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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.