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Assessment of DNA methylation for the detection of cervical neoplasia in liquid-based cytology specimens

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ABSTRACT

Objective. DNA methylation is an early event in carcinogenesis. Testing for DNA methylation has potential in cancer screening. The aim of this study was to investigate the feasibility of methylated DNA detection as a screening tool for squamous cell carcinomas (SCC) and squamous intraepithelial lesions (SIL) in cervical scrapings.

Methods. A multiplex, nested, methylation-specific polymerase chain reaction approach was used to examine promoter methylation of 12 genes (CDH1, DAPK, GSTP1, HIC-1, HIN-1, hMLH1, MGMT, p16, RAR-β, RASSF1A, SHP-1, and Twist) in biopsy-proven SCC ($n = 69$), high-grade SIL (HSIL, $n = 67$), low-grade SIL (LSIL, $n = 32$), and negative ($n = 41$) liquid-based cytology samples.

Results. The methylation frequency in normal, LSIL, HSIL, and SCC was significantly different ($p<0.01$) for eight genes (DAPK, HIC-1, HIN-1, MGMT, RAR-β, RASSF1A, SHP-1, and Twist). There was a trend toward increasing methylation of HIN-1, MGMT, RAR-β, RASSF1A, and SHP-1 with increasing severity of cervical squamous lesions. The number of methylated genes increased with the severity of cervical squamous lesions ($p<0.001$). In receiver-operating characteristic analysis, the three-gene combination (RAR-β/Twist/MGMT) showed the best performance to distinguish HSIL/SCC from LCIS/negative samples. The estimated specificity of this three-gene panel for detecting HSIL/SCC was 82.2%, and its sensitivity was 78.7%.

Conclusion. Although aberrant DNA methylation has the potential to function as a molecular biomarker of HSIL and SCC in liquid-based cytology tests, additional genes that are selectively methylated in HSIL and SCC are needed to improve clinical performance.

Introduction

Cervical cancer is a major cause of death in women worldwide and the fourth most common malignancy in Korea [1]. In Korea and other developed countries, the incidence and mortality rates of invasive cervical squamous cell carcinoma (SCC) have decreased significantly over the past decades [2]. This decline, in large part, has been due to cervical cytology screening programs and the identification and treatment of women with precursor squamous intraepithelial lesions (SIL). However, identification of cervical SCC and its precursor SIL, which relies on microscopic examination of exfoliated cervical cells, is associated with many problems, including low test sensitivity [3,4]. Therefore, new objective diagnostic methods, based on molecular changes specific for cervical carcinogenesis, are needed.

DNA methylation is an enzyme-induced chemical modification that usually occurs in cytosine-guanine dinucleotide-rich areas (CpG islands) in the gene promoter regions. Aberrant promoter methylation is an important mechanism for loss of gene function in tumors, and it may be more common than coding region mutations are [5,6]. As promoter hypermethylation is an early event in carcinogenesis and is often present in the precursor lesions of a variety of cancers, DNA methylation could serve as a marker for the early diagnosis of cancer [7–9].

Recently, the role of promoter methylation of tumor suppressor genes has been examined in cervical carcinogenesis [10,11]. Aberrant promoter methylation analysis on exfoliated cell samples may be used as a diagnostic tool for cervical cancer screening. At present, although there is some evidence to suggest that increased rates of hypermethylation various genes may be associated with cervical cancer, little data are available regarding sensitivity and specificity of the detection of hypermethylated genes for the identification of women with biopsy-confirmed SCC and its precursor SIL using exfoliated cell samples [12–19]. The goal of the present study was to determine if detection of hypermethylated genes in exfoliated cell samples might be used for a screening assay to identify women with SCC or its precursor lesions. A multiplex, nested, methylation-specific polymerase chain reaction (MSP) approach was used to examine promoter methylation of 12 genes (CDH1, DAPK, GSTP1, HIC-1, HIN-1, hMLH1, MGMT, p16, RAR-β, RASSF1A, SHP-1, and Twist) in biopsy-proven SCC, high-grade SIL (HSIL), low-grade SIL (LSIL), and negative specimens from liquid-based cytology samples. These genes were

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Table 1
Concordance between nested and conventional methylation-specific PCR for methylated RAR-β gene.

	Conventional methylation-specific PCR		p-value
	Unmethylated (%)	Methylated (%)	
Nested methylation-specific PCR			0.000
Unmethylated	132 (98.5)	2 (1.5)	
Methylated	3 (4.0)	72 (96.0)	

selected because they are frequently methylated in cervical cancer or other malignancies.

Materials and methods

Samples

Randomly selected biopsy-confirmed SCC ($n=69$), HSIL ($n=67$), LSIL ($n=32$), and malignancy-negative ($n=41$)) residual, liquid-based samples were identified from the pathology archives of the Chonnam National University Hwasun Hospital after approval was granted by the institutional review board. Malignancy-negative cervical cytologic samples were obtained from patients who were planned to undergo a hysterectomy for benign gynecological diseases. We confirmed normal cytologic diagnosis by the histopathological examination of cervical specimens. Cervical samples were collected using the Thin Prep Cytology Collection System (CYTYC Corporation, Marlborough, MA) according to the manufacturer's protocol. The sample vials were stored at ambient temperature and used within 30 months of the collection date. Mean storage time was 17 months, with a range of 4–30 months. Genomic DNA was isolated from cells spun down from 5 ml of Thin Prep Cytology Collections.

Sodium bisulfite treatment and MSP

Sodium bisulfite treatment was scaled down to a micromethod, as described previously [20]. DNA (13.5 μl) was heated for 10 min to 99 °C, quickly chilled, and incubated with 1.5 μl of 2 mol/L NaOH for 30 min at 42 °C. Freshly prepared 3.6 mol/L sodium bisulfite containing 1 mmol/L hydroquinone was mixed with the DNA, overlaid with oil, and incubated at 55 °C for 5 h in the dark. The sample was desalted using ion exchange columns (MicroSpin S200, Amersham, MA). DNA was precipitated with 200 μl absolute alcohol, washed with 70% ethanol, air dried, and resuspended in water (20 μl). Samples were aliquoted and stored at −80 °C until use.

The methylation status of the 12 different genes was analyzed by MSP using a multiplex, nested PCR approach, as described previously [21]. Step 1 of the nested MSP was performed with primer sets (sense and antisense) for all five individual genes in each reaction. Step 1 primers flanked the CpG-rich promoter regions of their respective targeted genes and contained no CpG dinucleotides. Hence, these primers did not discriminate between methylated and unmethylated nucleotides after bisulfite treatment. A negative control for the assay (water only) and unmethylated (human sperm) and methylated (MDA-MB-231 breast cancer cells) DNA controls were included in each set of multiplex step 1 reactions. Depending upon the starting amount of DNA, PCR products from step 1 reactions were diluted 1:5 to 1:1000 in water and subjected to the second step consisting of the MSP reaction. This incorporated two sets of primers for each gene (labeled as unmethylated or methylated) that were designed to recognize differences arising from bisulfite-induced modifications of unmethylated cytosines. All the primer sequences and PCR conditions for this nested MSP approach have been published previously [13, 22–24]. The PCR products underwent electrophoresis on 2% agarose gel and were visualized under UV illumination using ethidium bromide staining. Any tumor sample that reliably yielded a PCR product in the methylated reaction was considered positive for promoter hypermethylation.

To validate the reliability of the nested MSP, we performed conventional MSP using RAR-β primers for methylated sequence in all of the specimens, in accordance with previously reported protocol [25].

Statistical significance

Concordance between nested MSP and conventional MSP were determined using χ^2 tests. The linear by linear association for the trend was used to assess the statistical significance of the trend in the proportion of samples in which methylation was detected with increasing severity of cervical squamous lesions. The trend of increasing methylation with increasing severity of cervical squamous lesions was determined using Spearman's rank correlation test. Clinicopathologic features of the patients with SCC were compared with methylation using χ^2 tests.

Because the goal of cervical cancer screening programs that utilize the Papanicolaou test is to identify and treat women with precancerous HSIL and SCC, we calculated the sensitivities and specificities of methylation markers alone or in sequential combinations for the diagnosis of HSIL/SCC. Candidate genes were chosen by maximizing the sensitivity with a least 90% specificity. Sensitivity was defined as the percentage of HSIL or SCC cases in which gene methylation was detected. Specificity was defined as the percentage of negative or LSIL samples in which all genes were unmethylated. Receiver operating characteristic (ROC) analysis, with area under the ROC curve as a measure of test performance, was also used to identify a panel of genes in which positive methylation status could distinguish HSIL/SCC samples from LSIL/negative samples.

For all statistical analyses, the SPSS system for the personal computer (version 13.5 for Windows; SPSS Inc., Chicago, IL) was used, with $p<0.05$ regarded as statistically significant.

Results

Frequency of promoter hypermethylation

The correlation between nested and conventional MSP was statistically significant ($p<0.001$) with an agreement rate of 97.6% (Table 1, Fig. 1). There were five discrepant cases, three of them representing nested MSP methylated, conventional MSP unmethylated, and two the converse.

Representative examples of the MSP products obtained from step 2 of the nested MSP analysis for the RAR-β and RASSF1A genes are shown in Fig. 2.

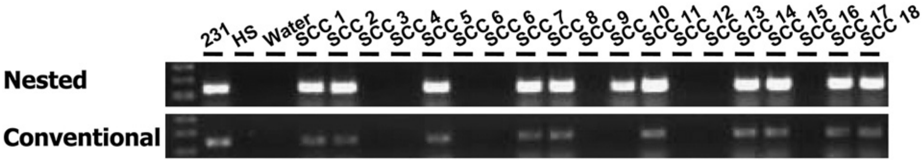

Fig. 1. Representative results of nested and conventional methylation-specific PCR analysis for methylated RAR-β genes in squamous cell carcinoma (SCC) samples. DNA from MDA-MB-231 (231) breast cancer cell lines and human sperm (HS) served as positive and negative controls for methylated genes, respectively. Water served as a negative control.