Application of a *Not*I subtraction and methylation-specific genome subtractive hybridization technique in the detection of genomic DNA methylation differences between hydatidiform moles and villi

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Abstract. Previous studies indicate that epigenetic modifications play an important role in transcriptional regulation and contribute to the pathogenesis of gestational trophoblastic disease, including complete hydatidiform moles (CHMs). However, the underlying mechanisms and the critical genes have not been clearly identified. In the present study, we developed a novel technique, *Not*I subtraction and methylation-specific genome subtractive hybridization (MS-G-SH), as a method of screening for methylation changes between hydatidiform moles and villi. Following *Not*I subtraction and hybridization, three different positive DNA clones were found in 110 random clones of DNA samples. Most importantly, two DNA clones having long CpG islands and high homology with exons of insulin-like growth factor 2 (IGF2) and transforming growth factor-β (TGF-β) were identified using bioinformatic tools. After bisulfite treatment and methylation-specific PCR, the specific methylation of certain exons of IGF2 and TGF-β was identified. In addition, the mRNA expression levels of these two genes were markedly different. In conclusion, this novel MS-G-SH technique is an alternative and effective approach for the detection of specific DNA methylation.

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Introduction

The epigenetic programming of the gametes and early post-fertilization embryos is essential for the development of a new organism. This programming involves the intricate interaction of the three layers of epigenetics: DNA methylation, histone modification and non-coding RNAs (1). Increasing evidence supports the importance of DNA methylation in the regulation of new organism development. 5-Methylcytosine (5mC) in the DNA from mammalian cells is found to be located almost entirely within CpG dinucleotides (2,3) and 70-80% of cytosine in CpG dyads is methylated on both strands in human cells. In general, CpG methylation is highly prevalent in repetitive sequences and in gene bodies, but rare at CpG islands within housekeeping promoters (2). Consistent with the functional importance of DNA methylation, it is non-random, well-regulated and tissue-specific (4-6). DNA methylation studies have attracted increasing interest in the field of epigenetics which is now a dynamic area of research challenging and revising traditional paradigms of gene expression and behavior (7).

Complete hydatidiform mole (CHM) is one of the most frequent abnormalities occurring during pregnancy (8). Although a number of studies have indicated that CHM is a maternal-effect autosomal recessive disorder in which recurrent pregnancy failure with molar degeneration occurs (9), the detailed mechanism of CHM genesis is not fully understood. The occurrence of CHM may have an epigenetic link. Hayward *et al* revealed a methylation defect at imprinted gene NLRP7 (NALP7) loci in tissue from four new familial biparental hydatidiform moles (9) and the differential methylation of SGCE/PEG10 was preserved in these four cases. Furthermore, differential methylation at the H19 locus (an imprinted gene) was observed in two hydatidiform mole cases (10). Li *et al* investigated the expression and methylation profiles of SOX2 in hydatidiform moles and choriocarcinoma and demonstrated that epigenetic mechanisms may be important in the transcription regulation of SOX2 and contribute to the pathogenesis of hydatidiform moles (11). It is becoming clear that distinct

epigenetic markers are essential for the pathogenesis of hydatidiform moles.

In the present study, we developed methylation-specific genome subtractive hybridization (MS-G-SH), a novel method to screen for methylated biomarkers for the early diagnosis of CHM. We found several methylated sequences in the whole genomes from tissue samples which differed between hydatidiform moles and villi. The methylation of two candidate genes, insulin-like growth factor 2 (IGF2) and transforming growth factor-β (TGF-β), was revealed to be related to the pathogenesis of CHM.

Materials and methods

Clinical sample collection. The trophoblastic tissues (hydatidiform moles and villi) were collected from the Shanghai First Maternity and Infant Hospital (Shanghai, China) between June 2009 and March 2010. Cases suspected by clinical and ultrasonographic analysis to be hydatidiform moles were suction evacuated. All human samples were obtained after obtaining approval from the Ethics Review Board of the Shanghai First Maternity and Infant Hospital and after obtaining written informed consent from the subjects. Due to material limitations, we could only analyze a limited number of hydatidiform moles and villi (3 of each).

Modified NotI-CODE and SSH procedure. The construction of *Not*I linking libraries has been previously described (12). Plasmid DNA was purified using the Axygen plasmid DNA extra kit (Axygen Biosciences, Union City, CA, USA). A standard protocol was used to prepare nylon filter replicas of a grid of *Not*I linking-specific *Not*I linking clones and five random unmapped human *Not*I linking clones. For hybridization to the nylon filters, the *Not*I representing probes were biotin-labeled by PCR. Sequencing gels were run on ABI 310 automated sequencers (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions (12). Two oligonucleotides, Not-X: 5'-AAAAGAATGTCAGTGTGTCAC GTATGGACGAATTCGC-3' and Not-Y: 5'-AAACTT ACAGTGTGTGTCACGTATGGCTGCTTAAGCGCCGG-3', were used to create the *Not*I linker. DNA A (tester) and DNA B (driver) (2 mg) at a concentration of 50 μg/ml were digested with 20 units *Bam*HI and 20 units *Bgl*II (Roche Molecular Biochemicals, Mannheim, Germany) at 37˚C for 5 h. After heat-inactivating for 20 min at 85˚C, 0.4 μg digested DNA was circularized overnight with T4 DNA ligase (Roche Molecular Biochemicals) in the appropriate buffer in a 1-ml reaction mixture. The DNA was then concentrated with ethanol, partially filled in and digested with 10 units *Not*I at 37˚C for 3 h. After digestion, *Not*I was heat-inactivated and the DNAs were ligated overnight in the presence of a 50 M excess of *Not*I linker at room temperature. All further steps were performed as previously described (12), with the modification that Not-X primer was used for PCR amplification and only two cycles were performed. These PCR-amplified tester and driver amplicons are known as NRs.

Quantitative real-time PCR (qRT-PCR) analysis of mRNA expression. Total RNA from each tissue was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA samples were treated with DNase I (Sigma-Aldrich, St. Louis, MO, USA), quantified and reverse-transcribed into cDNA using the ReverTra Ace-α first strand cDNA synthesis kit (Toyobo, Osaka, Japan). qRT-PCR was conducted using a realplex4 real-time PCR detection system from Eppendorf Co., Ltd., (Hamburg, Germany), using SYBR-Green real-time PCR Master mix (Toyobo) as the detection dye. qRT-PCR amplification was performed over 40 cycles with denaturation at 95˚C for 15 sec and annealing at 58˚C for 45 sec. The target cDNA was quantified using the relative quantification method. A comparative threshold cycle (Ct) was used to determine gene expression relative to a control (calibrator) and steady-state mRNA levels are reported as an n-fold difference relative to the calibrator. For each sample, the Ct values of the marker genes were normalized using the formula $\Delta Ct = Ct_{gene} - Ct_{18SRNA}$. To determine relative expression levels, the following formula was used $\Delta\Delta Ct = \Delta Ct_{hydatidiform\_moles} - \Delta Ct_{villi}$. The values used to plot the relative expression levels of the markers were calculated using the expression $2^{-\Delta\Delta Ct}$. The mRNA levels were calibrated based on the levels of 18S rRNA. The cDNA of each gene was amplified using primers as follows: TGF-β forward: 5'-CCCTGGACACCAACTATTGC-3'; TGF-β reverse: 5'-CTTCCAGCCGAGGTCCTT-3'. IGF2 forward: 5'-GTT CGGTTTGCGACACG-3'; and IGF2 reverse: 5'-AGAAGC ACCAGCATCGACTT-3'.

Bisulfite conversion of genomic DNA and methylation-specific PCR (MS-PCR). The cells were lysed with DNA lysis buffer (0.5% SDS, 0.1 M EDTA, 10 mM Tris-HCl pH 8.0 and 100 ng/ml Proteinase K, all from Sigma-Aldrich) and incubated at 55˚C for 2 h. The treatment of genomic DNA and the MS-PCR assay were performed as previously described (13). The specific primers for TGF-β and IGF2 were designed as follows: TGF-β methylated forward: 5'-TTTTGTATAATA GTATTCGCGATCG-3'; TGF-β methylated reverse: 5'-TAA CCTCCTTAACGTAATAATCGAC-3'; TGF-β unmethylated forward: 5'-TTTGTATAATAGTATTTGTGATTGG-3'; TGF-β unmethylated reverse: 5'-TAACCTCCTTAACATAAT AATCAAC-3'. IGF2 methylated forward: 5'-GTGTTTTTT ATTAATTTGGCGTTC-3'; IGF2 methylated reverse: 5'-ACT AAAAAATATCCACCAACTCCG-3'; IGF2 unmethylated forward: 5'-TGGTGTTTTTTATTAATTTGGTGTTT-3'; and IGF2 unmethylated reverse: 5'-ACTAAAAAATATCCACCA ACTCCAC-3'. The PCR products were separated by agarose gel electrophoresis with 12 g/l ethidium bromide containing 1X Tris-Acetate EDTA (TAE) buffer and visualized under UV illumination.

Statistical analysis. Each experiment was performed as least three times and data are shown as the mean ± SE where applicable and differences were evaluated using the Student's t-test. $P<0.05$ was considered to indicate a statistically significant result.

Results

DNA samples subtracted by NotI and MS-G-SH treatment. If a particular *Not*I site is present in a DNA sample, the self-ligated cyclized DNA molecule will be opened with *Not*I and labeled;

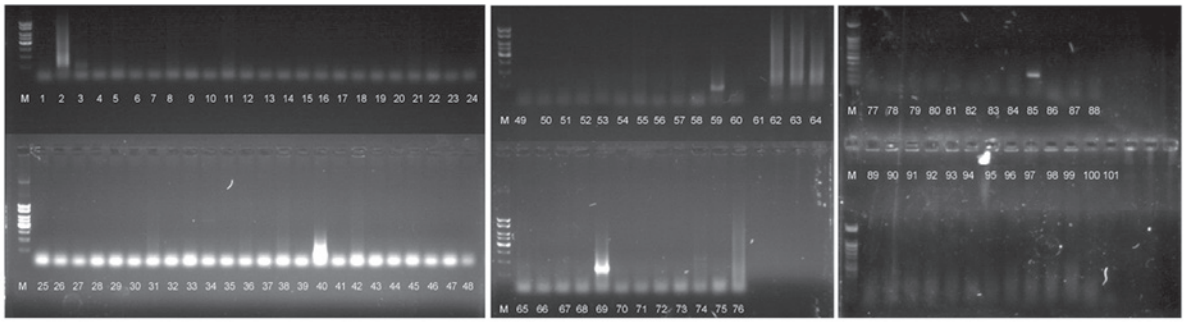

Figure 1. DNA samples were subtracted by *Not*I and MS-G-SH treatment. Following *Not*I subtraction and MS-G-SH treatment, we isolated DNA from 101 random clones and sequenced them. Eight of these clones contained the *Not*I site (numbers 2, 40, 59, 62, 63, 64, 69 and 85). After sequencing, three different clones were determined to contain *Not*I sites (C3, A11 and G9). These results demonstrated the efficiency of subtraction using *Not*I-surrounding sequences.

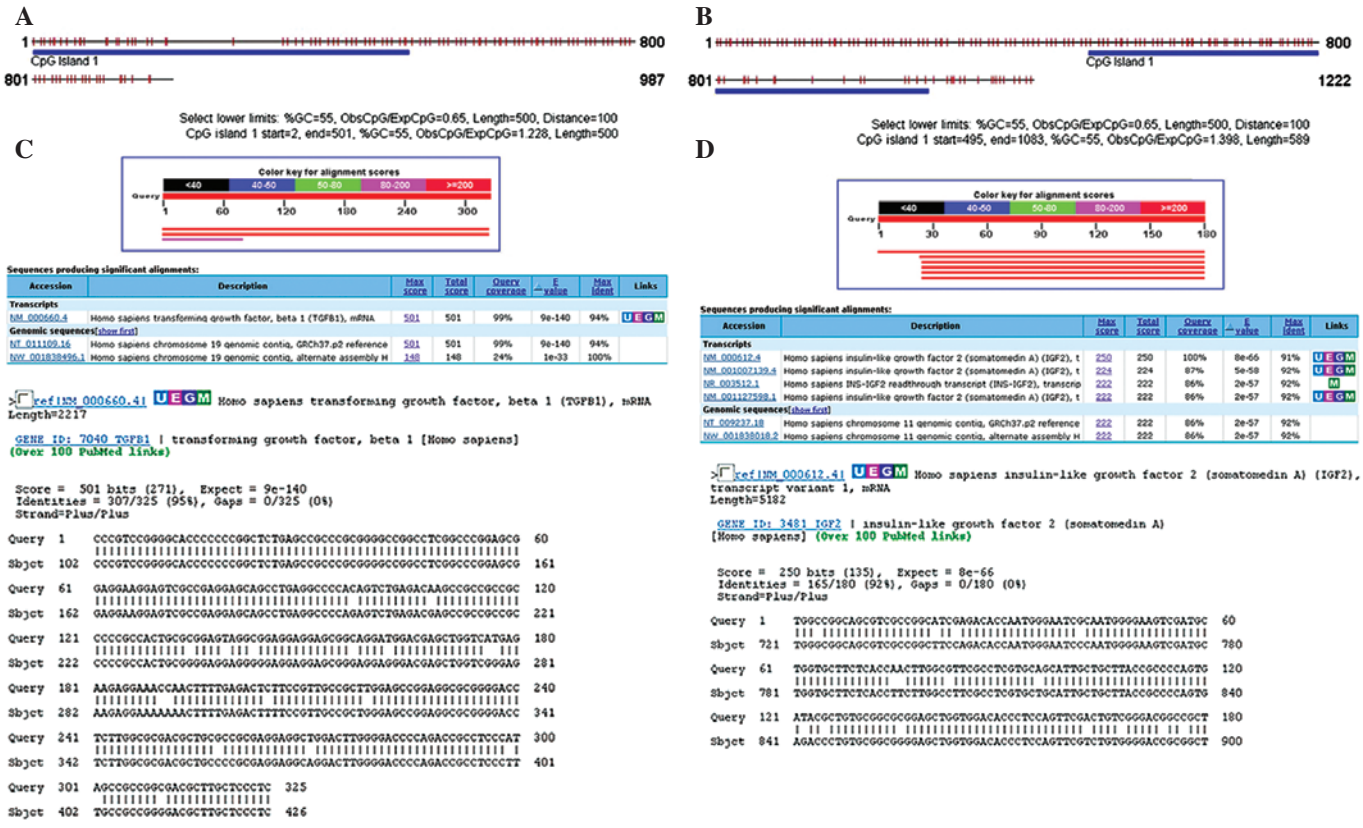

Figure 2. CpG islands and similarity in products of MS-G-SH were analyzed using bioinformatic tools. (A and B) The CpG islands were found on the gene locus. Analysis revealed the presence of long CpG islands in the G9 and A11 DNA segments. (C and D) The similarity in products of MS-G-SH and the human genome were analyzed using the Blast tool. The analysis results revealed that the nucleic acid sequence of G9 had high similarity to the primary exon of *Homo sapiens* transforming growth factor, β1 (TGF-β1) and the A11 sequence had high homology to the secondary exon of *Homo sapiens* insulin-like growth factor 2 (IGF2).

however, if this *Not*I site is deleted or methylated, the normal DNA will not contain the corresponding DNA sequence (12). Therefore, following *Not*I subtraction and MS-G-SH, the methylated *Not*I sites in the DNA samples will behave as deleted *Not*I sites since they will not be digested and therefore the procedure will simultaneously detect genes that are either deleted or methylated (12). In addition, the tester and driver DNA were digested with *Bam*HI and *Bgl*II and self-ligated at DNA concentrations too low for cyclization. Following *Not*I subtraction and MS-G-SH treatment, we isolated the DNA from 101 random clones and sequenced them (Fig. 1). Eight of these clones contained the *Not*I site (numbers 2, 34, 59, 62, 63, 64, 69 and 85). After sequencing, three different clones were determined to contain *Not*I sites (C3, A11 and G9). These results demonstrate the efficiency of subtraction using *Not*I-surrounding sequences.

CpG islands were located on the gene locus. After sequencing, we considered whether the CpG islands were located on the PCR products. The DNA methylation status of the three DNA segments was analyzed using CpG island searcher software (current version: 10/29/04, http://cpgislands.usc.edu/).

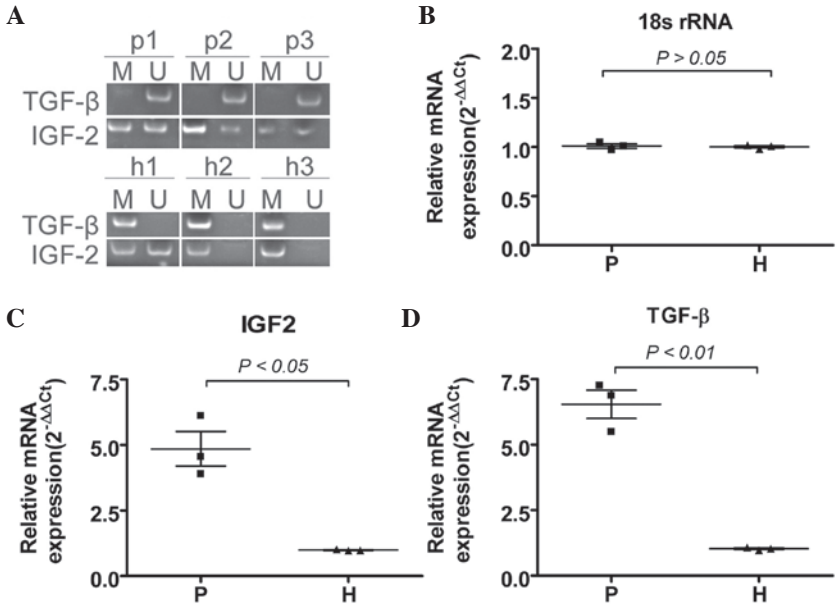

Figure 3. Methylation of the CpG islands of transforming growth factor-β (TGF-β) and insulin-like growth factor 2 (IGF2) and mRNA expression levels were assayed. (A) The positive integral genomic DNA bands were determined via agarose gel electrophoresis. The DNA methylation status of the TGF-β and IGF2 exon CpG islands was then investigated by sodium bisulfite treatment and sequencing of genomic DNA for each group. (B-D) mRNAs of TGF-β and IGF2 expressed differentially between hydatidiform moles and villi were assayed by quantitative real-time polymerase chain reaction (qRT-PCR). M, unmethylated; U, unmethylated; P, hydatidiform moles; H, normal villus.

This analysis revealed the presence of long CpG islands in the A11 and G9 DNA segments (Fig. 2). There were ~589 bp CpG islands (in the 495-1083 bp region) in the A11 DNA product and ~500 bp CpG islands (in the 2-501 bp region) in the G9 DNA product. However, CpG islands in the C3 DNA segments were not observed. The findings indicated that two positive CpG island DNA segments which were modified by DNA methylation would be found by performing MS-G-SH.

Similarity in products of MS-G-SH and the human genome were analyzed using the Blast tool. After sequencing and CpG island forecasting, the Blast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to determine which genes of the human genome had high similarity with the products of MS-G-SSH (Fig. 2). The analysis results revealed that the nucleic acid sequence of A11 had high similarity to the secondary exon of *Homo sapiens* IGF2 (somatomedin A, NM_000612.4). The rate of query coverage was 100% (for the 721-900 bp region of the IGF2 gene) and the percent identity was 92% (165/180) between the product of MS-G-SSH and the IGF2 gene. Blast tool analysis also revealed that the G9 sequence had high homology to the primary exon of *Homo sapiens* TGF-β1 (NM_000660.4). The rate of query coverage was 95% (for the 102-426 bp region of the TGF-β gene) and the percent identity was 95% (307/325). According to the results of the Blast tool and CpG island analysis, the TGF-β and IGF2 genes had specific modifications of methylation that differed between hydatidiform moles and villi.

CpG islands of TGF-β and IGF2 were modified by specific methylation that differed between hydatidiform moles and villi. Four pairs of primers (two pairs of unmethylated primers and two pairs of methylated primers) were designed to determine the methylation status of the primary exon of TGF-β and the secondary exon of IGF2. Genomic DNA from each group (3 hydatidiform moles and 3 villi) was extracted and positive integral genomic DNA bands were determined via agarose gel electrophoresis. The DNA methylation status of the TGF-β and IGF2 exon CpG islands was then investigated by sodium bisulfite treatment and sequencing of the genomic DNA from each group (Fig. 3). In the CpG island region of the primary exon of TGF-β, there was moderate hypermethylation in three normal samples of villi but marked hypomethylation in the three samples of hydatidiform moles. In the CpG island region of the secondary exon of IGF2, in the normal group, there was marked hypomethylation in two villi, while the locus of one villus was partially methylated. However, in the hydatidiform mole group, no significant changes were observed in the CpG island methylation status of the specific locus, and all hydatidiform mole samples displayed partial methylation. These differences between hydatidiform moles and villi suggest that the exons of TGF-β and IGF2 were epigenetically modified in a dynamic manner.

mRNAs of TGF-β and IGF2 are expressed differentially between hydatidiform moles and villi. To determine whether the mRNAs of TGF-β or IGF2 differed when their exon CpG had specific methylated modifications that differed between the hydatidiform moles or villi, the expression levels of mRNA were assayed by qRT-PCR. We found that when the primary exon of TGF-β was moderately hypermethylated in normal samples of villi, the mRNA expression levels of TGF-β were significantly lower than those in hydatidiform moles (Fig. 3). Regarding IGF2 expression, we found that there was marked hypomethylation in three samples of appreciably higher in hydatidiform moles than in villi. Relative mRNA expression is shown following normalization to 18S rRNA, which served as an internal control. These results suggest that when

DNA methylation differed, the mRNA expression level was likely to be affected.

Discussion

The role of the epigenetic modification of DNA and histone in the development of human diseases is just beginning to be understood (14). DNA methylation studies have attracted increasing interest in the field of epigenetics, which was poorly understood a few decades ago but is now a dynamic area of research challenging and revising traditional paradigms of gene expression and behavior (7). Methods for determining DNA methylation and histone modification have been developed. To date, the bisulfite treatment and MS-PCR, and chromatin immunoprecipition (ChIP) are two promising approaches for determining gene methylation and histone modification. Bisulfite treatment and subsequent MS-PCR enables the rapid assessment of the methylation status of virtually all CpG sites within a CpG island, independent of the use of methylation-sensitive restriction enzymes (15). In 1996, Herman *et al* identified promoter region hypermethylation changes associated with transcriptional inactivation in four important tumor suppressor genes (p16, p15, E-cadherin and von Hippel-Lindau) in human cancer by performing MS-PCR (15). In addition, ChIP is a powerful experimental approach that enables the identification of the proteins associated with specific regions of the genome (16). With the appropriate antibodies, it may be used to locate non-histone proteins and histones carrying specific covalent modifications, including acetylation, phosphorylation or methylation (16). It may also be used to study molecular mechanisms in transcription, DNA replication, DNA repair and chromatin remodeling during development and differentiation (16). However, each technology used to examine the epigenetic modification of DNA methylation has its advantages and disadvantages. The largest limitation of MS-PCR is that it is not suitable for examining DNA methylation in larger regions. Thus, methylation-sensitive, single-strand conformation analysis (MS-SSCA) and *Not*I subtraction and MS-G-SH have been developed to solve these problems (7,12). Previous studies have shown that *Not*I sites are almost exclusively located in CpG islands and are closely correlated with functional genes (3,18-23). Therefore, *Not*I sites may serve as very useful markers for physical and genetic mapping and also for the examination of DNA methylation. In 2002, Li *et al* used MS-G-SH in combination with microarrays to detect copy number and methylation changes in the whole genomes of human cancer cells (12). MS-G-SH was developed to screen a relatively large number of CpG sites on a CpG island; moreover, it may be targeted to regions of particular importance within the island.

In the present study, the MS-G-SH technique has been improved by performing MS-PCR to examine DNA product methylation instead of predigestion. MS-G-SH and MS-PCR have been applied to detect DNA methylation changes between hydatidiform moles and villi in whole genomes. Following *Not*I subtraction, MS-G-SH and sequencing, three positive DNA clones were obtained. Moreover, analysis of the CpG islands using searcher software indicated that two positive CpG island segments, which had been modified by DNA methylation, should be found by MS-G-SH. Following sequencing and CpG island forecasting, the results of Blast tool analysis revealed that these nucleic acid sequences had high similarities to the secondary exon of the human IGF2 gene and the primary exon of human TGF-β. According to these results, the TGF-β and IGF2 genes had specific modifications of methylation that differed between hydatidiform mole and villi. In addition, MS-PCR analysis indicated that specific modification of DNA methylation existed in the TGF-β and IGF2 exons of different samples. Furthermore, qRT-PCR showed that when the DNA methylation of TGF-β and IGF2 differed, their mRNA expression levels were affected. In conclusion, MS-G-SH is a useful method for the genome-wide screening of deleted, amplified and methylated *Not*I sites. This approach of screening and analyzing DNA methylation patterns based on the altered composition of PCR products with bisulfite treatment should facilitate future studies of methylation.

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