Abstract

**Purpose:** Abnormal DNA methylation is associated with many human cancers. The aim of the present study was to identify novel methylation markers in prostate cancer (PCa) by microarray analysis and to test whether these markers could discriminate normal and PCa cells.

**Experimental Design:** Microarray-based DNA methylation and gene expression profiling was carried out using a panel of PCa cell lines and a control normal prostate cell line. The methylation status of candidate genes in prostate cell lines was confirmed by real-time reverse transcriptase-PCR, bisulfite sequencing analysis, and treatment with a demethylation agent. DNA methylation and gene expression analysis in 203 human prostate specimens, including 106 PCa and 97 benign prostate hyperplasia (BPH), were carried out. Further validation using microarray gene expression data from the Gene Expression Omnibus (GEO) was carried out.

**Results:** Epidermal growth factor–containing fibulin-like extracellular matrix protein 1 (EFEMP1) was identified as a lead candidate methylation marker for PCa. The gene expression level of EFEMP1 was significantly higher in tissue samples from patients with BPH than in those with PCa (P < 0.001). The sensitivity and specificity of EFEMP1 methylation status in discriminating between PCa and BPH reached 95.3% (101 of 106) and 86.6% (84 of 97), respectively. From the GEO data set, we confirmed that the expression level of EFEMP1 was significantly different between PCa and BPH.

**Conclusion:** Genome-wide characterization of DNA methylation profiles enabled the identification of EFEMP1 aberrant methylation patterns in PCa. EFEMP1 might be a useful indicator for the detection of PCa. Clin Cancer Res; 17(13); 4523–30. ©2011 AACR.

Introduction

Although prostate cancer (PCa) continues to be a frequent cause of morbidity and death throughout the world, it can be diagnosed only by prostate biopsy. The indications for a prostate tissue biopsy include a finding of elevated serum prostate-specific antigen (PSA) and/or the results of a digital rectal examination. However, a significant proportion of biopsies are negative because of the low sensitivity and specificity of these tests (1, 2). Furthermore, the biopsy procedure itself can induce pain, discomfort, anxiety, and complications and increases medical costs (3). These limitations have spurred interest in other molecular parameters that could be used for more accurate, noninvasive diagnosis of PCa. To date, several potential biomarkers for the detection of PCa have been identified through molecular, biological, and genetic studies, but their predictive value remains to be conclusively verified (4–8).

Epigenetic alterations such as abnormal DNA methylation are associated with many types of human cancer. In fact, aberrant DNA methylation has been recognized as one of the early events in tumorigenesis (9–11). Thus, differences in methylation patterns between normal and tumor cells could potentially be exploited for the detection of tumor cells in biopsy specimens or to identify tumor-derived DNA in body fluids (7, 10). The advent of high-throughput microarray technology makes possible comprehensive insights into the molecular basis of human diseases (12). With this technology, genome-wide DNA methylation patterns and RNA expression levels in tumor specimens can be evaluated simultaneously, and molecular targets or gene classifiers that are specific for tumor cells can be identified (5, 13).

The ability to detect the presence or absence of PCa would be an invaluable tool for guiding appropriate management strategies. The aim of the present study was to...
identify novel epigenetic markers that can discriminate between normal and PCa cells by microarray analysis of DNA methylation and RNA expression patterns.

Materials and Methods

Cell lines and human tissue samples

The human PCa cell lines LNCaP-FGC (KCLB 21740), DU-145 (KCLB 30081), and PC-3 (KCLB 21435) and the normal human prostate epithelial RWPE-1 cell line (ATCC CRL-11609) were purchased from the Korean Cell Line Bank (Seoul, South Korea) and American Type Culture Collection, respectively. DU-145 cells were maintained in Dulbecco’s modified Eagle’s medium (WelGENE Inc.), LNCaP cells were maintained in RPMI 1640 medium (WelGENE Inc.), and PC-3 cells were cultured in Nutrient Mixture F-12 Ham medium (GIBCO). RWPE-1 cells were cultured in Defined Keratinocyte-SFM (GIBCO). All media were supplemented with 10% FBS (WelGENE Inc.) and L-glutamine, and cells were incubated in a humidified atmosphere with 5% CO2 at 37°C.

A total of 203 human prostate tissue samples were obtained from our institute and used for the validation of the screening results: 97 benign prostate hyperplasia (BPH) and 106 PCa (Table 1). Patients with PCa underwent radical prostatectomy (RP) or palliative transurethral resection (TUR); patients with BPH underwent TUR. Study design and validation strategies are outlined in Figure 1.

All tissues were macrodissected within 15 minutes of surgical resection. Each prostate specimen was confirmed by pathologic analysis of a part of the tissue sample in fresh frozen sections of the RP/TUR specimen and then frozen in liquid nitrogen and stored at −80°C until use. The collection and analysis of all samples were approved by the local Institutional Review Board, and informed consent was obtained from each subject.

DNA and RNA extraction

Genomic DNA was extracted by standard methods by using the Wizard Genomic DNA Purification System (Promega). Total RNA was extracted with TRIzol reagent (Life Technologies) according to the manufacturer’s protocol.

Table 1. Baseline characteristics of patients included in the study

<table>
<thead>
<tr>
<th></th>
<th>BPH (n = 97)</th>
<th>PCa (n = 106)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>Median (range)</td>
<td>67 (46–83)</td>
</tr>
<tr>
<td>PSA, ng/mL</td>
<td>Median (range)</td>
<td>1.6 (0.1–7.7)</td>
</tr>
<tr>
<td>Gleason score, n (%)</td>
<td>≤6</td>
<td>5 (4.7)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>41 (38.7)</td>
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<tr>
<td></td>
<td>8–10</td>
<td>60 (56.6)</td>
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<tr>
<td>T staging, n (%)</td>
<td>T2</td>
<td>54 (50.9)</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>30 (28.3)</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>22 (20.8)</td>
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</tbody>
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Translational Relevance

To identify novel methylation markers at the whole-genome level, rigorous selection criteria, as well as confirmatory studies and validation using human tissue specimens, are mandatory. In the current study, DNA methylation status and gene expression levels were considered simultaneously in a screen for candidate genes. Methylation status and expression levels of potential targets were then verified by various molecular techniques. The validity of the final candidate methylation marker was determined in a relatively large sample of human prostate tissues. Independent validation using microarray gene expression data from the Gene Expression Omnibus (series GSE6919) representing a Western population was also carried out. To the best of our knowledge, this investigation is the first to identify EFEMP1 as a novel methylation marker for prostate cancer. Furthermore, large-scale validation studies with human samples and functional investigation of EFEMP1 will improve our understanding of its role in the tumorigenic process and its clinical relevance.
The quality and integrity of the RNA were confirmed by agarose gel electrophoresis and ethidium bromide staining, followed by visual examination under UV light. cDNAs were synthesized from 1 μg of total RNA by random priming by using a First-Strand cDNA Synthesis kit (Amer sham Biosciences).

**DNA methylation profiling in cell lines**

Methylation patterns were assayed using the genome-wide Illumina Infinium HumanMethylation27 BeadChip array (Illumina Inc.), which enables interrogation of 27,578 Cpg dinucleotides covering 14,495 genes. Methylation assays were carried out according to the manufacturer's protocol. Bisulfite conversion of genomic DNA was carried out using the EZ DNA Methylation Kit (Zymo Research). Fluorescence signals corresponding to C- or T-nucleotides were measured, and the data were used to assign a quantitative measure of methylation level (β value). Each methylation data point represents the fluorescent signal from the methylated (M) and unmethylated (U) alleles. Background intensity was computed from a set of negative controls and subtracted from each analytic data point. The ratio of the fluorescent signals from the 2 alleles was then computed. The β value represents a quantitative measure of the DNA methylation level of specific Cpg islands, and ranges from 0 (completely unmethylated) to 1 (completely methylated).

**Gene expression profiling in cell lines**

For the integrated analysis of global methylation status and gene expression levels, we used the genome-wide HumanHT-12 Gene Expression BeadChip (Illumina Inc.), which covers 48,804 genes. Gene expression analysis was carried out according to the manufacturer's protocol. The details of the experimental method have been previously described (14, 15). In brief, the quality and integrity of the RNA were confirmed by agarose gel electrophoresis and ethidium bromide staining, followed by visual examination under UV light. Five hundred nanograms of total RNA was used for labeling hybridization according to the manufacturer's protocol. Arrays were scanned with an Illumina BeadArray Reader confocal scanner (BeadStation 500GXDW; Illumina Inc.), according to the manufacturer's instructions. Initial microarray gene expression data were obtained using the gene expression analysis module of Bead Studio software (version 3.1.3; Illumina Inc.).

**Real-time reverse transcriptase-PCR**

To verify the influence of methylation status on gene expression, real-time reverse transcriptase-PCR (RT-PCR) analysis of selected genes was carried out using a Rotor Gene 3000 PCR system (Corbett Research). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was analyzed in parallel as an internal control. RT-PCR reaction mixtures containing primers and SYBR Premix EX Taq (Takara Bio Inc.) were analyzed in microreaction tubes (Corbett Research). Spectral data were captured and analyzed using Rotor-Gene Real-Time Analysis Software 6.0 (Build 14; Corbett Research). Gene expression was normalized to the expression of GAPDH.

**5-Aza-2'-deoxycytidine treatment**

PCa cell lines were cultured until confluent and then treated with 5-aza-2'-deoxycytidine (AZA; Sigma Aldrich). Cells were exposed continuously to AZA (8.8 μmol/L) for 3 days. The duration and dosage of AZA treatment were based on previous results, showing that 8.8 μmol/L AZA induces the reexpression of silenced genes without excessive cell death (16, 17).

**Bisulfite genomic sequencing**

The methylation status of candidate genes was analyzed using bisulfite-modified genomic DNA, in which unmethylated, but not methylated, cytosine has been converted to uracil. Genomic sequencing of both strands of the candidate gene promoter was carried out after bisulfite treatment of genomic DNA. Following agarose gel electrophoresis, amplified fragments were excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen). Methylated nucleotides were verified by sequencing of the fragments with an ABI Prism 3700 XL DNA analyzer (Applied Biosystems). Cytosine peaks in the chromatogram with a height of 0.4 or more relative to the thymine peak height were considered as indicative of methylation in bisulfite genomic sequencing (BGS; ref. 18).

**Statistical analysis**

DNA methylation and gene expression profile data were normalized using quantile normalization in the R language environment (version 2.10.0; available at http://www.r-project.org/). Measured gene expression intensity was log_{2} transformed and median centered across genes and samples. As a means of comparing profile patterns between DNA methylation and gene expression arrays, the microarray data for DNA methylation were pooled with the microarray data set for gene expression by using lookup table information provided from the microarray platform manufacturer (Illumina Inc.). Two criteria were applied to detect genes whose methylation level differed significantly between PCa and control cells: (i) DNA methylation level (β) in cancer and control cells differed by at least 0.5; and (ii) gene expression level between cancer and control cells differed by at least 2-fold. To further validate genes identified in the initial cohort, we analyzed microarray data from prostate cancer tissues and organ donor prostate tissues, as reported by Yu and colleagues [available at Gene Expression Omnibus (GEO) genomics data repository: under the data series accession number GSE6919; ref. 19].

**Results**

**Identification of differentially methylated and expressed genes in PCa and normal cell lines**

Genome-wide methylation and expression profiles from 3 different PCa cell lines (LNCaP-FGC, DU-145, and PC-3)
were compared with control RWPE-1 prostate cells. The complete set of microarray data from the prostate cell lines is available online (http://www.ncbi.nlm.nih.gov/geo/) under the data series accession number GSE23388. Using highly stringent selection criteria (β difference >0.5), we identified 106 unique genes that were hypermethylated in all 3 PCa cell lines. Of these 106 unique hypermethylated genes, 13 exhibited at least a 2-fold difference in gene expression in all 3 cell lines as compared with control cells. To identify regulatory CpG islands in the promoters of these 13 candidate genes, we analyzed the locations of 5′-CpG islands (provided by the manufacturer) and putative transcriptional start sites using all available genome databases. The results of this analysis revealed that 5 of the 13 candidate genes contained CpG islands that were located upstream of the true transcriptional start site.

**Analysis of changes in gene expression levels by AZA treatment**

To confirm that DNA methylation resulted in gene silencing of the 5 identified candidate genes, we carried out gene expression analysis by RT-PCR. The expression levels of all 5 genes were significantly decreased in PCa cells as compared with control cells (each P < 0.05; data not shown). To determine whether the silencing of these genes could be reversed, PCa cells were treated with the DNA methyl transferase inhibitor AZA and then expression was analyzed by RT-PCR. Expression of 3 of the 5 candidate genes was significantly enhanced in response to AZA treatment in all 3 PCa cell lines as compared with the corresponding untreated control cells; the remaining 2 genes were either unchanged or their expression was altered in only one of the cell lines (data not shown). These 3 final candidate genes, identified as protein phosphatase 1 regulatory (inhibitor) subunit 14C (PPP1R14C), epidermal growth factor–containing fibulin-like extracellular matrix protein 1 (EFEMP1), and Islet1 (ISL1), were subjected to further analysis as putative methylation-silenced genes in PCa cells.

**Confirmation of CpG island hypermethylation by using BGS in prostate cell lines**

To determine whether the final 3 candidate genes were inactivated in PCa cells by promoter hypermethylation, we carried out BGS by using bisulfite-modified genomic DNA from prostate cell lines. BGS of CpG sites that were included in the array confirmed that the methylation patterns of PPP1R14C, EFEMP1, and ISL1 were significantly different in PCa and normal prostate cells. The majority of the CpG sites in the promoter regions of the 3 candidate genes were either completely or partially methylated in all 3 PCa cell lines, but not in control cells.

**Validation of methylated genes in human prostate tissues**

In a preliminary validation experiment, we analyzed the methylation status of the 3 candidate methylated gene markers in a small set of validated human prostate tissue samples (10 PCa and 10 BPH) by BGS. Of the 3 candidate markers, EFEMP1 was the best performing methylated marker in terms of sensitivity and specificity for PCa versus control BPH (data not shown). For further validation of EFEMP1, we analyzed 106 PCa and 97 control BPH tissue specimens (including those analyzed in the preliminary validation) by RT-PCR and BGS. The primer sequences used for RT-PCR and BGS and the results of BGS of EFEMP1 promoter CpG island methylation are illustrated in Figure 2. The level of expression of EFEMP1 was significantly higher in BPH than in PCa samples (P < 0.001; Fig. 3A). As presented in Table 2, EFEMP1 was methylated in 95.3% (101 of 106) and 13.4% (13 of 97) of PCa and BPH samples, respectively. Similar results were obtained even after stratification by stage, Gleason score, and PSA level. The validity of EFEMP1 as a methylation marker in PCa cells was also evaluated using an independent set of microarray data from a Western population, which included prostate cancer (n = 90) and organ donor prostate (n = 18) tissues (19). Similar to the current set of results, the expression level of EFEMP1 was significantly lower in PCa than in normal controls in the independent data set (P < 0.001; Fig. 3B).

**Discussion**

Using microarray analysis to detect novel epigenetic markers that are relevant to PCa, we identified significant differences in the methylation patterns of 3 genes in PCa and normal cell lines. Further analysis revealed an association between gene silencing due to promoter hypermethylation and reduced gene expression levels for all 3 genes in PCa cell lines. In validation experiments using human specimens, EFEMP1 emerged as the strongest candidate methylation marker, being methylated in most human PCa tissues but rarely methylated in noncancerous prostate tissue. Altered expression of EFEMP1 in PCa versus BPH was confirmed in an independent microarray data set.

Analysis of aberrant DNA methylation is gaining traction in the areas of cancer risk assessment, diagnosis, therapy monitoring, and novel drug targets for several different types of cancer (5, 11, 13, 20–23). Recently, the feasibility of detecting aberrant DNA methylation in tissue and body fluids and the potential role of DNA methylation as a tumor marker for PCa were reported (7, 20–22). Some methylation markers, such as GSTP1 and RASSF1A, exhibited high sensitivity and specificity for the detection and monitoring of PCa (7). Although histologically confirmed prostate tissues were used, the possibility of unrevealed PCa in BPH patients and undetected small fraction of methylated DNA molecule might affect our results of sensitivity and specificity. Nonetheless, in terms of methylation frequency, our results were comparable with previous reports (7, 20–22). The sensitivity and specificity of EFEMP1 methylation in distinguishing PCa and benign tissue reached 95.3% and 86.6%, respectively. Moreover, the frequency of EFEMP1 methylation in PCa was independent of serum PSA level, Gleason score, and stage. Although
Figure 2. BGS of EFEMP1 in cell lines and human tissues. A, schematic representation of EFEMP1 (accession number NM_018894.1) on chromosome locus 2p16, the location of the CpG island, and the region amplified for BGS. B, representative results of BGS of EFEMP1 promoter. CpG island methylation status in prostate cell lines (B) and human prostate samples (C). Unmethylated and methylated bisulfate-converted sequences are indicated above the chromatograms.
these findings are promising, large-scale clinical validation studies using primary human cancer tissue and body fluids are currently underway at our institute to confirm EFEMP1 as a diagnostic PCa methylation marker.

Because DNA methylation is reversible, it is considered a good therapeutic target. Drugs that target epigenetic alterations, such as DNA methylation inhibitors, restore the activity of genes by targeting aberrant heterochromatic regions, ultimately leading to the reactivation of tumor suppressor genes and/or other genes that are crucial for normal cellular function (24). EFEMP1 methylation was reversed by treatment with the DNA methyl transferase inhibitor AZA, which supports EFEMP1 as a putative therapeutic target for the treatment of PCa.

EFEMP1, also known as fibulin-3, belongs to the fibulin family, a widely expressed family of extracellular matrix proteins that regulate cell proliferation in a context-specific manner (25). EFEMP1 mediates cell-to-cell and cell-to-matrix communication, provides organization and stability to extracellular matrix structures, and seems to function as an antagonist of angiogenesis (26). Interestingly, EFEMP1 is a binding partner of tissue inhibitor of metalloproteinase-3 (TIMP-3; ref. 27). Fibulin-3 and another fibulin family member, fibulin-5, can antagonize tumor angiogenesis in vivo (26), which suggests that concerted deregulation of a set of antiangiogenic factors, such as fibulin-3 and TIMP-3, might contribute to tumorigenesis. Inactivation of EFEMP1 due to DNA hypermethylation has been reported recently in lung cancer and hepatocellular carcinoma (28, 29). To the best of our knowledge, the current study is the first to identify EFEMP1 as a methylation marker for PCa. Although the precise function of EFEMP1 and whether it has a causal relationship to PCa have yet to be determined, further large-scale validation studies with human samples and functional investigation of EFEMP1 will improve our understanding of its role in tumorigenesis and its clinical relevance.

Comprehensive insight into the molecular basis of human diseases is becoming a reality with microarray-based clinical research, and new molecular targets at the whole-genome level are rapidly emerging (5, 30, 31). The number of putative cancer-specific methylation markers is vast, and genome-wide techniques will likely bring to light new markers with improved sensitivity and

| Table 2. Frequency of EFEMP1 promoter methylation in human prostate tissues |
|-----------------|-----------------|-----------------|
|                  | BPH (n = 97)    | PCa (n = 106)   |
| Methylation rate | 13.4 (13/97)    | 95.3 (101/106)  |
| PSA level, ng/mL |                 |                 |
| <3               | 12.3 (8/65)     | 100 (3/3)       |
| 3–10             | 15.6 (5/32)     | 93.8 (15/16)    |
| 10–20            | –               | 93.8 (15/16)    |
| ≥20              | –               | 95.8 (68/71)    |
| T staging        |                 |                 |
| T2               | –               | 96.3 (52/54)    |
| T3               | –               | 93.3 (28/30)    |
| T4               | –               | 95.5 (21/22)    |
| Gleason score    |                 |                 |
| ≤6               | –               | 100 (5/5)       |
| 7                | –               | 95.1 (39/41)    |
| 8–10             | –               | 95.0 (57/60)    |

*Methylation rate is expressed as percentage (number of methylated samples/total number of samples).
Hodges E, Smith AD, Kendall J, Xuan Z, Ravi K, Rooks M, et al. High specificity. To date, genome-wide screening for regions that are differentially methylated in normal and tumor cells has typically resulted in the isolation of many differentially methylated DNA fragments (10, 32). However, to identify bona fide candidate methylation markers, rigorous selection criteria, confirmation of methylation status and expression level, verification of reexpression upon exposure to demethylating agents, and validation in human tissues are mandatory (10). In the current study, DNA methylation status and gene expression levels were considered simultaneously in a screen for candidate genes. Methylation status and expression levels of potential targets were verified by BGS and RT-PCR, and reversal of methylation-induced gene silencing by treatment with AZA was confirmed. The validity of the lead candidate methylation marker was determined using human prostate tissue samples from a relatively large patient population. The gene expression level of EFEMP1 was also independently verified using microarray data deposited in the online GEO database. The current results, derived from a single-genome analysis and confirmed by several independent techniques, strongly support EFEMP1 as a novel candidate epigenetic marker for the detection of PCAs and possibly a candidate therapeutic target as well. Continued effort along these lines will enhance the accuracy of PCA detection and diagnosis and lead to new therapies that target-specific molecular defects, thereby significantly lowering the morbidity associated with PCAs.

In conclusion, characterization of the DNA methylation profiles of PCAs at the genome-wide level resulted in the identification of EFEMP1, a gene that is aberrantly methylated in PCAs. This methylation marker could assist in more accurate detection and monitoring of PCAs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This research was supported by Basic Science Research Program of the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (2011-003333 and 2011-0001047).

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Received October 20, 2010; revised March 23, 2011; accepted April 30, 2011; published OnlineFirst May 13, 2011.

References

Clinical Cancer Research

**EFEMP1 as a Novel DNA Methylation Marker for Prostate Cancer: Array-Based DNA Methylation and Expression Profiling**

Yong-June Kim, Hyung-Yoon Yoon, Seon-Kyu Kim, et al.

*Clin Cancer Res* 2011;17:4523-4530. Published OnlineFirst May 13, 2011.

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