----------------|----------------------|--------------------|
| Gene           |                      |                    |
| CDX2           | F: TGGTGTGTTGTTTTTGTGTTATTAATAG |
|                | R: Biotin-CACCTCCTCCACCTCTAACTAATA |
|                | S: ATTATAGAGTTTGGTAAATAT |
|                | R: Biotin-AAATTTTCAATCCACTCTCTAT |
|                | S: TGTAACTGTTTATACTGTATTTAAAT |
| CEI            | F: TGGAAATGTAAGTGAATTTTGAATGTAT |
|                | R: Biotin-AAATTTTCAATCCACTCTCTAT |
|                | S: TGTAACTGTTTATACTGTATTTAAAT |
| DMC1           | F: GAGGGGGGTAGGTTGTTAAAA |
|                | R: Biotin-TCCCTCAAATCTCTAAATAT |
|                | S: GGGGTAGTTTGTATATTTTAATAG |
| IMP-1          | F: GATATTTTTAGTAAAGAG |
|                | R: Biotin-CCACTTAAACACTCTACTC |
|                | S: GGATTTTATATATGTTAGTG |
| PDE3A          | F: TGGGAATTTAGTGAAGAG |
|                | R: Biotin-CCACTTAAACACTCTACTC |
|                | S: GGATTTTATATATGTTAGTG |
| PENK           | F: ATATTTTTATGTTGTTTTTATAAG |
|                | R: Biotin-AAATTTTCAATCCACTCTCTAT |
|                | S: TGTAACTGTTTATACTGTATTTAAAT |
| SIM2           | F: Biotin-GTGGATTAGATTAGTTGGTTT |
|                | R: CACCCTCCCCAAATCTTT |
|                | S: CCTCCCCAAATCTCT |
| VSX1           | F: GGAGTGGGTTAGGAGAGATTT |
|                | R: Biotin-AGTAGTTGTTATGGAGGGAGT |
|                | S: TTTTTGAAATGTGTTGAGT |
| ZNF312         | F: AAAAGGGATTGTTGGAGGAGAA |
|                | R: Biotin-TCTCTATACAAAAACACAAAAATAC |
|                | S: GATTTGAGAGAGAGAG |

| mePENK-qMSP | Sequences (5’ → 3’)* | Concentration |
|-------------|----------------------|---------------|
| Gene        |                      |               |
| COL2A1      | F: GTAATTTAGATGGATTTGTTGTTAATAG |
|             | R: CTACCCCCAAAAAACACTCCATA |
|             | P: Cy5-AGAGAGGGGGGGTTAGGTAGGAGG |
| PENK        | F: TCGGGTTTTTATGTTGTTGGTCGC |
|             | R: AGAGACCAATCGGCTCAGG |
|             | P: Fam-TGGGGGCGATGCGCGCTTTCGG |

* F, R, S, and P represent forward, reverse PCR primers, sequencing primers, and PCR probe, respectively. Biotin, Cy5 or Fam indicates 5′ biotinylation, 5′Cy5 conjugation, and 5′Fam conjugation, respectively.
Statistical analysis
All statistical analyses were performed using MedCalc software, version 9.3.2.0 (Basel, Belgium). A P value of less than 0.05 was considered statistically significant. Receiver operating characteristic (ROC), area under ROC (AUC), and 95% confidence intervals (CI) were calculated to confirm the accuracy of diagnosis, sensitivity, and specificity. Samples were categorized as negative or positive based on the cutoff value determined by the ROC curve analysis of the assay results.

Results
Identification and confirmation of methylation candidate genes for BCa detection
To identify a subset of candidate genes differentially hypermethylated in BCa, methylation profiles were compared between primary bladder tumors and paired adjacent nontumor tissues using CpG microarray analysis (Additional file 1: Figure S1). Statistical analysis and fold change analysis identified nine top-ranking hypermethylated candidate genes (CDX2, CEI, DMC1, IMP-1, PDE3A, PENK, SIM2, VSX1, and ZNF312) in primary bladder tumors. To confirm microarray results, bisulfite-pyrosequencing was subsequently performed for tissues used in microarray. Results revealed that all nine candidate genes were significantly hypermethylated in most primary tumors (P < 0.05) (Fig. 1). Among them, three genes (DMC1, PENK, and SIM2) were selected for further independent validation with additional normal tissues and BCa tissues because the genes showed higher methylation levels in all cancer tissues than in normal tissues. It was confirmed that methylation levels of three genes were significantly higher in most tumor tissues than in normal bladder tissues (P < 0.05, Kruskal Wallis test) (Additional file 2: Figure S2).

Methylation status of DMC1, PENK, and SIM2 genes in urine sediments by bisulfite-pyrosequencing
To evaluate the possibility of using methylated genes DMC1, PENK, and SIM2 with urine-based DNA test for clinical application, bisulfite-pyrosequencing was performed on urine sediment DNA from patients with BCa (n = 16), patients with BUD (n = 23), or healthy subjects (n = 12) (Fig. 2). The overall MtIs of genes DMC1, PENK, and SIM2 in healthy controls were as low as 10.7 ± 9.0, 6.4 ± 5.4, and 7.5 ± 3.7, respectively. However, the overall MtIs of genes DMC1, PENK, and SIM2 in BCa patients were significantly elevated at 33.6 ± 25.4, 51.0 ± 23.2, and 45.4 ± 21.6, respectively (P < 0.05). The overall MtIs of genes DMC1, PENK, and SIM2 in urine samples from BUD patients were 20.9 ± 13.6, 13.3 ± 9.9, and 22.6 ± 14.2, respectively.

The overall MtIs of the DMC1 gene in BCa patients were significantly higher than those in healthy individuals (P < 0.05), but not significantly higher than those in BUD patients (P = 0.171). The overall MtIs of PENK and SIM2 genes were significantly higher across urine samples from BCa patients than those from patients with BUD or normal healthy subjects (P < 0.01) (Fig. 2). We focused on the PENK gene to pursue further clinical validation for urine-DNA test by methylation-specific real time PCR because it exhibited the best sensitivity and specificity.
for differentiating patients with BCa from control group such as patients with BUDs and healthy subjects (Additional file 3: Figure S3).

**Clinical validation of PENK methylation for detecting BCa in urine sediments by quantitative methylation-specific real time PCR**

To assess PENK methylation precisely, we established and optimized PENK methylation-specific real time PCR, mePENK-qMSP assay and tested the sensitivity and specificity of PENK methylation with urine DNA from 55 patients with BCa at various stages (Ta – T4), 25 patients with BUD, and 81 healthy individuals.

Results of mePENK-qMSP analysis showed that levels of PENK methylation in urine DNA from BCa patients were significantly higher than in urine DNA from controls composed of patients with BUD and normal healthy subjects ($P < 0.01$, Kruskal–Wallis test) (Fig. 3A). Next, we evaluated the clinical performance of mePENK-qMSP for differentiating Ta high-grade and advanced tumor stages (T1-T4) of BCa from controls (patients with BUD and healthy normal subjects) by constructing an ROC curve. Given an optimal cutoff value at 31.35 of 40-$\Delta$C_T, the AUC was 0.920 (95% CI: 0.863 – 0.959, $P < 0.001$) and the overall sensitivity for detecting BCa at all stages (Ta through T4) was 76.4% (95% CI: 63.0 – 86.8%) with a specificity of 92.5% (95% CI: 85.7 – 96.7%). Sensitivities

![Fig. 2](image-url) **Fig. 2** Methylation assessment of three genes in DNA from urine sediments by bisulfite-pyrosequencing. MtIs of samples are presented by box and whisker plots. Differences in MtI are statistically analyzed between BCa patients, BUD patients, and normal healthy subjects (N). *, $P < 0.05$ and **, $P < 0.01$ analyzed by Kruskal–Wallis test.

![Fig. 3](image-url) **Fig. 3** Methylation status of PENK in urine sediments by mePENK-qMSP. A mePENK-qMSP using an independent set of voided urine samples from BCa patients, BUD patients, and normal healthy subjects (N). Distribution of PENK methylation was expressed as 40-$\Delta$C_T value for each sample. A higher 40-$\Delta$C_T indicates a higher methylated level of PENK. Methylation status of PENK is plotted as box and whisker plots. TaLG: Ta Low-Grade; TaHG: Ta High-Grade; O.C.: Other urologic cancers. **, $P < 0.01$ analyzed by Kruskal–Wallis test. B ROC plots of PENK methylation for detecting TaHG and advanced tumor stages of BCa from BUD patients and normal healthy subjects. Cutoff value for methylation-positive, AUC, and $P$ value are indicated in the box.
for Ta low-grade, Ta high-grade, T1 and T2-T4 were 55.6, 83.3, 88.5, and 100%, respectively. Sensitivity for detecting Ta high-grade and advanced stages of BCa patients was 86.5% (95% CI: 71.2 – 95.5%) (Fig. 3B). PENVK methylation was not correlated with sex, age or stage (all P > 0.05, Fisher’s exact test). However, it was associated with tumor grade (P = 0.008, Fisher’s exact test) (Table 3). PENVK methylation was not detected in all samples of other cancers including all renal cancer patients (n = 6) and prostate cancer patients (n = 2).

Discussion
Aberrant DNA methylation of some genes is known to be an early event in tumorigenesis. Specific methylation sites have been considered as potential biomarkers for early detection of cancer [12, 13, 27]. While several urine- marker tests such as NMP22, Immunocyt, BTA stat, and UroVysion have been approved by US FDA, most assays have not been proven to have sufficient sensitivity and/or specificity to be utilized in clinical practice [8]. Some other studies have reported that multiple genetic and epigenetic biomarkers [5, 28–30]-based tests have been evaluated for detecting BCa but they showed various range of sensitivities of 41.6 to 92.0% and specificities of 73.0 to 91.0%.

Here, we identified candidates of methylation biomarkers such as DMC1, PENK, and SIM2 for BCa through comprehensive DNA methylation profiling analysis searching for differential methylation sites based on CpG microarrays and evaluation by pyrosequencing using clinical specimens. Based on a ROC curve and diagnostic model of BCa, we then selected PENK methylation as a biomarker for further clinical validation for the detection of BCa since PENK itself exhibited a highest sensitivity and specificity.

To assess the clinical performance of mePENVK-qMSP for early detection of BCa, patients with varying stages of BCa (Ta – T4) were included in this study. The mePENVK-qMSP test showed an overall sensitivity of 76.4% and a specificity of 92.5% in detecting BCa and the test had sensitivity of 86.5%, with high specificity of 92.5% when patients with Ta low-grade are not included in the analysis.

Since high-grade of T1 BCa and advanced stages are aggressive and have poor prognosis, early detection of high-grade T1 in BCa patients is crucial for decreasing morbidity and mortality [28, 31]. The mePENVK-qMSP test in this study showed a good sensitivity of 89.5% (17/19) for high-grade T1 BCa while the sensitivity for low-grade Ta patients only was as low as 55.6% (10/18). The low sensitivity could be attributed to the cohesive nature of low-grade tumor cells, which may decrease the number of exfoliated cells in the urine [7].

In order to increase the sensitivity of the test, it is necessary also to improve the analytical sensitivity of the detection method to measure accurately trace amounts of PENK methylation in urine-DNA. Additionally, DNA integrity is greatly affected by the collection protocol and storage conditions of urine samples prior to downstream procedures for DNA methylation analysis [32]. Therefore, developing a better preservation buffer and optimizing urine collection procedures can enhance the assay sensitivity for methylated PENK DNA when detecting BCa in urine sediment.

We previously filed a patent application related to the detection of biomarkers for methylation in urine DNA such as PENK methylation which can be used for BCa diagnosis non-invasively. At that time, Chung et al. [26] had published clinical studies evaluating the clinical validity of PENK methylation in detection of BCa using urine sedimentation as PENK methylation had 81.3% sensitivity and 79.1% specificity for detection of BCa. In comparison to this study, our findings showed comparable sensitivity and higher specificity. Zhang et al. [33] also recently reported detecting BCa in urine samples by using PENK methylation combined with 6 additional methylation markers. In that study, however, the clinical performance of PENK methylation itself was not assessed. Despite this, taken together with previous studies indicate that PENK methylation has potential for the use of molecular biomarker in detection of BCa non-invasively.

### Table 3 The relationship between clinicopathological parameters and PENK methylation in urine sediments

| Parameters | No. of total samples | No. of PENK methylation positive (%) |
|------------|----------------------|-------------------------------------|
| Sex        |                      |                                     |
| Male       | 43                   | 32 (74.4)                           |
| Female     | 12                   | 10 (83.3)                           |
| P valuea   | 0.709                |                                     |
| Age        |                      |                                     |
| < 65       | 19                   | 12 (63.2)                           |
| ≥ 65       | 36                   | 30 (83.3)                           |
| P valuea   | 0.109                |                                     |
| Stage      |                      |                                     |
| Ta, T1     | 50                   | 37 (74.0)                           |
| T2 -T4     | 5                    | 5 (100)                             |
| P valuea   | 0.324                |                                     |
| Grade      |                      |                                     |
| Low        | 26                   | 16 (61.5)                           |
| High       | 28                   | 26 (92.9)                           |
| P valuea   | 0.008                |                                     |

* P value was calculated by Fisher’s exact test
Studies have shown that PENK hypermethylation is also associated with other cancers including hepatocellular carcinoma, colorectal cancer, and prostate cancer [34–36]. Therefore, we assessed whether PENK methylation was detectable in urine samples from other urologic cancers (two prostate cancer and six kidney cancer patients). However, we did not find aberrant PENK methylation in prostate cancer patients (0/2) or renal cancer patients (0/6). This indicates that PENK methylation in urine is highly specific for BCa.

The PENK gene encodes met-enkephalin (MENK), known as an opiate growth factor (OGF), has been reported in brain and prostate tumors. And it was previously reported as a tonically active inhibitory factor that can interact with opioid growth factor receptors [37]. These reports may support, PENK as a tumor suppressor gene in several human tumors, including pancreatic cancer. In addition, MENK is required, in part, for apoptosis induction through transcriptional repression of NF-kB- and p53-regulated genes [38, 39].

This study has several limitations. First, patients with BCa (mean age: 69.1 years) were older than those with BUD and healthy subjects (mean age: 56.7 years) ($P < 0.001$, Kruskal–Wallis test). In addition, numbers of female BCa patients and other urological samples were small, leading to insufficient statistical power. In this study, PENK methylation was identified as a potential molecular biomarker for non-invasive diagnosis of bladder cancer. Because of limitations including the small number of samples, male-to-female ratio, and age-matching between BCa and non-BCa groups, it is not possible to draw any definitive conclusions at this time. Consequently, well-designed, large-scale clinical studies are required in order to determine whether this biomarker test using urine specimens is fully specific and accurate in detecting bladder cancer in clinical practice.

Conclusions

We identified a specific aberrant PENK methylation in BCa through CpG microarray analysis and stepwise filtering procedures. This study showed that BCa can be detected noninvasively using a real-time PCR-based PENK methylation assay based on urinary DNA. However, a large scale prospective clinical trial utilizing the PENK methylation test for urine will need to be conducted before this method can be employed in clinical practice.

Abbreviations

BCa: Bladder cancer; BUD: Benign urologic disease; qMSP: Quantitative methylation-specific PCR; MeDIA: Methylated DNA Isolation Assay; Mti: Methylation index; PENK: Proenkephalin; ROC: Receiver operating characteristic; AUC: Area under ROC.

Acknowledgements

We thank staff members at Genomictree, Inc. for their technical support. We also thank ChungNam National University Hospital for help in urine collection.

Authors’ contributions

Study concept and design: SA. Acquisition, analysis and interpretation of data: EL. Analysis and interpretation of the patient data: JHS, YGN, JSL, KHS. Drafting of the manuscript: TJO. Critical revision of the manuscript for important intellectual content: SA, BRB, JSL. Statistical analysis: TJO. Administrative, technical, or material support: TJO, EL. Study supervision: SA. All authors read and approved the final manuscript.

Funding

This work was supported by a grant (Project No. 10040174) funded by the Ministry of Trade, Industry and Energy, South Korea. The funding body had no role in the study design, data collection, analysis and interpretation of these data and in writing the manuscript.

Availability of data and materials

Raw methylation microarray data were submitted to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) with accession number GSE171369.

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Chungnam National University Hospital (IRB No. CNUH 2011–10–015–003). Written informed consent was obtained from all of participants, and the study adhered to local ethics guidelines.

Consent for publication

Not applicable.

Competing interests

TaeJeong Oh, Eunkyoung Lim, and Sungwhan An are employees of Genomic-tree, Inc. TaeJeong Oh and Sungwhan An are shareholders of Genomic-tree, Inc. Bo-Ram Bang, Justin Junguek Lee and Sungwhan An are employees of Promis Diagnostics. Justin Junguek Lee and Sungwhan An are shareholder of Promis Diagnostics. Other authors have no conflicts of interest to disclose.
References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394–424.

2. Charpentier M, Gutierrez C, Guillaudeux T, Verhoest G, Pedrez R. Noninvasive urine-based tests to diagnose or detect recurrence of bladder cancer. Cancers. 2021;13:1650.

3. Severt KD, Arnell B, Nagele U, Schilling D, Bedike J, Horstmann M, et al. Economic aspects of bladder cancer: what are the benefits and costs? World J Urol. 2009;27(3):295–300.

4. Peter EC, Philippe ES, Neeraj A, Rick B, Stephen AB, Mark KB, et al. NCCN guidelines insights: bladder cancer, version 2. 2016. J Natl Comp Cancer Netw. 2016;14(10):1213–24.

5. Ruan W, Chen X, Huang M, Wang H, Chen, J, Liang Z, et al. A urine-based DNA methylation assay to facilitate early detection and risk stratification of bladder cancer. Clin Epigenet. 2021;13:91.

6. Mieremani J, Kyprianou N. The promise of novel molecular markers in bladder cancer. Int J Mol Sci. 2014;15(15):23287–908.

7. Sullivan PS, Chan JY, Levin MR, Rao J. Urine cytology and adjunct markers for detection and surveillance of bladder cancer. Am J Transl Res. 2010;2(4):412–40.

8. Soria F, Droller MJ, Lotan Y, Gontero P, D'Andrea D, Gust KM, et al. Comprehensive genome methylation analysis in bladder cancer; identification and validation of novel methylated genes and application of these as a urine based FGFR3 mutation assay. J Urol. 2011;186:707–12.

9. Nishiyama N, Arai E, Chihara Y, Fujimoto H, Hosoda F, Shibata T, Kondo T, et al. Identification and validation of the methylated loci by the pyrosequencing technology. Methods Mol Biol. 2008;367:1–42.

10. Hughes S, Jones I. The use of multiple displacement amplified DNA as a control for methylation specific PCR. pyrosequencing, bisulfite sequencing and methylation-sensitive restriction enzyme PCR. BMC Mol Biol. 2007;8:91.

11. Hajdijak T. UroVysion FISH test for detecting urothelial cancers: meta-analysis after real-time-methylation specific PCR (SMART-MSP): high-throughput and probe-free quantitative DNA methylation detection. Nucleic Acid Res. 2008;36(7):e42.

12. Chen X, Zhang J, Ruan W, Huang M, Wang C, Wang H, et al. Urine DNA methylation assay enables early detection and recurrence monitoring for bladder cancer. J Clin Invest. 2020;130(2):678–89.

13. Zhang N, Chen S, Wu L, Wu Y, Jiang G, Shao J, et al. Identification of cancer-specific gene mutation of combination for the diagnosis of bladder cancer. J Cancer. 2019;10(26):6761–6.

14. Shiomi H, Iwai A, Kunitake T, Nishimura T, et al. Comprehensive genome methylation analysis in bladder cancer; identification and validation of novel methylated genes and application of these as urine tumor markers. Clin Cancer Res. 2011;17(7):5582–92.

15. Hermann T, Savio A, Olikow-Mitsel E, Mari A, Wettstein MS, Saba K, et al. A noninvasive urine-based methylation biomarker panel to detect bladder cancer and discriminate cancer grade. Urol Oncol. 2020;38(6):e603–e603.

16. Nishiyama N, Arai E, Chihara Y, Fujimoto H, Hosoda F, Shibata T, Kondo T, et al. Comprehensive genome methylation analysis in bladder cancer; identification and validation of novel methylated genes and application of these as urine tumor markers. Clin Cancer Res. 2011;17(7):5582–92.

17. Hermann T, Savio A, Olikow-Mitsel E, Mari A, Wettstein MS, Saba K, et al. A noninvasive urine-based methylation biomarker panel to detect bladder cancer and discriminate cancer grade. Urol Oncol. 2020;38(6):e603–e603.

18. Nishiyama N, Arai E, Chihara Y, Fujimoto H, Hosoda F, Shibata T, Kondo T, et al. Comprehensive genome methylation analysis in bladder cancer; identification and validation of novel methylated genes and application of these as urine tumor markers. Clin Cancer Res. 2011;17(7):5582–92.

19. Hermann T, Savio A, Olikow-Mitsel E, Mari A, Wettstein MS, Saba K, et al. A noninvasive urine-based methylation biomarker panel to detect bladder cancer and discriminate cancer grade. Urol Oncol. 2020;38(6):e603–e603.

20. Beukers W, Kandimalla R, van Houwelingen D, Kovacic H, Chin JF, Lingsma HF, Dijkstra J, Zwarthoff EC. The use of molecular analyses in voided urine for the assessment of patients with hematuria. PLoS ONE. 2013;8(10):e77657.

21. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, Danenberg PV, Laird PW. MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res. 2000;28:E32.

22. Moon YH, Oh T, Kim NY, Kim MS, An S. Methylated DNA isolation assay-mediated DNA methylation detection and whole-genome methylation profiling. Am Biotechnol Lab. 2009;27(10):23–5.

23. Dejeux E, El abdalaoui H, Gut IG, Tost J. Identification and quantification of differentially methylated loci by the pyrosequencing technology. Methods Mol Biol. 2009;507:189–205.

24. Kristensen LS, Mikeska T, Krupny M, Dobrovic A. Sensitive melting analysis after real-time-methylation specific PCR (SMART-MSP): high-throughput and probe-free quantitative DNA methylation detection. Nucleic Acid Res. 2008;36(7):e42.

25. Hughes S, Jones I. The use of multiple displacement amplified DNA as a control for methylation specific PCR. pyrosequencing, bisulfite sequencing and methylation-sensitive restriction enzyme PCR. BMC Mol Biol. 2007;8:91.

26. Chung W, Bondaruk J, Jelinek J, Lotan Y, Liang S, Czerniak B, et al. Detection of bladder cancer using novel DNA methylation biomarkers in urine sediments. Cancer Epidemiol Biomarkers Prev. 2011;20(7):1483–91.

27. Delphy YE, Cordelier P, Cho WC, Torrisani J. DNA methylation and cancer. Int J Mol Sci. 2013;14:15029–58.

28. Cretelli R, Fassanelli F, Oderda M, Poldorso S, Assumma BM, Viberti C, et al. Detection of multiple mutations in urinary exfoliated cells from male bladder cancer patients at diagnosis and during follow-up. Oncotarget. 2016;7(41):67435–48.

29. Larsen LK, Lind GE, Guldberg P, Dahl C. DNA-methylation-based detection of urological cancer in urine: Overview of biomarkers and considerations on biomarker design, source of DNA, and detection technologies. Int J Mol Sci. 2013;19:202657.

30. Chen X, Zhang J, Ruan W, Huang M, Wang C, Wang H, et al. Urine DNA methylation assay enables early detection and recurrence monitoring for bladder cancer. J Clin Invest. 2020;130(2):678–89.

31. Zhang N, Chen S, Wu L, Wu Y, Jiang G, Shao J, et al. Identification of cancer-specific gene mutation of combination for the diagnosis of bladder cancer. J Cancer. 2019;10(26):6761–6.

32. Shiomi H, Iwai A, Kunitake T, Nishimura T, et al. Comprehensive genome methylation analysis in bladder cancer; identification and validation of novel methylated genes and application of these as urine tumor markers. Clin Cancer Res. 2011;17(7):5582–92.

33. Hermann T, Savio A, Olikow-Mitsel E, Mari A, Wettstein MS, Saba K, et al. A noninvasive urine-based methylation biomarker panel to detect bladder cancer and discriminate cancer grade. Urol Oncol. 2020;38(6):e603–e603.

34. Nishiyama N, Arai E, Chihara Y, Fujimoto H, Hosoda F, Shibata T, Kondo T, et al. Comprehensive genome methylation analysis in bladder cancer; identification and validation of novel methylated genes and application of these as urine tumor markers. Clin Cancer Res. 2011;17(7):5582–92.

35. Roperch JP, Incitti R, Forbin S, Bard F, Mansour H, Mesli F, et al. Aberrant DNA methylation profiles in urothelial carcinomas and urothelial at the precancerous stage. Cancer Sci. 2010;101(1):231–40.

36. Renard I, Joniau S, van Cleynenbreugel B, Collette C, Naëmé C, Wassenbroek I, et al. 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. Eur Urol. 2010;58(1):96–104.

37. Marist JC, Koestler DC, Christensen BC, Karagas MR, Houseman EA, Kelsey KT. DNA methylation array analysis identifies profiles of blood-derived DNA methylation associated with bladder cancer. J Clin Oncol. 2011;29(9):1133–9.

38. Ishii D, Pulverer W, Erlf IE, Lemberger U, Kimura S, Abulafia M, et al. Discovery of molecular DNA methylation-based biomarkers through genome-wide analysis of response patterns to BCG for bladder cancer. Cells. 2020;9(8):1839.