Clinical Implications and Prognostic Values of Prostate Cancer Susceptibility Candidate Methylation in Primary Nonmuscle Invasive Bladder Cancer

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DNA methylation is the most common and well-characterized epigenetic change in human cancer. Recently, an association between prostate cancer susceptibility candidate (PRAC) methylation and genitourinary cancer was proposed. The aim of the present study was to evaluate the association between PRAC methylation status and clinicopathological parameters and prognosis in long-term follow-up primary nonmuscle invasive bladder cancer (NMIBC). The clinical relevance of PRAC methylation was determined in 136 human bladder specimens (eight normal controls [NCs] and 128 primary NMIBCs) using quantitative pyrosequencing analysis. PRAC methylation was significantly higher in NMIBC patients than in NCs and was significantly associated with higher grade and more advanced stage of cancer. Kaplan-Meier estimates revealed significant difference in tumor recurrence and progression according to PRAC methylation status (both \( p < 0.05 \)). Multivariate Cox regression analysis revealed that the PRAC methylation status was a strong predictor of recurrence (hazard ratio [HR], 2.652; \( p = 0.012 \)) and progression (HR, 9.531; \( p = 0.035 \)) of NMIBC. Enhanced methylation status of PRAC was positively associated with a high rate of recurrence and progression in NMIBC patients, suggesting that PRAC methylation may be a promising prognostic marker of NMIBC.

1. Introduction

Because bladder cancer is a heterogeneous disease, pathologically similar tumors may behave differently. Numerous factors are likely involved in disease outcomes, and many patients with nonmuscle invasive bladder cancer (NMIBC) experience disease recurrence and progression after primary treatment [1, 2]. Therefore, identifying patients at high risk of recurrence and progression who would benefit from more aggressive treatment, as well as those at low risk who require less intensive surveillance after initial adequate therapy, is challenging. Currently, conventional clinicopathological factors are insufficient to predict the outcome of patients with NMIBC. Thus, additional biomarkers are needed to predict the prognosis of NMIBC patients.

As in most other human cancers, bladder cancer originates from multiple combinations of genetic, epigenetic, and environmental factors [3–6]. DNA methylation, which inactivates tumor suppressor genes, is the most common and well-characterized epigenetic change in human cancer and may be a potential biomarker for cancer [6, 7]. Recent research proposed an association between the prostate cancer susceptibility candidate (PRAC) gene and human tumors, including prostate and renal cell carcinoma (RCC) [8–12]. In particular, PRAC hypermethylation is the hallmark methylation phenotype in RCC, and its alterations in precancerous
Table 1: Baseline characteristics of subjects.

| Variables                        | NC (n = 8) | NMIBC (n = 128) |
|----------------------------------|------------|-----------------|
| Age, yrs (mean)                  | 59.0 ± 22.2| 64 ± 13.4       |
| Gender, number of patients (%)   |            |                 |
| Male                             | 6 (75.0)   | 107 (83.6)      |
| Female                           | 2 (25.0)   | 21 (16.4)       |
| Number of tumors (%)             |            |                 |
| Single                           | —          | 76 (59.4)       |
| Multiple                         | —          | 52 (40.6)       |
| Tumor size (%)                   |            |                 |
| <3 cm                            | —          | 76 (59.4)       |
| ≥3 cm                            | —          | 52 (40.6)       |
| Grade, number of patients (%)    |            |                 |
| G1                               | —          | 38 (29.7)       |
| G2                               | —          | 72 (56.2)       |
| G3                               | —          | 18 (14.1)       |
| T Stage                           |            |                 |
| Ta                               | —          | 47 (36.7)       |
| T1                               | —          | 81 (63.3)       |
| Intravesical treatment, number of patients (%) | — | 58 (45.3) |
| Recurrence-free survival, months (median) | 44.5 (6.2–160.7) | 70 (54.7) |
| Recurrence, number of patients (%) |            |                 |
| No                               | —          | 90 (70.3)       |
| Yes                              | —          | 38 (29.7)       |
| Progression-free survival, months (median) | 51.0 (6.6–160.7) |                  |
| Progression, number of patients (%) |            |                 |
| No                               | —          | 113 (88.3)      |
| Yes                              | —          | 15 (11.7)       |

NC: normal control; NMIBC: nonmuscle invasive bladder cancer.

stages may be associated with tumor aggressiveness and patient prognosis [11, 12].

To our knowledge, no study has evaluated the prognostic role of the PRAC gene in bladder cancer. The aim of the present study was to evaluate the impact of PRAC methylation status on clinicopathological parameters and prognosis in long-term follow-up primary NMIBC patients.

2. Materials and Methods

2.1. Subjects and Sample Collection. A total of 136 human bladder tissues (eight normal controls [NC] and 128 NMIBC) were used for pyrosequencing (PSQ) analyses (Table 1). We selected available bladder tissues from Biobank which was utilized in our previous study [13]. NMIBC tissues were obtained from primary NMIBC patients who underwent transurethral resection (TUR) for histologically diagnosed transitional cell carcinomas between 1995 and 2010 at our institute. To exclude the possibility of incomplete resection or factors that may affect analyses, patients who were followed for less than 6 months or those that experienced disease relapse within 6 months were excluded from this study. NC tissues were obtained from individuals with benign prostate hyperplasia or bladder injury.

All tumors were macrodissected within 15 minutes of surgical resection. Each NMIBC specimen was confirmed by pathological analysis of a section of the tissue that was obtained from the TUR specimens, immediately frozen in liquid nitrogen, and stored at −80°C. The specimens were provided by the Chungbuk National University Hospital, a member of the National Biobank of Korea, which is supported by the Ministry of Health, Welfare, and Family Affairs. The collection and analysis of all samples were approved by the Chungbuk National University Hospital Institutional Review Board (GR2010-12-010), and informed consent was obtained from each subject.

Tumor staging was classified according to the 2002 TNM classification and the 1973 World Health Organization grading systems [14]. A second TUR was performed 2–4 weeks after the initial resection if a bladder cancer specimen did not include proper muscle or if a high-grade tumor was detected. Patients with intermediate- or high-risk NMIBC received one cycle of intravesical instillation therapy. Each patient was followed up and managed according to standard recommendations [1, 2]. Recurrence was defined as the return of primary NMIBC at a lower or equivalent pathologic stage (Ta/T1), and progression was defined as muscular invasion (TNM stage T2 or higher) or nodal/distant metastatic disease.

2.2. DNA Extraction and PSQ Analysis. Genomic DNA was extracted by standard methods using the Wizard Genomic DNA Purification System (Promega). Bisulfite conversion of genomic DNA was carried out using the EZ DNA Methylation Kit (Zymo Research). The DNA methylation status of PRAC was assessed by PSQ using PyroMark Q96 ID (Qiagen, Valencia, CA). The primer sequences and amplification conditions are described in Table 2. PCR reactions were conducted using 20 ng of bisulfite-converted genomic DNA. A biotin-labeled primer was used to purify the final PCR product using streptavidin-coated Sepharose beads (GE Healthcare, Buckinghamshire, UK). The PCR product was bound to Sepharose beads, purified, washed, denatured using a 0.2 mol/L NaOH solution, and washed again. Subsequently, 0.3 μmol/L PSQ sequencing primer was annealed to the purified single-stranded PCR product and PSQ was performed on a PyroMark Q96 ID (Qiagen, Valencia, CA). Target CpG sites were evaluated using the instrument software (PSQ96MA 2.1, Qiagen, Valencia, CA), which converts programs to numerical values for peak heights and calculates the proportion of methylation at each base as a C/T ratio. Data analysis was performed using PyroMark Q96 ID Software v1.0 software (Qiagen, Valencia, CA).
whether methylation marker was correlated with prognosis, in the favorable prognosis group (Table 3). To determine poor prognosis group (recurrence or progression) than methylation level of tumor grade and stage. The methylation status of the PRAC gene was significantly higher in the poor prognosis group (recurrence or progression) than in the favorable prognosis group (Table 3). To determine whether methylation marker was correlated with prognosis, the methylation levels of the PRAC gene were dichotomized (hypomethylation or hypermethylation) with the median defined as the cut-off point. Kaplan-Meier estimates revealed that the group with PRAC hypermethylation had significantly less time to recurrence and progression than did the PRAC hypomethylation group (Figure 1, log-rank test, each \( p < 0.05 \)). Univariate and multivariate Cox regression analyses showed that PRAC gene methylation was an independent predictive factor of recurrence (hazard ratio [HR], 2.652; 95% confidence interval [CI], 1.241–5.667; \( p = 0.012 \)) and

### Table 2: PRAC primers used for pyrosequencing analysis.

| Genes                     | PRAC                                  |
|---------------------------|---------------------------------------|
| Forward (5'-3')           | GGATTTTGTGGTTTATTTTTGTAGA             |
| Reverse (5'-3')           | (biotin)-CTCACCCCTCCCTATTTTC          |
| Sequencing primer (5'-3') | TGGTTTTTTTTTTTTAAAGGTAAA             |
| Amplicon location relative to TSS | ~35–209                         |
| Sequence to analyze       | TGATCGGTGGCAGCAGCGTTATCGGCGATCG      |
| Product size              | 244 bp                                |

Primer was designed using NCBI Reference Sequences build version 36.1. The PCR reaction contained 0.01 μM of each primers and Bioneer Taq (Bioneer, Daejeon, Korea), and 20 ng of bisulfite-treated DNA. The thermocycling parameters were as follows: denaturation at 94 °C for 5 minutes, followed by 45 cycles at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, 72 °C for 30 seconds, and a final extension at 72 °C for 5 minutes.

### Table 3: Association between PRAC methylation and clinicopathological characteristics.

| Variables                  | Methylation level (%) | \( p \) value |
|----------------------------|-----------------------|--------------|
| Normal versus cancer       |                       |              |
| Normal                     | 17.3 ± 4.0            | <0.001\footnote{1}|
| Cancer                     | 48.3 ± 24.3           |              |
| Number of tumors           |                       |              |
| Single                     | 45.6 ± 24.5           | 0.11\footnote{2}|
| Multiple                   | 52.4 ± 23.7           |              |
| Tumor size                 |                       |              |
| <3 cm                      | 54.5 ± 25.7           | 0.095\footnote{3}|
| ≥3 cm                      | 52.7 ± 21.7           |              |
| Grade                      |                       |              |
| G1                         | 33.7 ± 19.4           |              |
| G2                         | 50.5 ± 23.3           | <0.001\footnote{4}|
| G3                         | 70.8 ± 17.2           |              |
| T stage                    |                       |              |
| Ta                         | 38.6 ± 24.6           | <0.001\footnote{5}|
| T1                         | 54.0 ± 22.4           |              |
| Recurrence                 |                       |              |
| No                         | 43.6 ± 24.3           | 0.001\footnote{6}|
| Yes                        | 59.6 ± 20.5           |              |
| Progression                |                       |              |
| No                         | 45.6 ± 23.9           | <0.001\footnote{7}|
| Yes                        | 69.2 ± 16.4           |              |

\footnote{1}{\textsuperscript{1}} \( p \) value calculated using Student’s \( t \)-test. \footnote{2}{\textsuperscript{2}} \( p \) value calculated using ANOVA trend analyses test. PRAC: prostate cancer susceptibility candidate.

### 2.3. Statistical Analysis. The differences in continuous variables between two groups were assessed using a two-sample \( t \)-test or ANOVA trend analyses using polynomial contrasts. Median values were used as a cut-off point to divide patients into subgroups (hypomethylation or hypermethylation), and survival functions of PRAC genes were evaluated. The Kaplan-Meier curves were used to estimate time to recurrence or progression according to methylation status, and differences were evaluated using log-rank tests. For multivariate Cox proportional hazards regression analyses, the prognostic value of methylation status was evaluated and adjusted for well-known clinicopathological factors (sex, age, tumor size, tumor number, intravesical therapy, grade, and stage). Statistical analysis was performed using SPSS 20.0 software (IBM, Armonk, NY, USA). A \( p \) value <0.05 was considered statistically significant.

### 3. Results

#### 3.1. Baseline Characteristics. The baseline characteristics of the NC and NMIBC patients are presented in Table 1. Mean age was 64.0 ± 13.4 years old in patients with NMIBC. Mean recurrence-free survival and progression-free survival were 47.2 ± 40.4 months (median, 35.8; range, 6.1 to 183.3) and 61.1 ± 41.7 months (median, 50.9; range, 6.6 to 183.3), respectively.

#### 3.2. The Relationship between Methylation Levels and Clinicopathological Variables. As shown in Table 3, the methylation levels of PRAC genes were significantly higher in samples from NMIBC patients than in those from NC patients (\( p < 0.001 \)). To evaluate the relationship between methylation patterns and clinicopathological factors, methylation levels were examined in association with well-known prognostic factors such as tumor number, size, grade, and stage. High levels of PRAC methylation were significantly associated with tumor grade and stage.

#### 3.3. Methylation Status as a Predictor of Prognosis. The methylation level of PRAC was significantly higher in the poor prognosis group (recurrence or progression) than in the favorable prognosis group (Table 3). To determine whether methylation marker was correlated with prognosis,
Table 4: Multivariate Cox regression analysis of disease outcomes according to PRAC methylation in nonmuscle invasive bladder cancer (n = 128).

| Variables | Recurrence HR 95% CI | p value | Progression HR 95% CI | p value |
|-----------|----------------------|---------|-----------------------|---------|
| Age (<66 yrs versus ≥66 yrs) | 0.711 (0.364–1.390) | 0.319 | 0.884 (0.310–2.519) | 0.817 |
| Sex (male versus female) | 1.084 (0.441–2.662) | 0.860 | 1.148 (0.241–5.459) | 0.862 |
| Number of tumors (single versus multiple) | 1.099 (0.568–2.127) | 0.780 | 1.178 (0.420–3.301) | 0.755 |
| Tumor size (<3 cm versus ≥3 cm) | 1.270 (0.645–2.499) | 0.490 | 4.149 (1.184–14.540) | 0.026 |
| T Stage (Ta versus T1) | 0.963 (0.437–2.122) | 0.925 | 2.028 (0.233–17.623) | 0.521 |
| Grade (G1-2 versus G3) | 0.493 | | | 0.021 |
| G1 | 1 | | | |
| G2 | 1.124 (0.450–2.811) | 0.803 | 1.235 (0.074–20.544) | 0.883 |
| G3 | 1.825 (0.567–5.879) | 0.313 | 7.280 (0.397–133.445) | 0.181 |
| Intravesical therapy (no versus yes) | 1.248 (0.610–2.554) | 0.545 | 2.061 (0.558–7.612) | 0.558 |
| PRAC (hypomethylation versus hypermethylation) | 2.652 (1.241–5.667) | 0.012 | 9.531 (1.172–77.497) | 0.035 |

HR: hazard ratio; CI: confidence interval; PRAC: prostate cancer susceptibility candidate.

Recent research investigating prognostic factors for recurrence and progression in bladder cancer has focused on epigenetic changes [3, 5, 6]. The epigenetic silencing of tumor suppressor genes may be relevant clinically because epigenetic changes may be reversible and thus restore gene function [4, 5, 7]. In transitional cell carcinoma of the bladder, several studies revealed that methylated genes such as CDH1, FHIT, LAMC2, RASSFIA, TIMP3, SFRP1, SOX9, PMF1, and RUNX3 are associated with tumor characteristics and prognosis [3, 5, 15]. Because hypermethylation of the promoter occurs frequently in bladder cancer, detection of methylation status may be performed in exfoliated urinary cells or tumor tissues [5, 16]. Thus, markers of aberrant

4. Discussion

Our results showed that methylation level of PRAC was significantly higher in tissues from NMIBC than in those from NC patients and that the methylation status of PRAC was significantly associated with higher tumor grade and advanced pathological stage. Furthermore, PRAC methylation status was an independent prognostic indicator of recurrence and progression in long-term follow-up of primary NMIBC patients.
methylations may be a potential gateway for monitoring and determining the prognosis of bladder cancer. In the present study, the methylation of PRAC effectively discriminated bladder cancer from normal bladder tissues and was also associated with aggressive tumor features and poor prognosis. The results suggested that PRAC, a novel methylation marker identified in the present study, is specific to NMIBC and an appropriate tool to predict prognosis.

The PRAC gene is located on chromosome 17 at position 17q21 and is specifically expressed in prostate, rectum, and colon tissues [10]. However, little information is available about the function of the PRAC gene [8–12]. After the first identification of the PRAC gene in 2001, further research has been limited to specific organs such as prostate, rectum, and colon [11]. Recently, Arai et al. identified hypermethylation of the PRAC gene in RCC and showed a significant association between methylation status and prognosis [11]. These findings prompted our interest in identifying the prognostic role of PRAC methylation in NMIBC. To the best of our knowledge, the current study is the first to identify PRAC as a methylation marker related to bladder cancer.

Despite the prognostic significance of PRAC in NMIBC, our findings did not indicate a role in initiation or progression of bladder tumors. The lack of a clear association between these candidate markers and bladder cancer is a limitation of the present study, and this issue should be addressed in future studies. However, the objective of the present study was to identify markers related to NMIBC disease markers. Thus, we focused on the association between changes in methylation of specific markers and the associated disease phenotype rather than the effect of methylation status on gene transcription and function [4].

A key strength of our study was that we performed definitive subgroup analysis after selecting only the primary NMIBC patients. Because bladder cancer is a heterogeneous disease, and prognosis is affected by many factors, evaluating the effectiveness of a gene as a prognostic maker within a homogenous study population is important. Our findings indicate that promoter hypermethylation of PRAC was a reliable predictor of tumor recurrence and progression in primary NMIBC. Because methylation status was associated with an aggressive tumor phenotype, it may be used to identify patients at high risk of poor prognosis who require more aggressive treatment as well as those at low risk of recurrence and/or progression who require less intensive surveillance.

We analyzed the association between PRAC methylation status and prognosis in NMIBC patients. Kaplan-Meier analysis showed that NMIBC patients with hypermethylated PRAC had significantly decreased time to recurrence or progression. Multivariate analysis also showed that methylation of PRAC was an independent predictor of recurrence (HR, 2.652; p < 0.012) and progression (HR, 9.531; p < 0.035) in patients with NMIBC. Frequent recurrence is a major concern of NMIBC patients. NMIBC patients who experience progression to stage T2 during the surveillance period have a lower survival rate after cystectomy than patients who initially present with stage T2 disease [17]. Accordingly, early detection of progression to muscle invasive bladder cancer through frequent, extensive monitoring may provide a survival benefit for high-risk NMIBC patients. From a clinical point of view, epigenetic markers may be promising for early detection, prediction of treatment response, and indication of disease prognosis. The results presented herein are promising because the clinical significance was evaluated in a relatively large number of human tissue samples obtained from long-term follow-up primary NMIBC patients, and the selected methylation markers were independent predictors of disease outcome. Early cystectomy is recommended in NMIBC patients at high risk for recurrence and progression to improve survival outcomes. Therefore, our results may be useful to select the best treatment modality. However, further validation studies will be necessary to reduce false prediction rates, ensure reliable clinical relevance, and develop new therapies that target specific molecular defects, thereby reducing the morbidity associated with NMIBC.

5. Conclusions

Increased methylation of PRAC was significantly associated with a high grade and advanced stage of NMIBC. The methylation status of PRAC was an independent predictor of recurrence and progression in primary NMIBC. Thus, the methylation status of PRAC represents a useful parameter for predicting prognosis in patients with primary NMIBC.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution

Young-Won Kim and Hyung-Yoon Yoon contributed equally to this work.

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References

[1] M. Babjuk, M. Burger, R. Zigeuner et al., “EAU guidelines on non-muscle-invasive Urothelial carcinoma of the bladder: update 2013,” European Urology, vol. 64, no. 4, pp. 639–653, 2013.
[2] A. M. Kamat, P. K. Hegarty, J. R. Gee et al., “ICUD-EAU international consultation on bladder cancer 2012: screening, diagnosis, and molecular markers,” European Urology, vol. 63, no. 1, pp. 4–15, 2013.
[3] W.-J. Kim and Y.-J. Kim, “Epigenetic biomarkers in urothelial bladder cancer,” Expert Review of Molecular Diagnostics, vol. 9, no. 3, pp. 259–269, 2009.
[4] T. Ushijima, “Detection and interpretation of altered methylation patterns in cancer cells,” Nature Reviews Cancer, vol. 5, no. 3, pp. 223–231, 2005.
[5] A. Besaratinia, M. Cockburn, and S. Tommasi, “Alterations of DNA methylome in human bladder cancer,” Epigenetics, vol. 8, no. 10, pp. 1013–1022, 2013.

[6] S. Sharma, T. K. Kelly, and P. A. Jones, “Epigenetics in cancer,” Carcinogenesis, vol. 31, no. 1, pp. 27–36, 2010.

[7] S. B. Baylin and P. A. Jones, “A decade of exploring the cancer epigenome—biological and translational implications,” Nature Reviews Cancer, vol. 11, no. 10, pp. 726–734, 2011.

[8] G. Lenka, W.-H. Weng, C.-K. Chuang, K.-F. Ng, and S.-T. Pang, “Aberrent expression of the PRAC gene in prostate cancer,” International Journal of Oncology, vol. 43, no. 6, pp. 1960–1966, 2013.

[9] D. C. Koestler, J. Li, J. A. Baron et al., “Distinct patterns of DNA methylation in conventional adenomas involving the right and left colon,” Modern Pathology, vol. 27, no. 1, pp. 145–155, 2014.

[10] X. F. Liu, P. Olsson, C. D. Wolfgang et al., “PRAC: a novel small nuclear protein that is specifically expressed in human prostate and colon,” Prostate, vol. 47, no. 2, pp. 125–131, 2001.

[11] E. Arai, S. Chiku, T. Mori et al., “Single-CpG-resolution methylation analysis identifies clinicopathologically aggressive CpG island methylator phenotype clear cell renal cell carcinomas,” Carcinogenesis, vol. 33, no. 8, pp. 1487–1493, 2012.

[12] Y. Tian, E. Arai, M. Gotoh, M. Komiyama, H. Fujimoto, and Y. Kanai, “Prognostication of patients with clear cell renal cell carcinomas based on quantification of DNA methylation levels of CpG island methylator phenotype marker genes,” BMC Cancer, vol. 14, no. 1, p. 772, 2014.

[13] Y.-J. Kim, H.-Y. Yoon, J. S. Kim et al., “HOXA9, ISL1 and ALDH1A3 methylation patterns as prognostic markers for non-muscle invasive bladder cancer: array-based DNA methylation and expression profiling,” International Journal of Cancer, vol. 133, no. 5, pp. 1135–1142, 2013.

[14] M. Babjuk, W. Oosterlinck, R. Sylvester, E. Kaasinen, A. Böhle, and J. Palou-Redorta, “EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder,” European Urology, vol. 54, no. 2, pp. 303–314, 2008.

[15] S. Ali Hosseini, R. C. Sobti, K. Malekzadeh, S. K. Singh, and K. Joshi, “Frequency of PI6INK4a and PI4ARF genes methylation and its impact on bladder cancer cases in north Indian population,” Disease Markers, vol. 28, no. 6, pp. 361–368, 2010.

[16] R. Garcia-Baquero, P. Puerta, M. Beltran et al., “Methylation of a novel panel of tumor suppressor genes in urine moves forward noninvasive diagnosis and prognosis of bladder cancer: a 2-center prospective study,” The Journal of Urology, vol. 190, no. 2, pp. 723–730, 2013.

[17] C. T. Lee, R. L. Dunn, C. Ingold, J. E. Montie, and D. P. Wood Jr., “Early-stage bladder cancer surveillance does not improve survival if high-risk patients are permitted to progress to muscle invasion,” Urology, vol. 69, no. 6, pp. 1068–1072, 2007.