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Original Article

Analysis of promoter methylation of four cancer-related genes in samples of cervical tissue with high-grade squamous intraepithelial lesions, squamous cell carcinoma *in situ*, and early squamous cell carcinoma

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ABSTRACT

Objectives: Promoter methylation of some cancer-related genes may occur in many cancers and also in their precancerous lesions. This study examined adenomatous polyposis coli (APC), glutathione S-transferase, pi-class (GSTP1), prostaglandin-endoperoxide synthase 2 (PTGS2), and retinoic acid receptor beta (RARB) genes to assess if they are sensitive methylation markers when used to detect high-grade squamous intraepithelial lesions (HSIL) and early cancer in cervical tissues.
Materials and methods: DNA was obtained from 11 HSILs, 20 samples of squamous cell carcinoma (SCC) *in situ* (SCIS), and 16 samples of early SCC. The promoter methylation status of the selected genes was assessed using a methylation-specific polymerase chain reaction (MSP).
Results: One SCC sample was noninformative for all four genes. Five of the remaining samples were informative for three genes and 41 samples for all four genes. The rate of detection rate of at least one gene in the SCC group (60.0%, 9/15) was significantly higher than in the HSIL group (27.2%, 3/11) and the SCIS group (15.0%, 3/20) group ($p = 0.025$). The highest detection rate for PTGS2 was seen in the SCIS group (11.1%, 2/18) with the highest rates for APC (20.0%, 3/15), GSTP1 (7.1%, 1/14), and RARB (28.6%, 4/14) in the SCC group. Only RARB exhibited a significantly higher detection rate in the SCC group than in the other two groups ($p = 0.027$).
Conclusion: The results confirmed that promoter methylation of APC, GSTP1, PTGS2, and RARB is not prevalent in cervical tissues with HSIL or cancer. They are not sensitive methylation markers when used to detect these lesions in cervical tissues.

1. Introduction

It is evident that adequate screening combined with appropriate treatment of preinvasive lesions could significantly decrease the incidence and mortality of cervical cancer [1]. Screening programs have used broadly morphological assessment of cervical scrapings. Nonattendance is the primary limiting factor for the effectiveness of such screening programs. Moreover, invasive cervical cancer still occurs in women who have access to cancer screening and treatment services. The main causes of invasive cervical cancer in this patient group can be attributed to false-negative Pap smears and to poor follow-up of abnormal results [2].

Theoretically, the solution to these problems is to develop a sensitive screening test that could reliably identify self-sampling cervicovaginal samples with high-grade dysplasia or more severe disease [3]. Because cervicovaginal materials often contain a large amount of normal vaginal cells, the few abnormal cells might easily be missed in a morphology-based screening test. Screening for high-risk human papillomavirus (HPV) infection is highly sensitive, but not specific, for this purpose, because in most patients, a positive HPV test result indicates a transient infection rather than a risk of eventual invasive cervical cancer [4,5].

Conflicts of interest: none.

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More recently, detection of cancer-type specific genetic and epigenetic alterations is being widely considered as another attractive approach, because such alterations often increase gradually from precancerous lesions to invasive cancer [6]. Among these putative alterations, detection of aberrant methylation seems to have the greatest potential in screening tests to detect cancer at the precancerous and early invasive stages [7]. The excellent stability of methylated DNA in most clinical specimens allows them to be analyzed using a variety of detection methods [8].

In addition to the methodology used to detect methylation markers, the genes selected for the test are also important in determining the usefulness of a screening program. Theoretically, in the search for these marker genes, the simplest strategy would be a two-stage approach. The first stage of research focuses on finding candidate genes that can detect the targeted lesion with high sensitivity. From these candidate marker genes, the second-stage study is designed to verify the gene(s) that can distinguish test samples with or without the targeted lesion with high specificity.

Genome-wide analysis seems to be an effective way to identify some genes with high sensitivity and specificity. However, the genes identified so far by different research teams based on this approach have not shown any overlap in any gene [9–13]. An alternative approach used in this study was to search the PubMeth database, in which the reported detection frequencies of numerous genes with promoter methylation in human cancers are collected [14]. Among the genes with a reported prevalence ranging from 60% to 80%, there were at least four genes reported in cervical cancers with detection frequencies that varied widely or were reported in only a single study. These genes were adenomatous polyposis coli (APC), glutathione S-transferase, pi-class (GSTP1), retinoic acid receptor beta (RARB), and prostaglandin-endoperoxide synthase 2 (PTGS2) [15–20]. This first-stage study tried to elucidate the methylation prevalence of these four genes in cervical tissues with high-grade squamous intraepithelial lesions (HSIL) up to early cancer. Based on detection rates determined by methylation-specific polymerase chain reaction (MSP), the goal of this study was to verify if any of these four genes has a detection frequency > 60%, thus meriting further evaluation in a second-stage study.

2. Materials and methods

2.1. Sample selection

From the pathological file at Chi Mei Medical Center, Tainan, Taiwan, all cervical specimens resected through conization or hysterectomy between 2004 and 2006 were reviewed. There were a total of 47 samples with an adequate amount of the targeted lesion, including 11 samples with HSIL, 20 with squamous cell carcinoma (SCC) *in situ* (SCIS), and 16 with invasive SCC at an early stage (pT1a2 or pT1b1). The morphological diagnostic criteria followed those defined by the World Health Organization, while the pathological staging adhered to the definition of the American Joint Committee on Cancer/International Union for Cancer Control. The Institutional Review Board of Chi Mei Medical Center reviewed and approved this study (09912-004).

In tissue sections stained with hematoxylin and eosin, areas of the representative paraffin-embedded tissue blocks rich in the above-targeted lesions were punched out using a bone marrow punch instrument (BM 11-10, Gallini Medical Devices, Mirandola, Italy). Each selected tissue core was 3 mm in diameter and 2 mm thick.

2.2. DNA preparation

The punched tissues were first treated by xylene to remove the paraffin, followed by washes with 100% alcohol. The tissue samples were then incubated in a mixture containing 500 μL of the cell lysis solution from the Puregene kit (D-50K2, Minneapolis, MN, USA) and 5 μL of proteinase K solution (Sigma-Aldrich, St. Louis, MO, USA; 10 mg/mL in 10mM Tris, pH 7.8, 5mM EDTA, and 0.5% sodium dodecyl sulfate) at 55°C overnight or longer if needed. Subsequent DNA isolation followed the Puregene procedures using D-50K3 solution for protein precipitation, 100% isopropanol to form DNA pellets, and 70% alcohol for a final washing. After vacuum drying, the DNA pellet was dissolved in an appropriate amount of 1 X Tris-EDTA (TE) buffer and stored at −20°C for long-term storage. The DNA concentration was determined using a fluorometer (Hoefer Scientific Instruments, San Francisco, CA, USA).

2.3. MSP

Prior to the analysis, DNA samples were modified using bisulfite treatment to convert unmethylated (but not methylated) cytosines to uracil using protocols modified from those reported by other researchers [21,22]. Briefly, 1 μg of each DNA sample was treated in 0.3M sodium hydroxide (NaOH) at 37°C for 30 minutes and then incubated in 3.0M sodium bisulfite ($NaHSO_3$; S8890; Sigma-Aldrich, St. Louis, MO, USA) and 0.5 M hydroquinone ($C_6H_4(OH)_2$; H9003; Sigma-Aldrich) at 55°C overnight. After desalination using the Wizard DNA Clean-Up System (Promega, Madison, WI, USA), samples were denatured using 0.3 M NaOH at room temperature for 5 minutes and then neutralized using 2.5 M ammonium acetate ($NH_4C_2H_3O_2$). Subsequently, the DNA in the sample suspension was precipitated using 100% alcohol, followed by washing with 75% alcohol and then air drying. Finally, the DNA pellet of each sample was dissolved in 50 μL of 1 X TE and stored at 4°C for several weeks or at −20°C for long-term storage.

These modified DNA samples were then subjected to polymerase chain reaction (PCR) using either unmethylated (Um) primers or methylated (M) primers to amplify the corresponding unmethylated and methylated promoter sequences, respectively. Table 1 lists the sequences of all primers used in this study, coupled with the related annealing temperatures and PCR product sizes. Each test was performed in a 30 μL PCR mixture, containing 1X PCR buffer (Green GoTaq Flexi Buffer, Promega), 0.6 U GoTaq Host Start Polymerase (Promega), 75 ng bisulfite modified DNA, and 100mM of both primers. The PCR started with an initial

Table 1
Gene symbol, name, primer sequences, annealing temperature (Ta) for the methylation-specific PCR, and sizes of PCR product.

Gene symbol	Primer sequence	Product (bp)	Ta (°C)
APC	UF: GTGTTTTATTGTGGAGTGTGGGTT	108	58
	UR: CCAATCAACAAACTCCCAACAA		
	MF: TATTGCGGAGTGCGGGTC	98	58
	MR: TCGACGAACTCCCGACGA		
GSTP1	UF: GATGTTTGGGGTGTAGTGGTTGTT	97	58
	MF: TTCGGGGTGTAGCGGTCGTC	91	58
	MR: GCCCCAATACTAAATCACGACG		
PTGS2	UF: TTTAATTTTATTTGTTTTAGTTTGTTTTGATGTGATTTTTTTG	136	56
	UR: TCCAAAAATCTAAACAACCCTAAAATCCAAAAACA		
	MF: TTAATTTTATTCGTTTTAGTTTGTTTCGACGTGATTTTTTC	125	56
	MR: TAAACGACCCTAAAATCCGAAAACG		
RARB	UF: TTGAGAATGTGAGTGATTTGA	146	54
	UR: AACCAATCCAACCAAAACAA		
	MF: TCGAGAACGCGAGCGATTCG	146	58
	MR: GACCAATCCAACCGAAACGA		

APC = adenomatous polyposis coli; GSTP1 = glutathione S-transferase, pi-class; PTGS2 = prostaglandin-endoperoxide synthase 2; RARB = retinoic acid receptor beta.

denaturation at 95°C for 15 minutes, followed by 40 cycles at 95°C for 45 seconds, 55°C for 45 seconds, 72°C for 1 minute, and then a final extension at 72°C for 10 minutes. Finally, the PCR products were subjected to electrophoresis using 5% polyacrylamide gels and then visualized under ethidium bromide staining and ultraviolet illumination.

Each PCR experiment included two control DNA samples, in which the whole genomic sequences were either universally methylated (positive control) or unmethylated (negative control). The methylated (positive) control DNA was prepared by treating lymphocyte DNA with *M.Sss*I (New England Biolabs, Beverly, MA, USA) [23]. The unmethylated (negative) control DNA was prepared by nested whole genome amplification of the same DNA sample using phi29 DNA polymerase (GenomiPhi V2 DNA Amplification Kit, Illustra, GE Healthcare, Buckinghamshire, HP7 9NA, UK) [24]. When MSP using both Um and U primer sets worked well for control samples but not for a testing sample, the result was categorized as noninformative.

2.4. Statistical analysis

Fisher's exact test was applied to analyze the results of differences between different testing groups. Values of $p < 0.05$ were considered statistically significant.

3. Results

One sample in the SCC group failed in the MSP amplification for all four genes using both Um and M primer sets. All 11 samples with HSIL were informative for the four genes. Three samples with SCIS and two with SCC were informative for only three genes, including one which failed for GSTP1, two for PTGS2, and another two for RARB as shown in Table 2. Notably, the storage time of all those paraffin-embedded tissues selected for DNA isolation was 10 years. Compared with the samples with HSIL, failure of MSP amplification seemed to occur more often in samples with SCIS (3/20) and SCC (3/15).

The MSP results of all informative samples that showed a positive finding for promoter methylation for one or more of the four genes are depicted in Fig. 1. Table 2 shows the frequencies of the four genes with promoter methylation detected, either individually or in different combinations, in the three groups of samples. Remarkably, among all samples with informative results, none of the four genes exhibited a detection rate $\geq$ 60%, the preliminary criteria for acceptance as a promising candidate.

Table 2
Detection rates of promoter methylation of four genes using methylation-specific polymerase chain reaction (PCR) in the cervical tissues with lesion at different stages of squamous cell carcinogenesis.

Gene	Total ($n = 46$)	HSIL ($n = 11$)	SCIS ($n = 20$)	SCC ($n = 15$)	p^*
APC	4/46 (8.7)	0/11 (0)	1/20 (5.0)	3/15 (20.0)	0.197
GSTP1	1/45 (2.2)	0/11 (0)	0/20 (0)	1/14 (7.1)	0.556
PTGS2	4/44 (9.1)	1/11 (9.1)	2/18 (11.1)	1/15 (6.7)	> 0.99
RARB	6/44 (13.6)	2/11 (18.2)	0/19 (0)	4/14 (28.6)	0.027
Any gene	15/46 (32.6)	3/11 (27.2)	3/20 (15.0)	9/15 (60.0)	0.025
APC+PTGS2+RARB	14/46 (30.4)	3/11 (27.2)	3/20 (15.0)	8/15 (53.3)	0.062
APC+PTGS2	8/46 (17.4)	1/11 (9.1)	3/20 (15.0)	4/15 (26.6)	0.546
PTGS2+RARB	10/46 (21.7)	3/11 (27.2)	2/19 (10.5)	5/15 (33.3)	0.270

Data are presented as *n*/*N* (%).
*p value determined using Fisher's exact test.
APC = adenomatous polyposis coli; GSTP1 = glutathione S-transferase; HSIL = high-grade squamous intraepithelial lesion; PTGS2 = prostaglandin-endoperoxide synthase 2; RARB = retinoic acid receptor beta; SCC = squamous cell carcinoma, pi-class; SCIS = squamous cell carcinoma *in situ*.

There were two other significant findings. One was that the detection rate of one or more genes with promoter methylation in the SCC group (60.0%, 9/15) was significantly higher than those in the HSIL (27.2%, 3/11) and SCIS (15.0%, 3/20) groups ($p = 0.025$). The other finding was that the detection rate of methylated RARB in the SCC group (28.6%, 4/14) was significantly higher than those in the HSIL (18.2%, 2/11) and SCIS (0%, 0/19) groups ($p = 0.027$).

4. Discussion

One SCC sample among the 47 samples examined in this study failed in MSP amplification of all four genes. Five of the remaining 46 samples were noninformative for one gene, including one SCC sample with failed analysis of GSTP1, two SCIS samples with failed analysis of PTGS2, and one each of SCIS and SCC sample with failed analysis of RARB, as shown in Table 2. It is of note that the chromosomal loci of GSTP1, PTGS2, and RARB were 11q13, 1q25.2-q25.3, and 3p24.2, respectively. The failure rates of MSP amplification seemed to be higher in the SCIS and SCC samples than the HSIL samples. This phenomenon may be partly due to chromosomal deletions that often occur during progression of most cancers [25].

In this first-stage study of a limited number of samples, there were two significant findings. One was that the overall frequency of the promoter methylation detectable in the four genes was significantly higher in the SCC group than in the HSIL and SCIS groups ($p = 0.025$). This is consistent with a well-demonstrated phenomenon in human cancers, i.e., the overall number of methylated tumor-related genes often increases gradually along the course of tumor progression [8]. Another significant finding was that methylated RARB occurred significantly more frequently in the SCC group than in the other two groups ($p = 0.027$). These results are compatible with those found in another study that suggested RARB methylation seems to be a prognostic factor in cervical carcinogenesis [26].

RARB is the only one of the three isoforms (alpha, beta, and gamma) of the nuclear retinoic acid receptor that plays a central role in growth regulation of epithelial cells and related tumorigenesis [27]. The data collected in the PubMeth website revealed that RARB methylation occurs frequently in SCCs arising from the skin and oral cavity, with reported frequencies ranging from 60% to 80% [14]. The highest frequency of methylated RARB detected in this study was in the SCC group, 28.6% (4/14), which is consistent with results from other studies of cervical cancers, with reported frequencies ranging from 26% to 54% [14–16,18–20].

PTGS2, also called cyclooxygenase-2, is responsible for the prostanoid biosynthesis involved in inflammation and mitogenesis/carcinogenesis, and is presumably inactivated in apoptosis [28]. Data from several large studies collected in the PubMeth website revealed that PTGS2 methylation is highly prevalent in cancers arising from the breast, prostate, and urinary bladder, with reported frequencies ranging from 60% to 80% [14]. The frequencies of methylated PTGS2 detected in the three sample groups in this study did not differ significantly, and ranged from 6.7% to 11.1% (Table 2). Only one study has reported cervical SCCs with PTGS2 methylation of 6.7% (1/15) [17].

APC is a well-characterized tumor suppressor involved in downregulating Wnt/beta-catenin signaling and is central to development and the mature organism. Initially identified in colorectal cancer, APC is inactivated in various malignancies by genetic and epigenetic mechanisms [14,29]. Data from several large studies collected in the PubMeth website revealed that APC methylation is highly prevalent in cancers arising from the esophagus and stomach, with reported frequencies ranging from 60% to 80% [14]. The highest detection rate among the three sample groups in this study was 20% (3/15) in the SCC group. The

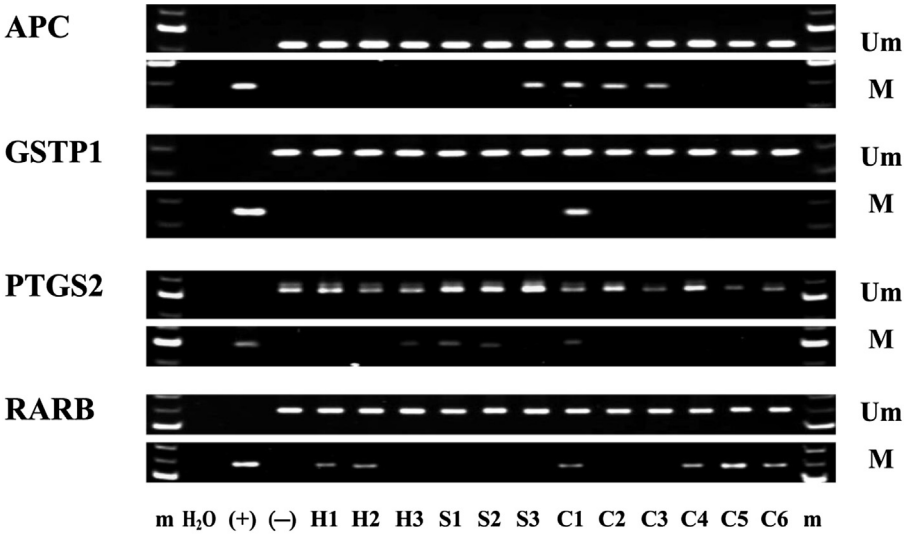

Fig. 1. Promoter methylation status of four cancer-related genes determined with methylation-specific polymerase chain reaction (MSP). APC = adenomatous polyposis coli; C1–C6 = samples with early-stage squamous cell carcinoma; GSTP1 = glutathione S-transferase, pi-class; H1–H3 = samples with high-grade squamous intraepithelial lesions; M = MSP using methylated primers; m = size markers; PTGS2 = prostaglandin-endoperoxide synthase 2; RARB = retinoic acid receptor beta; S1–S3 = samples with squamous cell carcinoma *in situ*; Um = MSP using unmethylated primers; (+) = methylated positive control; (–) = unmethylated negative control.

frequencies of APC methylation in cervical SCC in other studies ranged from 16% to 55% [14,15,17].

GSTP1 is an isoform of the GSTs, a major group of detoxification enzymes. These isoenzymes contribute to resistance to carcinogens, antitumor drugs, environmental pollutants, and products of oxidative stress [30]. Data from several large studies in the PubMeth website revealed that GSTP1methylation is highly prevalent in prostate cancers, with reported frequencies > 85% [14]. However, among the 45 samples with informative MSP results in the present study (Table 2), only one sample in the SCC group was positive for GSTP1 methylation. The reported frequencies of cervical SCCs in other studies varied from 0% to 21% [15–17,20].

In conclusion, the aim of this first-stage study was to assess if the promoter methylation of four genes, APC, GSTP1, PTGS2, and RARB, could be sensitive markers in the detection of HSIL, SCIS, or early SCC in cervical tissues. MSP analysis of 46 samples revealed that none of these four genes exhibited a detection rate $\geq$ the defined criteria, 60%. Therefore, they are not ideal candidate genes for further evaluation in a second-stage study.

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