Urinary Nucleic Acid TSPAN13-to-S100A9 Ratio as a Diagnostic Marker in Prostate Cancer

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INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed malignancy in Western men (1), and the number of newly diagnosed PCa cases in Korea is increasing (2). The widespread use of prostate-specific antigen (PSA) testing led to a dramatic increase in the incidence and survival of PCa patients (3). However, PSA is an excellent organ-specific but not cancer-specific marker, and its specificity for the detection of PCa is low (4). Transrectal prostate biopsy, which is necessary to confirm the diagnosis of PCa, has a limited value because of its low detection rate and uncomfortable nature of the procedure. Therefore, accurate, specialized, and convenient diagnostic biomarkers for PCa are needed.

Cell-free nucleic acid-based biomarkers in body fluids have recently become a topic of interest (5). Fragmented DNAs and RNAs exist as circulating nucleic acids and can be isolated from body fluids such as blood, feces and urine (6-8). Circulating (cell-free) nucleic acids (CNA) are released from apoptotic and necrotic cells, although their exact origin is poorly understood (9). Despite the fact that their source remains unclear, CNAs reflect the molecular changes occurring in specific tissues (10). Furthermore, they can be isolated from body fluids using non-invasive procedures, which make them superior as cancer biomarkers to tissue markers that are obtained by invasive procedures.

Despite their value as cancer biomarkers, the quantification of CNAs is difficult. Therefore, we adopted the two-gene expression ratio method previously described by Ma et al. (11). The two-gene expression ratio consists of the comparison of one up-regulated gene to one down-regulated gene. In our previous study (10), urinary expression levels of S100A9 nuclear acids were significantly lower in PCa than in benign prostatic hyperplasia (BPH). Therefore, S100A9 was selected as the down-regulated gene for the two-gene expression ratio.

In the present study, the up-regulated gene for the two-gene
expression ratio was identified, and the mRNA expression levels were assessed. The ratio of up-regulated to down-regulated gene was assessed in PCa and BPH tissues to confirm our finding at the tissue mRNA level, and in urine samples from PCa and BPH to determine the value of urinary nucleic acids as diagnostic biomarkers of PCa.

MATERIALS AND METHODS

Study design
A schematic of the study design, which included four different stages, is shown in Fig. 1. Candidate genes were selected from tissue mRNA micro-array data and evaluated using the two-gene expression ratio method in urinary nucleic acids. The genes selected for two-gene expression ratio were validated in tissue mRNA and urinary nucleic acid cohorts.

Candidate gene selection from micro-array data
The gene expression profiles for GSE2618 (12), GSE6099 (13), GSE6608 (14), GSE6919 (14), and GSE23388 (15) were downloaded from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) database and differentially expressed genes (DEGs) were selected using Gene Spring GX 7.3 software (Agilent Technology, Santa Clara, CA, USA). The DEGs were identified using a t-test with a threshold P value < 0.05 (Benjamini and Hochberg False Discovery Rate) and a fold-change ≥ 2; PCa-associated genes were obtained from NCBI’s Entrez Gene (http://www.ncbi.nlm.nih.gov/gene/) and GeneCards (http://www.genecards.org/) databases.

Study population and samples
A total of 95 urine samples and 234 prostate tissue samples obtained from patients with PCa and BPH treated at our institute were used in the study. In detail, 12 PCa cases and 5 BPH controls were used in the two-gene expression ratio selection study; and 129 PCa and 105 BPH tissue samples and 37 PCa and 31 BPH urine samples were used in the validation cohort (tissue mRNA validation cohort and urinary nucleic acid validation cohort) (Fig. 1). All of the urine samples were collected prior to surgery on the first morning and centrifuged at 25,000 rpm for 15 min, and the supernatants were stored at -20°C until use. The tissue study included patients with PCa who underwent palliative transurethral resection (TUR) or radical prostatectomy, and patients with BPH who underwent TUR. All prostate tissue samples were macro-dissected within 15 min of surgical resection. Each prostate specimen was confirmed by pathological analysis of fresh-frozen sections, and the remaining tissue was frozen in liquid nitrogen and stored at -80°C until use. The controls were matched by age; and subjects were screened to ensure that their laboratory values were within the normal range and that they had no history of cancer. Controls with serum PSA levels ≥ 3 ng/mL underwent transrectal prostate biopsy before transurethral resection of the prostate (TURP) to rule out the presence of cancer. Patients who received neoadjuvant therapies, such as radiation therapy or androgen deprivation, were not included in the study. Gleason grades were measured in 12-core transrectal biopsies, TURP or radical prostatectomy specimens. Tumor stage was estimated from specimens obtained from radical prostatectomy or from magnetic resonance imaging, computed tomography, or bone scans.

Nucleic acid extraction from urine
Urinary nucleic acids were extracted using the QIAquick® gel extraction kit (Qiagen GmbH, Hilden, Germany). Each frozen urine sample (1 mL) was melted down at room temperature and treated with 500 µL of QG buffer (contained in QIAquick® gel extraction kit). After incubation for 10 min at 50°C, 500 µL of isopropanol was added and the sample was mixed. The sample was transferred onto a QIAquick column, which binds nucleic acids, and the column was placed into a 2 mL collection tube and centrifuged for 1 min at 13,000 rpm. The aqueous flow-through was discarded and the QIAquick column was placed into the same collection tube. After addition of 500 µL of QG buffer, the column was centrifuged for 1 min at 13,000 rpm, and bound nucleic acids were washed with 750 µL of PE buffer (contained in QIAquick® gel extraction kit) and centrifugation for 1 min. The aqueous flow-through was discarded and the
QIAquick column was centrifuged for an additional 1 min at 13,000 rpm and placed into a clean 1.5 mL microcentrifuge tube, and nucleic acids were eluted by addition of 50 µL of EB buffer (contained in QIAquick® gel extraction kit) to the center of the QIAquick membrane and centrifugation for 1 min at 13,000 rpm. The nucleic acids dissolved in EB buffer were stored at -20°C until use.

RNA extraction from tissues and cDNA synthesis
RNA was extracted from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as described in a previous report (10). The cDNA was synthesized from 1 µg of total RNA using the first strand cDNA synthesis kit (Amersham Biosciences Europe GmbH, Freiburg, Germany), according to the manufacturer's protocol.

Real-time PCR
To quantify urinary nucleic acid and tissue mRNA expression, real-time PCR was performed using a Rotor Gene 6000 instrument (Corbett Research, Mortlake, Australia). The Real-time PCR were performed in micro-reaction tubes (Corbett Research) using SYBR Premix EX Taq (Takara Bio Inc., Otsu, Japan). The nucleotide sequences of gene-specific primers for real-time PCR are shown in Table 1. In the urine study, a standard plasmid was included in real-time PCR to produce a standard curve using copy number (with copy numbers of 10^5, 10^4, 10^3, and 10) and threshold cycle (Ct) values. To construct standard plasmids, 360 bps of each gene including the PCR amplified target region were synthesized and ligated into pUC57 plasmid DNA (GenScript, Piscataway, NJ, USA). The synthesized target regions of each gene were confirmed by capillary sequencing. The Ct values of each gene from the real-time PCR run were plotted on the standard curve to calculate copy number. For the relative quantification of gene expression, the comparative Ct method was used to calculate the two-gene expression ratio. The final two-gene expression ratio values expressing the ratio of up-regulated gene to down-regulated gene (∆Ct = Ct up-regulated gene - Ct down-regulated gene) were calculated using the following formula: 2^-∆Ct, which allowed for the comparison of the different samples of our study. Finally, the formula 2^-[Ct up-regulated gene-Ct down-regulated gene] was used for the calculation of the ratio of up-regulated to down-regulated gene in urinary nucleic acids. In tissue study, GAPDH was used as an endogenous RNA reference gene and the expression of each gene of in-

| Target gene | Primer sequences for the urinary nucleic acids study | Primer sequences for the tissue mRNA study |
|-------------|-----------------------------------------------------|------------------------------------------|
| ACSL3       | Sense, 5´-CACAAGTGAGATCCCAGAC-3´                     | Anti-sense, 5´-TGAAATCTCTTCCCACTCC-3´   |
| AGR2        | Sense, 5´-CCCTGCTCGAAGTTTACAG-3´                    | Anti-sense, 5´-AGGCTTGTTAGCTGGTTC-3´   |
| C19orf48    | Sense, 5´-TGGCTCAACAGGGCTTCC-3´                     | Anti-sense, 5´-CTTCAGGCTGCTGACTGAC-3´  |
| CANT1       | Sense, 5´-GAACTTCTCTACAGTTTACAG-3´                 | Anti-sense, 5´-GCACTGCTGGTCAATGGAC-3´  |
| HPN         | Sense, 5´-GCTGCAATGCGGGCTGAC-3´                     | Anti-sense, 5´-TGCGAGGTACGTGCAC-3´     |
| OR51E2      | Sense, 5´-GTGGCTCTCTGATAGAAGAC-3´                   | Anti-sense, 5´-GCCAGGTCAATGGCTGAA-3´   |
| PCBP2       | Sense, 5´-TGCTACCAACTGCACTGAG-3´                    | Anti-sense, 5´-CCAACATGACACGCGATC-3´   |
| RASD1       | Sense, 5´-GGAGGCTCAACGGCCTAC-3´                     | Anti-sense, 5´-GATGTCAGCTGAGTAGAC-3´   |
| SLC45A3     | Sense, 5´-GATTGGCACTGACAGCTG-3´                     | Anti-sense, 5´-ACTCTTCGAGCAGCATGG-3´   |
| SPON2       | Sense, 5´-ATATGGCTGACTACAGCATG-3´                   | Anti-sense, 5´-CATATGCTGACTACAGCATG-3´ |
| SYT7        | Sense, 5´-TAGCTGGTCACTGACAGCTG-3´                   | Anti-sense, 5´-TATGGCTCCAGCCAGCAGATC-3´|
| WISP1B       | Sense, 5´-TCTGCTCAACGGCCAGCAAG-3´                  | Anti-sense, 5´-CAACATGACACGCGATC-3´    |
| TSPAN13     | Sense, 5´-GTTAGTCTCTGCTGCTTTAGT-3´                 | Anti-sense, 5´-CTGGAAATGACATCCAGAG-3´  |
| S100A9      | Sense, 5´-GGAAGGACCTGGACAAATG-3´                    | Anti-sense, 5´-TGAGGAGCCGAGCAGATG-3´   |
| GAPDH       | Sense, 5´-CATGTGCTGACTGAGGCT-3´                     | Anti-sense, 5´-ATGGCATGGACGTGCTGATC-3´ |
terest was normalized to that of GAPDH. All samples were run in triplicate.

Statistical analysis
Mann Whitney U-test nonparametric analysis was applied to assess the urinary nucleic acid levels and mRNA expression levels. Statistical analysis was performed using SPSS 21.0 software (IBM, Armonk, NY, USA), and P < 0.05 was considered statistically significant.

Ethics statement
The study protocol was approved by the institutional review board of Chungbuk National University (IRB approved number: GR2010-12-010). Informed consent was obtained from each subject.

RESULTS

PCa-specific candidate diagnostic gene classifiers
In the micro-array analysis, 13 candidate genes, namely, ACSL3, AGR2, C19orf48, CANT1, HPN, OR51E2, PCBP2, RASD1, SL-C45A3, SPON2, SYT7, TSPAN13, and VPS13B, were selected from the Venn diagram. These genes showed significantly higher mRNA expression levels in PCa than in BPH controls and were used in the two-gene expression ratio selection study.

Selection of genes for two-gene expression ratio
The baseline characteristics of the PCa patients and BPH controls enrolled in the two-gene expression ratio selection study are listed in Table 2. The mean age of the PCa patients was 70.83 yr (range, 64-81) and that of the BPH controls was 69.3 yr (range, 46-85 yr). The serum PSA levels were 47.52 ± 81.98 and 2.89 ± 1.81 in PCa and BPH patients, respectively. The analysis showed that the TSPAN13-to-S100A9 urinary nucleic acid expression ratio was significantly different between PCa and BPH samples, with a P value of 0.037 (Table 3). Therefore, the TSPAN13-to-S100A9 ratio was selected for the validation study.

TSPAN13 and S100A9 tissue mRNA expression levels in PCa cases and BPH controls
Table 3 lists the baseline characteristics of the 129 PCa cases and 105 BPH controls. The mean age of PCa patients was 69.5 yr (range, 48-87 yr), and that of the BPH controls was 69.3 yr (range, 46-85 yr). The serum PSA level was higher in PCa patients than in BPH controls (100.47 ± 239.65 ng/mL vs. 4.17 ± 7.79 ng/mL; P < 0.001). Of the 129 PCa cases, 66 (51.2%) underwent radical prostatectomy using open or laparoscopic procedures. The expression of TSPAN13 was significantly higher in PCa patients than in BPH controls (6.52 ± 13.87 vs. 20.64 ± 25.73; P < 0.001).
PCa than in BPH, whereas the expression of S100A9 was significantly lower in PCa than in BPH (each $P < 0.001$) (Table 4). The TSPAN13-to-S100A9 mRNA expression ratio, which was analyzed with the same methods used in urine study, was significantly higher in PCa than in BPH ($P < 0.001$) (Table 4 and Fig. 2). Receiver operating characteristics (ROC) analysis was performed to assess the predictive value for PCa, which showed that the area under the curve (AUC) was 0.898 for the TSPAN13-to-S100A9 expression ratio (Fig. 3). No differences in the TSPAN13 and S100A9 mRNA expression levels and ratio were observed.
The ratio of urinary TSPAN13-to-S100A9 nucleic acid levels in the urinary nucleic acid validation cohort

Table 4 shows the clinicopathological characteristics of 37 PCa cases and 31 BPH controls included in the urinary nucleic acid validation cohort. The TSPAN13-to-S100A9 ratio was significantly higher in PCa cases than in BPH controls (P = 0.013) (Table 4 and Fig. 2). ROC analysis showed that the AUC value was 0.676 for the urinary nucleic acid TSPAN13-to-S100A9 ratio (Fig. 3). These results were consistent with those of the tissue mRNA expression study and demonstrated the diagnostic value of the urinary TSPAN13-to-S100A9 nucleic acid ratio for PCa. In the analysis of TSPAN13-to-S100A9 ratio according to clinical characteristics, there were no differences based on clinicopathological variables such as age, PSA, grade, and stage (data not shown).

DISCUSSION

The results of the present study indicated that the urinary TSPAN13-to-S100A9 nucleic acid ratio has diagnostic value as a biomarker for PCa. The TSPAN13-to-S100A9 ratio of urinary nucleic acids was significantly higher in PCa cases than in BPH controls (P = 0.013), and ROC curve analysis showed an AUC value of 0.676, which was consistent with the AUC value in the tissue mRNA expression study (AUROC = 0.898, P < 0.001). These results demonstrate the possibility of finding diagnostic markers for PCa, since this outcome was obtained using urine supernatants and a new method, namely, the two-gene expression ratio.

Increasing research efforts have focused on identifying new non-invasive diagnostic markers for solid tumors. Significant advances have been made in certain malignancies, such as colon cancer (16). In urological cancer, numerous articles have reported results on genetic and epigenetic modifications in urine sediment as biomarkers for early diagnosis (17-19); however, few studies have investigated markers in the urine supernatant (20,21). In the present study, we extracted CNAs from the urine supernatant rather than using the urine sediment as a source of material. The urine supernatant is more suitable for identifying cancer biomarkers than the urine sediment because the sediment contains normal DNA derived from non-cancerous cells which might hamper the analysis. This was supported by Szarvas et al. (22), who suggested that the detection rate of genetic changes is higher in CNAs isolated from the urine supernatant than in those from the urine sediment. However, the origin of CNAs in the urine supernatant remains poorly understood. It is possible that circulating DNA or RNA is released from either necrotic or apoptotic cells (9). Zancan et al. (23) reported the usefulness of cell-free DNA measurements from urine supernatants to differentiate between patients with bladder cancer and control cases. In addition, two studies showed reliable discrimination between cancer and non-cancer patients using urine supernatant (21,24). These studies support the use of urine supernatants for the analysis of nucleic acids for PCa detection.

Although the CNAs extracted from the urine supernatant are valuable as cancer biomarker, the numeric values obtained by real-time PCR from urinary nucleic acids are difficult to normalize, whereas in tissue mRNA real-time PCR studies, housekeeping genes such as GAPDH can be used as reference materials. Therefore, in the present study, we used a plasmid standard and the two-gene expression ratio. Plasmids were used as reference materials in urinary nucleic acid real-time PCR to generate standard curves. The PCR fragments of target genes were cloned into suitable plasmid vectors and experiments were performed using the same dilutions of each plasmid standard. The use of the same copy number of the plasmid standard allowed the adjustment of data obtained from real-time PCR. Furthermore, plasmid standards have additional advantages. One advantage of using plasmid standards is that once they are constructed by cloning the target PCR fragment into a suitable plasmid vector, they can be easily prepared in large amounts. In addition, plasmids can be stored for long periods and aliquoted at -20°C without significant degradation. Therefore, plasmid standards can be used as reference material to minimize inter-assay variation, and may offer a solution to the normalization problem associated with the use of urinary nucleic acids.

In the present study, the two-gene expression ratio was used. In a tissue mRNA study, Ma et al. (11) identified the two-gene
expression ratio, \( \text{HOXB13-to-IL17BR} \), as a predictor of breast cancer outcome during the early period. The \( \text{HOXB13} \) gene was up-regulated and the \( \text{IL17BR} \) gene was down-regulated in breast cancer patients with disease recurrence. Reid et al. (25) failed to validate this two-gene ratio on frozen samples from 58 patients; however, a large scale (1,252 primary breast tumor specimens) study confirmed the reliability of the two-gene ratio as a predictor of breast cancer outcome (26). The two-gene expression ratio in individual patients was also identified as a biomarker signature for lung cancer diagnosis and prognosis (27,28). Although these studies were based on tissue mRNA expression analyses, they highlight the value of the two-gene expression ratio as a reliable biomarker, and suggest that it could be adapted to the analysis of CNAs. The objective of the present study was to identify a two-gene expression ratio in urinary nucleic acids consisting of one up-regulated and one down-regulated gene as a diagnostic marker for PCa. The \( \text{TSPAN13-to-S100A9} \) ratio showed the same pattern in tissue mRNA and in urinary nucleic acids. These results demonstrate the reliability of urinary nucleic acid \( \text{TSPAN13-to-S100A9} \) ratio as a biomarker.

\( \text{S100A9} \), the down-regulated gene in the two-gene expression ratio in our study, has been investigated extensively. \( \text{S100A9} \) is a member of the \( \text{S100} \) family of proteins containing two EF hand calcium-binding motifs (29,30). \( \text{S100A9} \) forms a complex with \( \text{S100A8} \), another member of the \( \text{S100} \) family, and the \( \text{S100A9/S100A8} \) heterodimer is released in virtually all inflammatory disorders (31). Tissue mRNA expression analyses showed elevated levels of \( \text{S100A9} \) in many malignancies such as breast, lung, gastric, colorectal, and pancreatic cancer (32). However, in PCa, the expression of \( \text{S100A9} \) was reported to be up-regulated, whereas our previous study showed the opposite result (10,33). Our previous study analyzed a greater number of samples than that of the study reporting the up-regulation of \( \text{S100A9} \) in PCa, which increases the accuracy of our results. Moreover, our study demonstrated the down-regulation of \( \text{S100A9} \) in PCa using both tissue mRNA expression and urinary nucleic acid analyses. Therefore, in the present study, we used \( \text{S100A9} \) as the down-regulated gene in the two-gene expression ratio to examine the reliability of urinary nucleic acids as biomarkers of PCa.

The present study had several limitations. First, the number of samples used in the validation study was relatively small; further investigation with a greater number of cases is needed to confirm the diagnostic value of the \( \text{TSPAN13-to-S100A9} \) ratio. In addition, the micro-array data used for the selection of candidate genes in this study was derived from Western patients, and differences between Western and Korean patients were not taken into consideration. Future studies should include the selection of candidate genes from a Korean cohort to improve the accuracy of our findings. Finally, additional studies are necessary to confirm the relationship between \( \text{S100A9} \) and \( \text{TSPAN13} \). Although \( \text{S100A9} \) is a well studied gene, \( \text{TSPAN13} \) as a member of the transmembrane-4 superfamily has not been investigated extensively, although it is known to be involved in signal transduction events that play a role in the regulation of cell development, activation, growth and motility.

In patients with urothelial carcinoma, urine is a particularly desirable source of diagnostic marker, since its collection is more convenient and less invasive than that of blood. In the present study, analysis of urinary nucleic acids using the two-gene expression ratio yielded positive results regarding the identification of urinary diagnostic markers for PCa. In addition, the procedure of detecting the two-gene expression ratio in nucleic acids isolated from the urine supernatant is simple and applicable to the clinical setting. However, the AUC value in the urinary nucleic acid cohort did not show sufficient detection power, despite the fact that the \( \text{TSPAN13-to-S100A9} \) ratio pattern was the same in urinary nucleic acids and in the tissue mRNA validation cohort. The identification of additional two-gene expression ratios in urinary nucleic acids is necessary to develop an accurate diagnostic marker for PCa using the methods described in the present study.

In conclusion, the urinary nucleic acid \( \text{TSPAN13-to-S100A9} \) ratio shows potential as a diagnostic marker for PCa. The results of the present study indicate that analysis of the urine supernatant is a simple diagnostic method for PCa that could be adapted to the clinical setting in the future.

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DISCLOSURE

The authors have no potential conflicts of interest to disclose.

AUTHOR CONTRIBUTION

Conception and design of the study: Yan C, Kim YH, Yun SJ. Acquisition of data: Kim YH, Seo SP, Jeong P, Lee IS. Statistical analysis: Seo SP, Kim D, Kim JM. First draft of manuscript: Yan C, Kim YH, Kang HW. Revision and critical review of the manuscript: Choi YH, Moon SK, Yun SJ, Kim WJ. Manuscript approval: all authors.
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