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Pyrosequencing Analysis of *APC* Methylation Level in Human Prostate Tissues: A Molecular Marker for Prostate Cancer

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Purpose: Epigenetic alterations such as abnormal DNA methylation are associated with many human cancers. Differences in methylation patterns between neoplastic and normal cells can be used to detect cancer. The aim of the present study was to evaluate the effectiveness of detecting *Adenomatous polyposis coli* (*APC*) hypermethylation by quantitative pyrosequencing for discriminating between normal and prostate cancer (PCa) cells and for predicting tumor behaviors.

Materials and Methods: A total of 218 human prostate tissues obtained from our institute were assessed: 106 specimens of benign prostatic hyperplasia (BPH) and 112 specimens of PCa. The methylation status of *APC* was analyzed by quantitative pyrosequencing. The association between the *APC* methylation level and clinicopathological parameters was explored.

Results: The level of *APC* methylation was significantly higher in PCa specimens than in BPH specimens (33.3%±20.7% vs. 1.3%±1.8%, $p < 0.001$). The sensitivity and specificity of *APC* methylation status in discriminating between PCa and BPH reached 89.3% and 98.1%, respectively. Similar results were obtained after stratification by stage, Gleason score, and prostate-specific antigen level. The *APC* methylation level correlated positively with Gleason score (p trend=0.016). There was no association between the *APC* methylation level and the PSA level or staging.

Conclusions: Our results demonstrate that *APC* methylation is associated with PCa and its aggressive tumor features.

Keywords: *Adenomatous polyposis coli; Biological markers; DNA methylation; Prostatic neoplasms; Sequence analysis*

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INTRODUCTION

DNA hypermethylation-induced silencing of tumor suppressor and DNA repair genes is a frequent phenomenon in cancer [1-4]. These hypermethylation markers are promising tools for detecting cancer cells in tissues and body fluids, and various methods for detecting aberrant DNA methylation have been developed in recent years. Which of these analytical methods is best suited for use in the clinical setting, however, remains unclear [2-6]. The majority of previous studies used methylation-specific polymerase chain reaction (MSP) or quantitative methylation-specific polymerase chain reaction (QMSP) to assess methylation and often artificially classified the data into simplistic hypermethylated or hypomethylated categories [2-4]. Despite common use, these methods have known disadvantages, including the generation of false-positive and false-negative results [7,8]. Pyrosequencing (PSQ) is one of the most accurate methods available for quantifying DNA methylation [3,7,8]. It is a sensitive and highly reproducible method that is uniquely suited to the analysis of clinical specimens from which only small amounts of DNA can be isolated [7,8]. To the best of our knowledge, relatively few studies of methylation in PCa have used the

PSQ method [8,9].

Adenomatous polyposis coli (APC) is a well-characterized tumor suppressor gene that regulates the Wingless-type (Wnt) signaling pathway via ubiquitin-mediated beta-catenin degradation. *APC* is consistently hypermethylated during PCa development, and *APC* hypermethylation has well-established diagnostic and prognostic significance [10-16]. The aim of the present study was to evaluate the relevance of *APC* methylation in discriminating between benign prostatic hyperplasia (BPH) and PCa and to determine whether *APC* methylation is associated with clinicopathological parameters by use of quantitative PSQ analysis.

MATERIALS AND METHODS

1. Human tissue samples

A total of 218 human prostate tissues obtained from our institute were assessed: 106 BPH and 112 PCa specimens. Patients with PCa underwent radical prostatectomy or palliative transurethral resection (TUR). Patients with BPH underwent TUR. All tissues were macrodissected within 15 minutes of surgical resection. Each prostate specimen was confirmed by pathological analysis of fresh-frozen sections, and the rest of the tissue was frozen in liquid nitrogen and stored at -80°C until use. The biospecimens for this study 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 local Institutional Review Board (GR 2011-04-006), and informed consent was obtained from each subject.

2. DNA extraction and PSQ analysis

Genomic DNA was extracted by standard methods by use of the Wizard Genomic DNA Purification System (Promega, Madison, WI, USA). Bisulfite modification of genomic DNA (500 ng) was performed by using the EZ DNA methylation kit (D5006, Zymo Research, Irvine, CA, USA) per the manufacturer's instructions. *APC* methylation was assayed by PSQ. Polymerase chain reaction (PCR) and sequencing primers were designed by using PyroMark Assay design software ver. 2.0.1.15 (Qiagen, Valencia, CA, USA). The PSQ assay was designed to evaluate the methylation status of four CpG sites. The primer sequences and amplification conditions are described in Table 1. A two-step PCR reaction was conducted by using 20 ng of bisulfite-converted genomic DNA. A biotin-labeled primer (reverse primer) was used to purify the final PCR product by using streptavidin-coated Sepharose beads (10041200; GE Healthcare, Milwaukee, WI, USA). The PCR product was bound to Sepharose beads, purified, washed, denatured by use of 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) per the manufacturer's instructions. To provide an internal control for total bisulfite conversion, a non-CG cytosine was included in the region targeted for PSQ where possible. Target CpG sites were evaluated by using the instrument software (PSQ96MA 2.1, Qiagen), which converts pyrograms to numerical values for peak heights and calculates the proportion of methylation at each base as a C/T ratio. Data analysis was performed by using PyroMark Q96 ID software v.1.0 (Qiagen).

3. Statistical analysis

Quantitative *APC* methylation levels were compared to clinicopathological characteristics. The methylation level of each sample was expressed as a mean value (the sum of each CpG site methylated level [%]/total number of CpG sites [n=4]). Differences in continuous variables between groups were assessed by the two-sample t-test. Categorical variables were compared by the chi-square test. For discriminating between PCa and BPH, receiver operator characteristic (ROC) curve analysis was applied (MedCalc ver. 12.0; MedCalc Software, Mariakerke, Belgium). The area under the ROC curve and the methylation threshold yielding optimal sensitivity and specificity for the prediction of PCa were calculated. Using the same thresholds, the sensitivity, specificity, positive predictive value, and negative predictive value were also calculated. Pearson's correlation was used to evaluate the relationship between *APC* methylation level and clinicopathological parameters. Tests for trend were performed by analysis of variance trend analyses by use of polynomial contrasts. For statistical purposes, PCa specimens were divided into sub-

TABLE 1. Primer sequences used in the pyrosequencing analysis

	Forward	Reverse	Sequencing primer
First step[a]	5'-GGTAAGGGGTTAGGGTTAGGTAG-3'	5'-ACAACACCTCCATTCTATCT-3'	
Second step[b]	5'-GGTAAGGGGTTAGGGTTAGGTAG-3'	5'-Biotin-ACTACACCAATACAACCACATATC-3'	AGGGTTAGGTAGGTT

[a]:The first step polymerase chain reaction (PCR) reaction contained 0.01 μM primers, bioneer Taq (Bioneer) and 20 ng of bisulfite-treated DNA. The thermocycling parameters were as follows: denaturation at 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds; and a final extension at 72°C for 5 minutes. [b]:The second step PCR reaction contained 0.01 μM primers, bioneer Taq (Bioneer) and 1 μL of first step PCR product. The thermocycling parameters were as follows: denaturation at 94°C for 5 minutes; 40 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds; and a final extension at 72°C for 5 minutes.

groups according to clinicopathological parameters as follows: 1) Gleason score (≤6, 7, ≥8), 2) clinical tumor-node-metastasis stage at diagnosis (T1-2, T3, T4; N0, N1; M0, M1), and 3) PSA level at diagnosis (<3, 3–10, ≥10 ng/mL). Statistical analysis was performed by using the SPSS ver. 12.0 (SPSS Inc., Chicago, IL, USA). Values of $p<0.05$ were considered statistically significant.

RESULTS

1. Baseline characteristics

The baseline characteristics of the study sample are presented in Table 2. There were no significant differences in mean age or total prostate volume between the BPH and PCa patients. PCa patients had elevated levels of PSA compared with BPH patients.

2. Methylation level in prostate tissues

The *APC* methylation level was significantly higher in the PCa samples than in the BPH samples (33.3%±20.7% vs. 1.3%±1.8%, $p<0.001$) (Fig. 1A). By ROC analysis, the area under the curve of the *APC* methylation level for the prediction of PCa reached 0.960 (95% confidence interval [CI], 0.934 to 0.987) (Fig. 1B). To ensure well-balanced sensitivity and specificity, an *APC* methylation threshold of 6.07% was used. That threshold showed 89.3% sensitivity (95% CI, 82.0 to 94.3), 98.1% specificity (95% CI, 93.4 to 99.8), 98.0% positive predictive value (95% CI, 93.1 to 99.8), and 89.7% negative predictive value (95% CI, 92.6 to 94.6). By use of that threshold, the patients were divided into two groups: hypermethylated and unmethylated. As presented in Table 3, *APC* was methylated in 89.3% (100/112) and 1.9% (2/106) of PCa and BPH samples, respectively. Similar results were obtained after stratification by PSA level, stage, and Gleason score.

TABLE 2. Baseline characteristics of patients

Characteristic	BPH (n=106)	PCa (n=112)	p-value
Age (y)	69.4±7.9	68.9±7.1	0.590
Prostate-specific antigen (ng/mL)	4.4±5.7	135.4±216.2	<0.001
Total prostate volume (mL)	40.7±23.7	41.9±22.8	0.723
Source of tissue			
Transurethral resection	106 (100)	55 (49.1)	
Prostatectomy		57 (50.9)	

Values are presented as mean±standard deviation or number (%).
BPH, benign prostate hyperplasia; PCa, prostate cancer.

3. Association between methylation level and clinicopathological parameters in PCa

To evaluate the relationship between *APC* methylation level and clinicopathological parameters, a correlation analysis was performed. No correlation was found between *APC* methylation level and age (r=−0.103, p=0.281) or serum PSA level (r=−0.085, p=0.372). By contrast, a positive correlation between *APC* methylation level and Gleason score was observed in PCa patients (r=0.190, p=0.045). *APC* methylation levels were also compared to clinicopathological parameters including Gleason score, stage at diagnosis, and PSA level at diagnosis. The *APC* methylation level was positively associated with the Gleason score (p trend=0.016) (Fig. 2), whereas no trend was found between the *APC* methylation level and PSA level or stage (p trend>0.05).

DISCUSSION

Our results showed that *APC* methylation levels are significantly higher in PCa patients than in BPH patients.

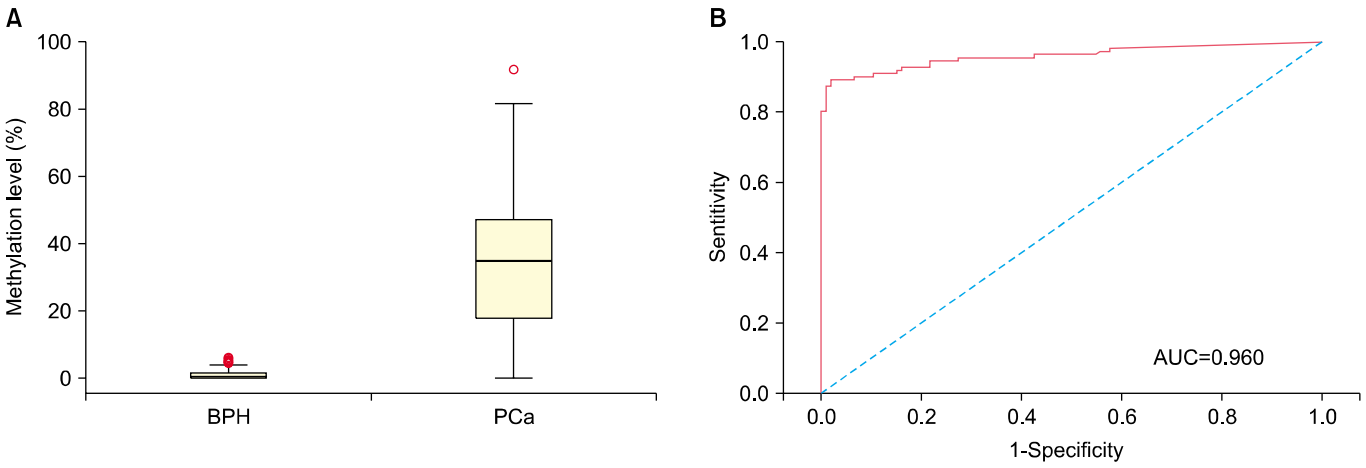

FIG. 1. (A) *Adenomatous polyposis coli (APC)* methylation level in benign prostatic hyperplasia (BPH) and prostate cancer (PCa). Differences (p-value) between BPH and PCa were evaluated by the two-sample t test. The Y axis indicates the quantitative *APC* methylation level. (B) ROC curve analysis of *APC* methylation for the prediction of PCa. The broken line represents the reference line. AUC, area under the curve.

TABLE 3. Frequency of *Adenomatous polyposis coli* promoter methylation in human prostate tissues

Variable	BPH (n=106)	PCa (n=112)
Methylation rate	2 (1.9)	100 (89.3)
PSA level (ng/mL)		
<3	0/55 (0)	1/1 (100)
3–10	5/32 (4.8)	23/26 (88.5)
≥10	0/9 (0)	76/85 (89.4)
Clinical T staging		
cT1-2	-	49/53 (92.5)
cT3	-	31/36 (86.1)
cT4	-	20/23 (87.0)
Clinical N staging		
N0	-	74/81 (91.4)
N+	-	26/31 (83.9)
Clinical M staging		
M0	-	71/81 (87.7)
M+	-	29/31 (93.5)
Gleason score		
≤6	-	4/6 (66.7)
7	-	40/44 (90.9)
8–10	-	56/62 (90.3)

Values are presented as no. of methylated samples/total no. of samples (%).
BPH, benign prostate hyperplasia; PCa, prostate cancer; PSA, prostate-specific antigen.

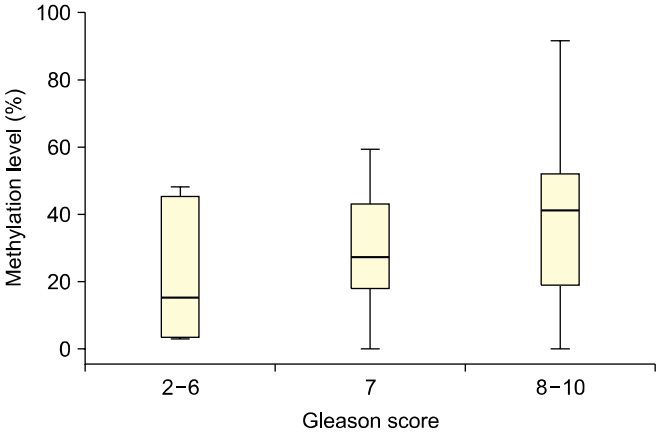

FIG. 2. Association between the *Adenomatous polyposis coli (APC)* methylation level and the Gleason score. Differences (p-value) between groups were evaluated by analysis of variance trend analysis. The Y axis indicates the quantitative *APC* methylation level.

Moreover, *APC* hypermethylation was not only associated with the increased incidence of PCa but was also positively correlated with increased Gleason score. By contrast, no significant relation was detected between APC methylation and age, stage, or PSA level in PCa patients.

Epigenetic alterations, such as abnormal DNA-methylation patterns, are associated with many human tumor types [2-4]. Differences in methylation patterns have also emerged as markers for cancer risk assessment, cancer diagnosis, and therapy monitoring in several different types of cancer [2-4]. DNA methylation is quite useful in cancer detection owing to the inherent stability of DNA compared with RNA or proteins [4]. As a disease marker, methylation is useful regardless of whether it functions in gene silencing or not, as long as it is specific to tumor cells or is associated with clinically important information [3,4]. In this regard, the present results suggest that the detection of *APC* methylation by PSQ has promising diagnostic value in PCa owing to its high sensitivity (89.3%) and specificity (98.1%).

Recently, several methodologies have become available to detect the methylation status of certain genes in clinical samples [3,4]. However, the results depend on the detection method, including the primer design, reagents, detectors, equipment, and protocols, which influences sensitivity and specificity. The use of conventional MSP is limited in cancer detection because benign lesions can be weakly positive and cannot be distinguished from cancer cases. Moreover, the results of MSP in any particular DNA region are reported simply and perhaps subjectively as methylated or unmethylated, without allowing the quantitation or identification of partial methylation. Quantitative analysis of DNA methylation status with appropriate methods might improve the accuracy of interpretation obtained from small amounts of DNA in clinical samples. In that respect, PSQ might be a better method because it provides quantitative information for each target CG site instead of qualitative information. In the present study, PSQ was used to measure methylation, and an optimal threshold level was identified to discriminate between PCa and normal controls. The threshold was relatively low (6.07%) and might therefore be misleading with other techniques [3,7,8]. One of the major advantages of PSQ is the ability to compare samples quantitatively and to segregate various pathologic covariates accurately on the basis of methylation levels. Without PSQ, no significant correlation between *APC* methylation level and Gleason score could be detected in the present study.

APC is a well-characterized tumor suppressor. It downregulates Wnt signaling by targeting the transcriptional coactivator beta-catenin for proteasomal degradation, thereby preventing its association with the nuclear transcription factor T-cell factor/lymphoid enhancer factor [10]. The Wnt pathway plays a central role in tumorigenesis. Its inappropriate activation is a common feature of many human cancers, leading to the deregulation of cell proliferation and differentiation [17]. Initially identified in colorectal cancer, *APC* is inactivated in various malignancies, including PCa, by genetic and epigenetic mechanisms [11-16]. The methylation status of *APC* can be used to distinguish benign tissues from PCa [13-16]. Furthermore, the methylation status of *APC* correlates significantly with clinicopathological variables, including tumor stage, grade, and poor prognosis [11-14]. Our findings are consistent with previous results and indicate that *APC* hy-

permethylation is a reliable predictor of PCa and of its aggressive features [13,14].

Although histologically confirmed prostate tissues were used, the possibility of unrevealed PCa in BPH patients and an undetected small fraction of methylated DNA might affect our results for sensitivity and specificity. Additionally, determination of the Gleason score with different surgical methods such as TUR and prostatectomy may be a limitation of our study. Nonetheless, promising methylation frequency results were obtained. The sensitivity of *APC* methylation analysis by PSQ and its specificity for PCa over benign tissue reached 89.3% and 98.1%, respectively. Moreover, the frequency of *APC* methylation in PCa was independent of serum PSA level, Gleason score, and stage. Although these findings are promising, these kinds of studies must be performed with body fluids (urine and blood) to have clinical relevance. For these reasons, multicenter, large-scale clinical validation studies using primary human cancer tissues and body fluids are currently underway at our institute to confirm *APC* as a diagnostic methylation marker for PCa. These studies will improve our understanding of the biological role and clinical relevance of *APC* methylation in tumorigenesis.

CONCLUSIONS

Our study demonstrates that *APC* methylation is associated with PCa and its aggressive tumor features.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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