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Taiwanese Journal of Obstetrics & Gynecology 51 (2012) 43–49

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Original Article

New primers for methylation-specific polymerase chain reaction enhance specificity of detecting STAT1 methylation

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Accepted 2 November 2011

Abstract

Objective: Signal transducer and activator of transcription (STAT)1 is a key tumor suppressor, which is always methylated in a variety of human cancers. However, nonspecific primers for the detection of specific promoter hypermethylation of STAT1 gene can lead to false-positive or false-negative results for gene methylation.

Materials and Methods: We designed new primers for the detection of STAT1 methylation and compared the sensitivities and specificities of these new primers with prior published primers by methylation-specific polymerase chain reaction (PCR) from ovarian clear cell carcinomas. The mRNA expression levels of STAT1 in these cancerous tissues were also evaluated by reverse-transcriptase PCR and correlated with the results of promoter methylation of STAT1 gene.

Results: Nine (39%) of the 23 samples detected by the new primers and 13 samples (56%) detected by prior published primers showed STAT1 methylation. A direct DNA sequencing test revealed that four of the 13 samples (30.8%) showed false positivity for STAT1 methylation using the prior published primers. In contrast, none of the nine samples was false-positive for the detection of STAT1 methylation using the new primers. The new primers for the detection of STAT1 methylation showed 100% specificity and 100% sensitivity without false positivity.

Conclusion: Specific primers for methylation-specific PCR are mandatory for the accurate detection of STAT1 gene methylation. Besides, specific primers can generate correct interpretation of STAT1 gene methylation, and its correlation with the clinicopathological characteristics and outcome of cancer patients.

Keywords: methylation; polymerase chain reaction; signal transducer and activator of transcription 1

Introduction

Hypermethylation in the promoter region of genomic DNA is now believed to be an important epigenetic alteration occurring in the early stage of carcinogenesis of many cancers [1–3]. Cytosine–guanine (CpG) island methylation is one of the best-understood epigenetic changes in human cancers, and it is also an important epigenetic mechanism in gene transcriptional regulation [4]. Aberrant methylation of tumor suppressor genes plays an important role in carcinogenesis, as a result of altering the DNA secondary structure and inducing chromosome remodeling, even transcriptional repression [5,6]. Although the methylation status of the CpG island in tumor

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1028-4559/$ - see front matter
doi:10.1016/j.tjog.2012.01.009

suppressor genes has been studied extensively for its potential to predict cancer behavior [7], this epigenetic alternation has not proven to be an entirely reliable prognostic marker for cancer prognosis, due to the exact methods used for detecting methylation in the CpG island.

Most assays for gene methylation changes rely on the treatment of genomic DNA with sodium bisulfite, which could convert unmethylated but not methylated cytosine residues to uracil, resulting in the generation of noncomplementary, single-stranded DNA. Following this conversion process, we used methylation-specific polymerase chain reaction (MS-PCR) with a pair of specific PCR primers to detect the epigenetic alternation of maternal and paternal alleles. MS-PCR is a frequently used, inexpensive method for detecting gene methylation. Following bisulfite modification, we performed PCR using two sets of primers designed to amplify either methylated or unmethylated alleles.

The design of the primers is the crucial issue in obtaining reliable MS-PCR results for the detection of gene hypermethylation. The methylated and ummethylated primer sets need to be designed for the same CpG sites and include multiple CpG sites at the 3′ ends. For this study, we designed a set of new primers for the MS-PCR to detect signal transducer and activator of transcription (STAT)1 methylation. The mRNA expression levels of STAT1 in the ovarian samples with or without STAT1 gene hypermethylation were also checked and correlated with the results of the MS-PCR. We further compared the sensitivity, specificity, and false-positive and false-negative rates in the detection of STAT1 methylation by using new and prior published primers. The new primers that we designed were more accurate in detecting the STAT1 methylation in ovarian cancer than the prior published primers.

Materials and methods

Patients and specimens

The study was approved by the Institutional Review Board of the National Taiwan University Hospital. All women were informed and gave their written consent to participate in the study. Twenty-three women with three benign ovarian tumors and 20 with ovarian clear cell carcinomas were enrolled. Ovarian specimens were obtained intraoperatively and frozen immediately at −70 °C until analysis.

DNA extraction

The genomic DNA of the ovarian tissues was isolated using the Qiagen EZ1 DNA Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions, as described previously [8].

MS-PCR

The MS-PCR was performed as described previously with some modifications [9]. Briefly, genomic DNA was first treated with sodium bisulfite, then desulforated with NaOH, precipitated with ethanol, and resuspended in distilled water. After the treatment with sodium bisulfate, the methylation status of the DNA was checked by primers specific for the methylated and unmethylated alleles of the STAT1 gene. The processes of MS-PCR in the isolated genomic DNA of ovarian samples were performed using the EZ DNA Methylation Kit™ (Zymo Research Corporation, Orange, CA, USA), following the manufacturer's instructions, as described previously [10–12].

There were two sets of primers for STAT1 MS-PCR. The first set of primers were originally designed and reported by Xi [13] and have been defined here as the prior published primers. The primers for detecting unmethylated STAT1 gene were 5′-AAATTTGTTTTTTGTTTGGATTTTT-3′ (sense) and 5′-ACCAATTAAACACAACTATTCCATA-3′ (antisense) to generate a 269-bp product. To detect the methylated STAT1 gene, 5′-AAATTTGTTTTTTGTTTGGATTTTC-3′ (sense) and 5′-AATTAAACGCGACTATTCCGTA-3′ (antisense) were used to generate a 266-bp product [13].

The second set of STAT1 methylated or unmethylated specific primers that we designed were defined as the new primers. These primers were designed from the web site http://www.urogene.org/methprimer/index1.html and used a promoter sequence published on the Pubmed (accession number: AF182311.1) website. The primers for the unmethylated STAT1 gene were 5′-ATTTTTGGTATTTGGAATTTTATGG-3′ (sense) and 5′-AACCAAACAAAAACAACCCAACA-3′ (antisense) to generate a 116-bp product. The primers for the methylated STAT1 gene were 5′-TATTTTCGGTATTCGGAATTTTAC-3′ (sense) and 5′-GAACAAAAACGACCCAACGC-3′ (antisense) to generate a 113-bp product.

As shown in Fig. 1, the detection of methylation status of the STAT1 gene was on the CpG island located at nucleotides 626–895 (prior published primers) and at nucleotides 121–233 (new primers) upstream of the start site of the exon. The prior primers designed by Xi could detect methylation at nucleotides 626–895 upstream of the start site of the exon.

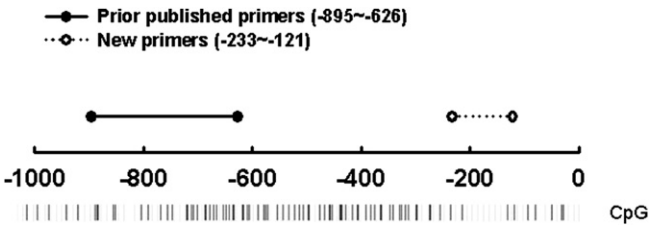

Fig. 1. A diagrammatic representation of the MS-PCR specific primers for the detection of STAT1 promoter methylation. Two sets of methylation-specific primers for the STAT1 promoter region were used. The prior published primers could detect the methylation from promoter −895 to −626 with 266-bp methylated and 269-bp unmethylated PCR products. The new primers could detect the methylation from promoter −233 to −121 with 113-bp methylated and 116-bp unmethylated PCR products. The number ranges above indicate the nucleotide position relative to the translational start site (ATG). Short vertical bars on the bottom line show the CpG sites. CpG = cytosine–guanine; MS-PCR = methylation-specific polymerase chain reaction; STAT1 = signal transducer and activator of transcription 1.

The 266-bp and 269-bp PCR products indicated methylation and nonmethylation of the STAT1 gene by using the prior published primers. Our new primers could detect methylation at nucleotides 121–233 upstream of the start site of the exon. The 113-bp PCR product indicated methylation of the STAT1 gene. In contrast, the 116-bp PCR product indicated nonmethylation of the STAT1 gene.

The PCR products, whether from the prior published or the new primers, were further separated by capillary electrophoresis (CE) or 3% agarose gel electrophoresis (AGE), with the results being visible after staining with ethidium bromide (EtBr). We performed direct DNA sequencing to check the accuracy of the CE and AGE.

CE and direct DNA sequencing

We used CE to analyze the MS-PCR products via the HDA System with a GCK-5000 cartridge kit (eGene, Irvine, CA, USA), as described previously [14]. Briefly, the gel-matrix in the gel cartridge was composed of proprietary linear polymer with EtBr dye. The PCR products were diluted 20-fold with deionized water and placed in the sample chamber of the instrument. DNA samples were then injected into the capillary channels and subjected to electrophoresis based on the manufacturer's protocol [15–17]. BioCalculator Graphing software (eGene) was used to label the peak sizes automatically [14].

The PCR products for CE were also checked by direct DNA sequence analysis using an automated ABI sequencing system (ABI3730, Applied Biosystems, Foster city, CA) to confirm the results of the CE.

RNA extraction

Ovarian tissue specimens were collected, frozen, and stored as described earlier. The total RNA of the ovarian tissues was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, as described previously [18,19].

Reverse-transcriptase PCR

RNA was first reverse-transcribed to cDNA using the Moloney murine leukemia virus reverse transcriptase kit (Invitrogen Life Technologies, San Diego, CA, USA) [18,19]. A set of primers, 5′-TTCAGAGCTCGTTTGTGGTG-3′ and 5′-AGAGGTCGTCTCGAGGTCAA-3′, were used for 30 cycles to determine the expression levels of STAT1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene to compare with the target gene STAT1. A set of primers, 5′-ACCCAGAAGACTGTGGATGG-3′ and 5′-TGCTGTAGCCAAATTCGTTG3′ was also used for 30 cycles to generate GAPDH. The PCR products were then analyzed in 1% agarose gel with EtBr staining in Tris/borate/EDTA solution. Differences in the transcription levels of STAT1 between the samples with methylated and unmethylated STAT1 gene were compared using the electrophoresis images.

Results

Prior published primers for MS-PCR showed a 30% false-positive rate in the detection of STAT1 gene methylation

We evaluated whether methylation could be observed within the CpG island of the promoter of the STAT1 gene. Representative figures of the STAT1 gene methylation by MS-PCR when using the prior published primers in CE analysis (Fig. 2A) and in AGE (Fig. 2B) are shown. Thirteen (56.5%) of the 23 samples showed methylation in the cancerous tissues and 0/3 in the normal tissues, and the results were consistent between the CE and AGE analyses.

Direct DNA sequencing was also performed to confirm the methylation status of the STAT1 using MS-PCR with the prior published primers. Fig. 2C1, sample S3, revealed unmethylation of the STAT1 gene methylation by direct DNA sequencing, but it showed STAT1 gene by MS-PCR using the prior published primers when analyzed by CE and AGE. In addition, there were another three samples of unmethylated STAT1 gene that revealed STAT1 gene methylation by MS-PCR with prior methylation. A total of four out of 13 samples (31%) showed false-positive results for STAT1 gene methylation as detected by MS-PCR using the prior published primers. None of the samples showed false-negative results by MS-PCR using the prior published primers when checked by direct DNA sequencing.

New primers for MS-PCR showed no false-positive or false-negative results for detection of STAT1 gene methylation

We used our new primers to detect methylation of the STAT1 gene. The primers that we designed are shown in Fig. 1. The representative figures of MS-PCR using the new primers by CE and AGE analyses are shown in Figs. 3A and 3B. Nine of the 20 samples (45%) showed methylation in cancerous tissues. No methylation of the STAT1 gene was detected in the three normal ovarian tissues. The results were also consistent between the CE and AGE analyses.

Direct DNA sequencing was performed to confirm the methylation status of STAT1 using MS-PCR with the new primers. Sample S3, which revealed nonmethylation of the STAT1 gene as detected by MS-PCR and CE and AGE analyses, can be seen in Fig. 2B. Direct DNA sequencing also revealed an unmethylated STAT1 gene, as shown by MS-PCR with CE and AGE analyses (Fig. 3C2). In addition, the other three samples that were false-positive for STAT1 gene methylation using the prior published primers showed nonmethylation of the STAT1 gene using the new primers, and were confirmed to be unmethylated STAT1 gene by direct DNA sequencing. When we used our new primers, none of the 23 samples showed false-positive or false-negative results in the methylation status of the STAT1 gene by MS-PCR. Our results indicate that the new primers provide more accuracy in detecting the methylation of the STAT1 gene.

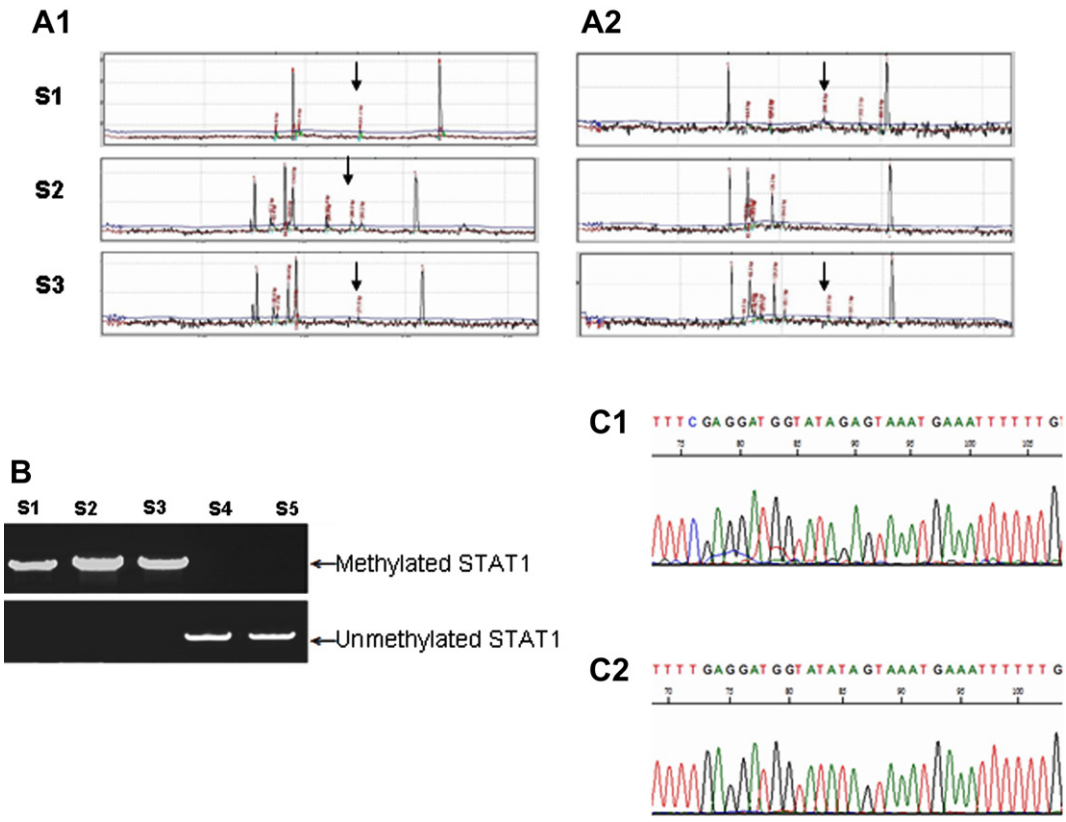

Fig. 2. STAT1 promoter methylation detected by the prior published primers in various ovarian cancerous samples. (A) CE analysis of PCR products with the prior published primers. A1: methylated PCR products as indicated by the arrows. A2: Unmethylated PCR products as indicated by the arrows. (B) AGE analysis of the PCR products using the prior published primers. The S1, S2 and S3 samples showed STAT1 methylation, but the S4 and S5 samples showed STAT1 non-methylation. (C) Direct DNA sequencing analysis of the STAT1 gene. C1: S3 sample. C2: S4 sample. Both the S3 and S4 samples showed nonmethylation. The results were incompatible between the CE, AGE and direct DNA sequencing analyses using the prior published primers. AGE = agarose gel electrophoresis; CE = capillary electrophoresis; PCR = polymerase chain reaction; STAT1 = signal transducer and activator of transcription 1.

RNA expression levels of STAT1 in ovarian cancerous and normal tissues as detected by RT-PCR

To investigate further the correlation between RNA expression and methylation status of the STAT1 gene, RT-PCR was performed. Fig. 4 shows the representative figures of the RNA expression levels of STAT1 in the tissue samples. The RNA expression levels of STAT1 (Samples 1 and 2) were lower in the methylated STAT1 samples than in Samples 3–5, the unmethylated STAT1 samples. Our results indicated that the samples with STAT1 gene methylation showed low mRNA expression of STAT1.

Discussion

MS-PCR is a PCR-based method for identifying known allelic mutations in nucleic acid sequences. MS-PCR is a simple, sensitive and specific method for determining the methylation status of CpG-rich regions [20,21]. This method is based on the introduction of artificial mutations into the PCR-primer binding regions of amplified DNA in an allele-specific manner. By using allele-specific primers with mutagenic positions, the artificially introduced mutations can anneal to the target alleles more specifically. All of the nine STAT1 methylation ovarian cancerous samples could be detected by MS-PCR in the present study, whether we used the prior published or the new primers. The MS-PCR products are specific for their respective alleles and can be directly identified by AGE or CE without further manipulation [22–24]. Nevertheless, methylation could not be detected by the MS-PCR using methylation-specific primers when the regions of the CpG sites were not methylated.

MS-PCR is currently widely used to study the promoter methylation of specific genes. However, gene hypermethylation detection by MS-PCR amplification can produce false-positive results as we have shown here. In the current study, four samples without STAT1 gene methylation showed STAT1 methylation with MS-PCR when using the prior published primers. The false-positive results can be attributed to several reasons. The annealing temperature is the first important step in an MS-PCR reaction. If the annealing temperature is too low or too many cycles are used, amplification can occur across the 3′ mismatch [25]. Our new primers for detecting STAT1 methylation could efficiently amplify the methylated alleles (Fig. 3). The new primers contained three CpG sites with the designed annealing temperature at 57 °C during PCR, so the new primers could reduce the wrong PCR products. In contrast, the prior published primers contained

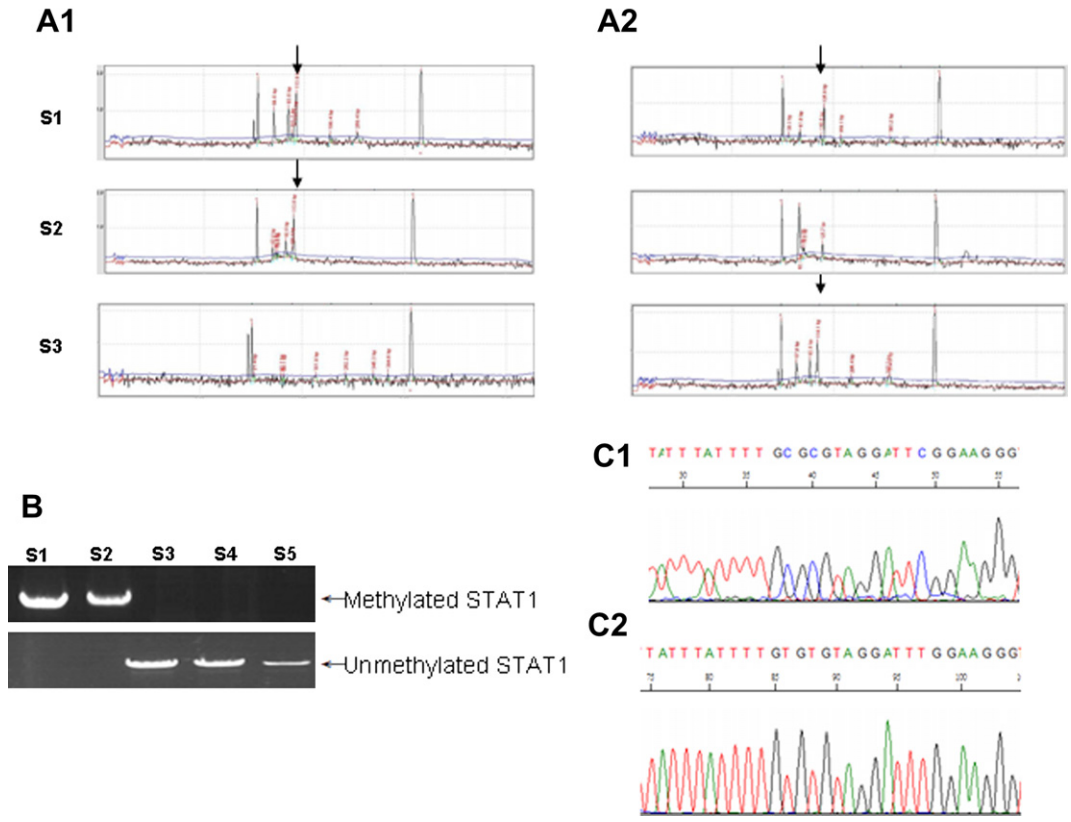

Fig. 3. STAT1 promoter methylation detected by the new primers in various ovarian cancerous samples. (A) CE analysis of the PCR products using the new primers. A1: methylated PCR products as indicated by the arrows. A2: Unmethylated PCR products as indicated by the arrows. (B) AGE analysis of the PCR products using the prior published primers. The S1 and S2 samples showed STAT1 methylation, but the S3, S4 and S5 samples showed STAT1 nonmethylation. (C) Direct DNA sequencing analysis of STAT1 gene. C1: S1 sample. C2: S3 sample. S1 was STAT1 methylation and S3 was STAT1 unmethylation. The results were compatible between the CE, AGE and direct DNA sequencing analyses using the new primers. AGE = agarose gel electrophoresis; CE = capillary electrophoresis; PCR = polymerase chain reaction; STAT1 = signal transducer and activator of transcription 1.

only one CpG site with a relatively low annealing temperature at 54 °C, so the PCR products of the prior published primers could only have comparable specificity. Raising the annealing temperature resulted in a significantly more efficient amplification of the methylated template. By adjusting the annealing temperature of the PCR amplification, we were able to improve the specificity of the STAT1 methylation samples.

Another critical parameter affecting the specificity of methylation-specific PCR is the design of the primers. Suitable and specific primers can enhance the success rate of the entire PCR. In general, primers should be designed to amplify a region that is 80–250 bp in length, and should contain enough cytosines in the original sequence to assure that unmodified DNA will not serve as a template for the primers [26]. The new primers that we designed contained three CpG sites to amplify a 113-bp DNA fragment. In contrast, the prior published primers contained only one CpG site to amplify 266-bp nucleotides. Low characterization efficacy in the primer could be the key issue leading to false-positive results. By increasing the CpG sites in the primer design, we approached the appropriate DNA length and were able to improve the specificity of our assay for STAT1 methylation.

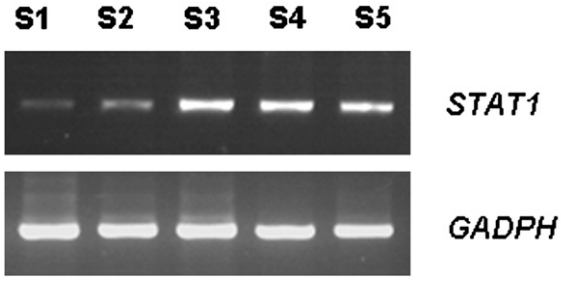

Fig. 4. Expression of STAT1 mRNA in various ovarian cancerous samples. The expressions of STAT1 mRNA were lower in the S1 and S2 samples than in the S3, S4 and S5 samples. STAT1 = signal transducer and activator of transcription 1.

DNA hypermethylation of gene-associated CpG islands results in either downregulation or complete abrogation of gene expression. Gene expression should be checked to confirm the biological effect of gene methylation. Suzuki et al have reported that the methylation of promoters correlates with low or no transcription [27]. Many examples also have demonstrated that methylation of tumor suppressor genes such as BRCA-1 [28], RASSF1A [28], PTEN [29], and P16 [30] can result in gene silencing and then carcinogenesis. In the current study, we also used RT-PCR to confirm the mRNA expression level and to validate the post-transcriptional

modification of STAT1. The expression levels of STAT1 mRNA in these unmethylated STAT1 samples remained high (Fig. 4). In contrast, the samples with methylated STAT1 demonstrated downregulated expression of STAT1 mRNA (Fig. 4). Consequently, the STAT1 methylation has the biological function of reducing STAT1 gene expression.

Analysis of the hypermethylation of gene promoters has extraordinary importance for tumor biology and clinical applications. Methylated promoters have been detected in a variety of cancer patient tissues, as well as in ovarian cancer tissues [1,31−33]. Studies have demonstrated that profiles of specific promoter methylation in different tumors are associated with the clinical response and outcomes of cancer patients [2,34,35]. However, these methylation-related biomarkers are still limited in their clinical application, because of the lack of specificity and sensitivity. This highlights the critical need for the design of specific primers and probes to improve the sensitivity and specificity for the detection of gene methylation. With the identification of DNA methylation in cancer initiation and progression, distinct efforts should be made to develop strategies for facilitating clinical application.

AGE and CE are the common analytic methodologies for the analysis of PCR products. We utilized CE to demonstrate our MS-PCR results in this study. AGE is most commonly used for the separation of biological macromolecules and is a visual method for confirming the presence of nucleotides. It provides good resolution and separation of large molecules from small. However, the visualization of oligonucleotides requires the highly genotoxic agent EtBr. This test is time-consuming, requires a larger sample volume, and its high pollution level should be noted. CE showed technical advantages over AGE and previous gel methods in terms of the time and labor saved by the automated instrumentation [36]. The most important advantage is its ability to measure the size of PCR products with very high resolution [37]. Our data from the MS-PCR and CE revealed the same sensitivity and specificity with those from direct genomic DNA sequencing (Fig. 3). The major disadvantages of CE including non-covalent complexes are frequently disrupted results [38] and its expensive instrumentation. However, if considered as high-throughput equipment for further clinical application, CE is a powerful and attractive strategy for methylation analysis.

In the present study, new primers of MS-PCR could detect the methylation of STAT1 gene more accurately than the prior published primers. The mRNA expression levels of STAT1 gene detected by RT-PCR also confirmed the accuracy of the new primers of MS-PCR. The designation of specific primers is indeed an important issue for the accurate detection of STAT1 gene methylation. Correct detection of the methylation of tumor suppressor gene STAT1 can be efficiently utilized for the correlation of clinicopathological characteristics and for the prediction of the outcome of ovarian cancer patients.

Acknowledgments

This work was supported by a grant from National Taiwan University Hospital (NTUH100-S1609). We would like to thank the Second and Seventh Core Laboratories of the Department of Medical Research at National Taiwan University Hospital for technical assistance.

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