Allelic methylation status of CpG islands on chromosome 21q in patients with Trisomy 21

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Abstract. Trisomy 21 is a chromosomal condition caused by the presence of all or part of an extra 21st chromosome. There has been limited research into the DNA methylation status of CpG islands (CGIs) in trisomy 21, therefore, exploring the DNA methylation status of CGIs in 21q is essential for the development of a series of potential epigenetic biomarkers for prenatal screening of trisomy 21. First, DNA sequences of CGIs in 21q from the USCS database were obtained and 149 sequences and 148 pairs of primers in the BGI YH database were aligned. All 300 cases were analyzed by a heavy methyl-polymerase chain reaction (HM-PCR) assay and a comparison of the DNA methylation status of CGIs was made between trisomy 21 and the control. The HM-PCR assay results did not show a difference in the DNA methylation status between individuals with trisomy 21 and the control. In total, there were 11 CGIs that showed various DNA methylation statuses between Japanese and Chinese patients. Subsequently, bisulfite genomic sequencing found variations in the methylation status of CpG dinucleotides in CGIs (nos. 14, 75, 109, 134 and 146) between trisomy 21 and the control. The different DNA methylation status of CpG dinucleotides in CGIs may be a potential epigenetic marker for diagnosing trisomy 21. No difference was identified in the DNA methylation status of 21q CGIs among Chinese individuals with trisomy 21 and the control. The homogeneity of the DNA methylation status of 21q CGIs in Chinese patients indicates that DNA methylation is likely to be an epigenetic marker distinguishing ethnicities.

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Introduction

Down's syndrome (DS) or trisomy 21 is a chromosomal condition caused by the presence of all or part of an extra 21st chromosome. DS is a high-incidence birth defect that is often associated with impairment of cognitive ability and physical growth (1). Individuals with DS have a higher risk of congenital heart disease (CHD), dysfunction of the thyroid gland, Hirschsprung's disease, eye and hearing disorders, leukemia and testicular cancer; however, there is a wide range of phenotypic variation in DS (2-6). It is now more than half a century since DS was first shown to result from trisomy 21 (7). Although progress has been made by investigating genes to understand the complex phenotypes associated, the mechanisms remain far from clear.

DS disorders are the result of extra copies of the genes located on chromosome 21. In general, an overexpression of the genes arises and DNA hypomethylation is a possible mechanism to explain the altered gene expression. DNA methylation often occurs in a CpG dinucleotide, in which the cytosine gains a methyl group. Hypermethylation results in transcriptional silencing, for example genomic imprinting and X-chromosome inactivation, while hypomethylation is linked to chromosomal instability and loss of imprinting. Unmethylated CpGs are often grouped in CpG islands (CGIs), which are present in the 5' regulatory regions of a number of genes and acquire abnormal hypermethylation in numerous disease processes. Alterations of DNA methylation have been recognized as an important component of cancer development (8). Altered methylation status in peripheral blood lymphocytes (PBLs) has been linked to increased risk of several diseases and, in addition, PBLs are an easily accessible source to identify potential epigenetic biomarkers (9).

In the present study, a comprehensive CGI methylation analysis was performed using trisomy 21 and control cases to identify the allelic methylation status in CGIs of PBLs. The aim of the study was to determine the utility of CGI methylation analysis associated with human diseases, in particular, the DS complex phenotypes and to locate potential epigenetic biomarkers for prenatal diagnosis. To the best of our knowledge, there are no comprehensive CGI methylation analyses that have focused on chromosome 21q using a large number

of trisomy 21 samples (10), thus, the present study focused on chromosome 21, which is the major contributor of DS complex phenotype.

Materials and methods

Alignment of CGI data to the BGI YH database. The present analysis was based on the results of a comprehensive measurement of CGI methylation on human chromosome 21q (11). Yamada *et al* repeat-masked the chromosome sequence and computationally identified all non-repetitive CGIs using standard tools and parameters (GC content, >50%; ratio of observed versus expected number of CpG dinucleotides, >0.6; >400 base pairs in length). The authors designed primers for the 149 CGI identified and extracted corresponding DNA from samples of human PBLs. Finally, the authors determined the methylation status of each CGI using methylation-specific restriction enzymes (via *Hpa*II-*Mcr*BC-PCR). The DNA sequences of 149 CGIs were acquired from UCSC (11) and were aligned with the sequence in BLAST, the BGI YH database (http://yh.genomics.org.cn/search.jsp). The score of each alignment was indicated by one of five colors, in which the highest score was >200 and shown in red. Multiple alignments on the same database sequence were connected by a striped line. A continuous red line indicated a perfect match (100% match) and the alignment data was classified into ten groups based upon the percentage of red line. Subsequently, the primers of the 148 CGIs (11) were aligned in sequence in the BLAST BGI YH database (CGI no. 103 was investigated with bisulfite sequencing as it lacked *Hpa*II and Hha*I* sites and could not detected by HM-PCR). When a base differed to a sequence in the database, the primer was a 0% match. The alignment results were divided into three groups: Perfect match (a pair of primers was 100% matched); 50% match (one primer of a pair was 100% matched); and no match (a pair of primers was 0% matched).

Study subjects and diagnosis. A total of 150 control cases and 150 DS cases were obtained through the Children's Hospital of Chongqing Medical University (Chongqing, China) and participant's families or correspondents provided informed consent. The distribution of age and residential placement did not differ between the control and the patients. Confirmation of trisomy 21 was obtained by G-banded karyotypes, all patients had complete trisomy 21, with 100% concordance between cytogenetics and the clinical diagnosis of DS. Data for Japanese individuals were obtained from the study by Yamada *et al* (11).

Preparation of genomic DNA from human PBLs. Trisomy 21 and normal human lymphocytes were prepared from peripheral blood. Total genomic DNA was extracted by TIANamp Blood DNA kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. The concentration of DNA was determined using the GeneQuant pro RNA/DNA Calculator (GE Healthcare, Pittsburgh, PA, USA) and the integrity of DNA was determined by electrophoresis.

HpaII-McrBC PCR assay. Human genomic DNA (0.5 μg) was digested with 15 units *Hpa*II or *Hha*I (Promega Corp., Madison, WI, USA) or 100 units *Mcr*BC (New England Biolabs, Ipswich, MA, USA) overnight at 37˚C in 50 μl of the buffers recommended by the suppliers. Subsequently, the enzymes were inactivated at 65˚C for 20 min and the levels of digested DNA were determined by electrophoresis.

For PCR analysis, 0.5 μl (5 ng) genomic DNA digested with each enzyme was used in a 10 μl reaction mixture containing 0.25 units Ex-*Taq* DNA polymerase (Takara Bio Inc., Shiga, Japan), 4 nmol dNTP (Takara Bio Inc.) and 10 nmol each primer [Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China] in 5 μl 2X GC Buffer I or 2X GC Buffer II (Takara Bio Inc.). The thermal cycling parameters were recommend by Yamada *et al* (11). The amplified products were electrophoresed on a 2% agarose gel, stained with Goldview nucleic acid stain (SBS Genetech, Beijing, China) and visualized by the Molecular Imager Gel Doc XR+ System (Bio-Rad, Hercules, CA USA).

Bisulfite genomic sequencing. Human genomic DNA (2 μg) from PBLs was treated with sodium bisulfite according to the standard procedure. One-tenth of the bisulfite-treated DNA was used for PCR in a 50 μl reaction mixture, 10X PCR Buffer [100 mM Tris-HCl (pH 8.8), 500 mM KCl, 15 mM $MgCl_2$ and 0.8% (v/v) Nonidet P40], 10 nmol each dNTP, 10 nmol each primer and 4 units Ex-*Taq* DNA polymerase [all Sangon Biotech (Shanghai) Co., Ltd.]. The primer sequences are described in Table I. The amplified products were subsequently cloned into a pUC18-T vector [Sangon Biotech (Shanghai) Co., Ltd.] and sequenced using the Applied Biosystem 3730 DNA Analyzer (Life Technologies, Carlsbad, CA, USA). The results were further analyzed using the BDPC DNA methylation analysis platform (http://services.ibc.uni-stuttgart.de/BDPC/BISMA/).

Statistical analysis. Statistical analyses were performed using SPSS 10.0 software (SPSS Inc., Chicago, IL, USA). Comparisons between two groups were made using χ^2 tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CGI sequence alignment data from the BGI YH database. There are a total of 149 CGIs in chromosome 21q, according to criteria outlined by Yamada *et al* (11). The alignment data from the BGI YH database showed that 87% of CGIs (130 of 149) were >80% matched; 13.42% were a perfect match (20 of 149), 22.14% were >95% matched (33 of 149), 21.48% were >90% matched (32 of 149), 16.11% were >85% matched (24 of 149) and 14.09% were >85% matched (21 of 149). A match of <80% accounted for 13% of total CGIs (19 of 149); 2.01% were >75% matched (3 of 149), 8.05% were >70% matched (12 of 149), 0.67% were >65% matched (1 of 149), 1.34% were >60% matched (2 of 149) and 0.67% were >55% matched (1 of 149). The primers of the 148 CGIs were obtained from the experimental design of Yamada *et al* (11). The alignment data indicated that 98% (145 of 148) of the primers were a perfect match, two pairs of primers (CGI nos. 27 and 75) were a 50% match and one pair of primers (CGI no. 90) was not a match. New primers for CGI nos. 27, 75 and 90 were designed using Primer 3 (v0.4.0; http://primer3.wi.mit.edu/) as follows:

Table I. CGI DNA methylation statuses as determined by bisulfite genomic sequencing.

CGI no.	Methylation using bisulfite genomic sequencing		Methylation using HM-PCR		Bisulfite genomic sequencing primers	CGI-linked genes
	Chinese	Japanese	Chinese	Japanese		
14	Unmethylation		Unmethylation	Unmethylation	5'-GTAGATYGTGTAATTTTATTTATTAGTTAG-3' 5'-CAAAACTACRAAATTCCCAAAC-3'	ADAM metallopeptidase with thrombospondin type 1 motif 5 (ADAMTS5)
38	Unmethylation		Unmethylation	Unmethylation	5'-GAGTTTGTGGGATTTTGTAGTGAGT -3' 5'-CATCRCCTATCACCTAAACCC-3'	Regulator of calcineurin 1 (RCAN1)
41	Incomplete methylation		Incomplete methylation	Unmethylation	5'-GGAGGTYGTTTTAGAAAGTTGAGA -3' 5'-CAACCCCAACTTCCTCTACTCC-3'	Runt-related transcription factor 1 (RUNX1)
75	Unmethylation		Unmethylation	Complete methylation	5'-GGGATAAYGATATTTTTTGGGG-3' 5'-CCATACCRACTATTCTTTATTACATTC-3'	PR domain zinc finger protein 15 (PRDM15)
103	Complete methylation	Composite methylation			5'-GTAGTTGGGATTATAGTTATATGTTATTATG-3' 5'-CCTCTAAATCCTTATCCCAAAAC-3'	ES1 protein homolog, mitochondrial isoform Ib precursor
109	Incomplete methylation		Incomplete methylation	Unmethylation	5'-GATGGTTTYGYGGGGTTAGG-3' 5'-RCCCTACAACAACACCRAAACC-3'	Protein C21orf2 isoform 4 (C21orf2)
129	Incomplete methylation		Incomplete methylation	Complete methylation	5'-GTTTGAGGTTGGTTTAGGTTTTG-3' 5'-CATCTCCCRAATATAAAACTTACTCC-3'	Folate transporter 1 isoform 3
133	Complete methylation		Complete methylation	Complete methylation	5'-TATGGTGGTAGGTAAGAGAGTATGTG-3' 5'-CCCRCAAACCCTAAATCTTAAC-3'	Folate transporter 1 isoform 3
134	Incomplete methylation		Incomplete methylation	Complete methylation	5'-AAGATTTTGTAGTTGTAAGTGGTGTAG-3' 5'-CCAACTAAATACATATTCTTCCTTCTC-3'	Poly (rC)-binding protein 3 (PCBP3)
46	Complete methylation		Complete methylation	Complete methylation	5'-GATATTTTATATTTGTAGGGTTAGTGGA-3' 5'-CAAAACCCCAATCAATCACAC-3'	Pericentrin (PCNT)

CGI, CPG islands; HM-PCR, heavy methyl-polymerase chain reaction.

Figure 1. Human genomic DNA incubated with *Mcr*BC, *Hpa*II and *Hha*I enzymes. Lanes 1, human genomic DNA; 2, DNA incubated with *Mcr*BC; 3, DNA incubated with *Hpa*II; and 4, DNA incubated with *Hha*I.

Forward: 5'-CTCTCACCGCCGCAAGTCGGTCGC-3' and reverse: 5'-CGTTGTTGGGGAACTTTTACTGTG-3' for CGI no. 27; forward: 5'-CAGCTCAAGAATGCACTGCATTT-3' and reverse: 5'-AGTCAAAACCCGGCTGGATTTCC-3' for CGI no. 75; and forward: 5'-GTATGTGCCACCAAA TGATTATTCCT-3' and reverse: 5'-ACTCACTCTCCTAAC TTGAAGTTTTC-3' for CGI no. 90.

HpaII-McrBC PCR assay to evaluate 21q CGI allelic methylation status. The electrophoresed images of genomic DNA and DNA products digested by *Mcr*BC, *Hpa*II or *Hha*I enzymes, respectively, are shown in Fig. 1. When the genomic DNA were completely digested by the enzyme, the DNA products appeared as smeared bands in gel electrophoresis. Using the heavy methyl-polymerase chain reaction (HM-PCR) assay, results showed that there was almost no difference in the DNA methylation status of 21q CGIs among individuals with trisomy 21 and the control, as shown in Table II. A total of 148 CGIs in 21q were screened, including 102 null methylation, 26 complete methylation, 7 composite methylation and 13 incomplete methylation. However, 3 null methylation CGIs (nos. 12, 41 and 109), 2 complete methylation (nos. 129 and 134) and 1 composite methylation (no. 55) in Japanese patients were all incomplete methylation in Chinese patients. In addition, 1 incomplete methylation CGI (no. 68) and 1 complete methylation CGI (no. 75) in Japanese patients were also incomplete methylation in Chinese patients. Finally, 1 complete methylation CGIs (nos. 1 and 137) in Japanese patients were composite methylation in Chinese. In total, there were 10 CGIs that showed varying DNA methylation statuses among Japanese and Chinese patients, as presented in Fig. 2 and Table II.

Table II. Variations in DNA methylation statuses between Chinese and Japanese patients determined using HM-PCR.

	Methylation status detected using HM-PCR		Methylation status detected using bisulfite genomic sequencing		
CGI no.	Chinese	Japanese	Chinese	Japanese	CGI-linked genes
1	Composite methylation	Complete methylation			LON peptidase N-terminal domain and ring finger 2 (LONRF2)
12	Incomplete methylation	Unmethylation			ADAM metallopeptidase with thrombospondin type 1 motif 1 (ADAMTS1)
41	Incomplete methylation	Unmethylation	Incomplete methylation		Runt-related transcription factor 1 (RUNX1)
55	Incomplete methylation	Composite methylation		Composite methylation	Holocarboxylase synthetase (HLCS)
68	Unmethylation	Incomplete methylation			Tryptophan rich basic protein (WRB)
75	Unmethylation	Complete methylation	Unmethylation		PR domain zinc finger protein 15 (PRDM15)
109	Incomplete methylation	Unmethylation	Incomplete methylation		Protein C21orf2 isoform 4
129	Incomplete methylation	Complete methylation	Incomplete methylation		Folate transporter 1 isoform 3 (SLC19A3)
134	Incomplete methylation	Complete methylation	Incomplete methylation		Poly (rC)-binding protein 3 (PCBP3)
137	Composite methylation	Complete methylation		Complete methylation	Collagen α-1(VI) chain precursor (COL6A1)

HM-PCR, heavy methyl-polymerase chain reaction.

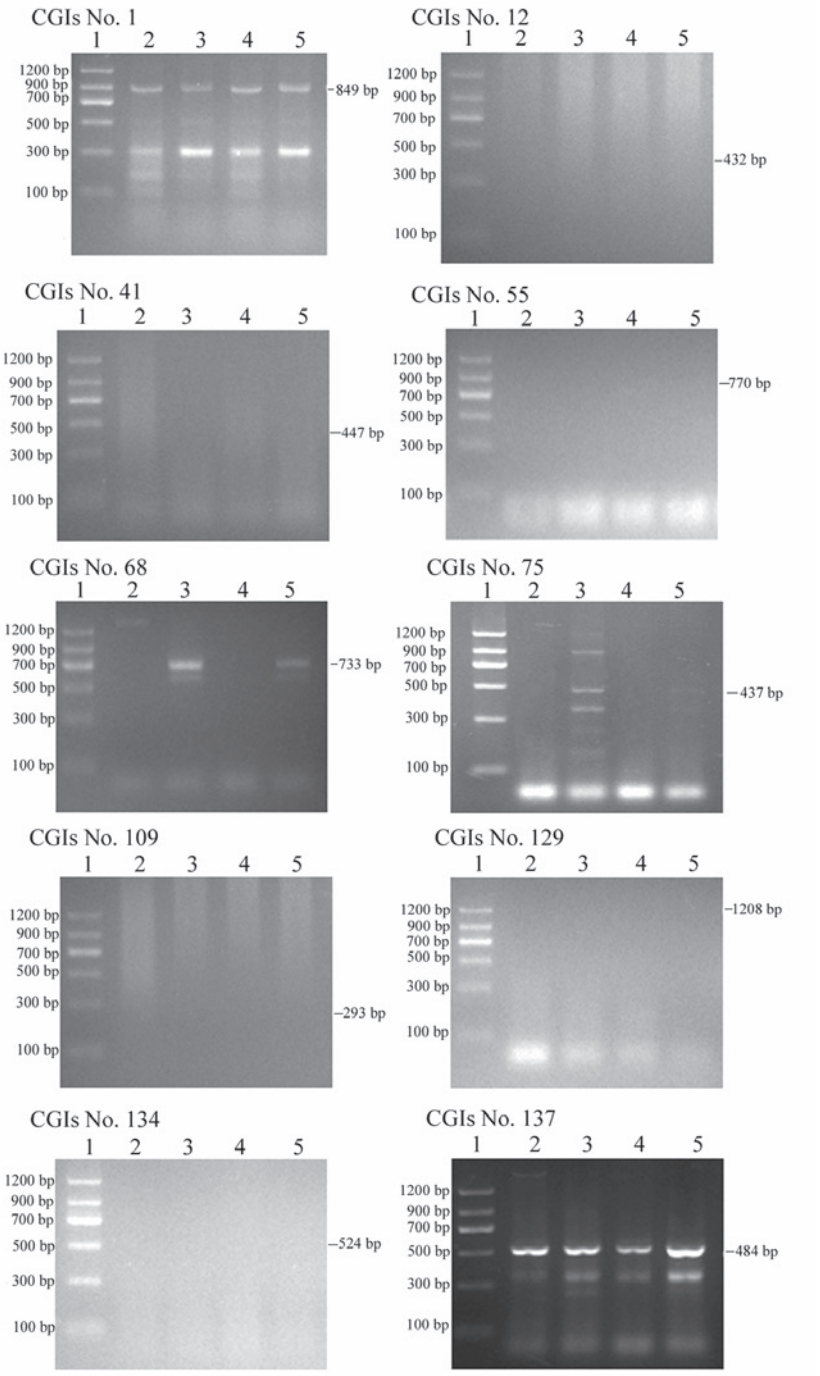

Figure 2. Representative agarose gel showing polymerase chain reaction (PCR) products of ten methylation CGIs on chromosome 21q. Lane 1, marker; 2, PCR products of *Hpa*II digested DNA from a individual with Down's syndrome; 3, PCR products of *Mcr*BC digested DNA from a individual with Down's syndrome; 4, PCR products of *Hpa*II digested DNA from the control; and 5, PCR products of *Mcr*BC digested DNA from the control. CGI, CpG island.

Bisulfite genomic sequencing to confirm allelic methylation status. Nine CGIs (nos. 14, 38, 41, 75, 109, 129, 133, 134 and 146) were selected to validate the HM-PCR assay data using bisulfite sequencing in trisomy 21 and the control, as shown in Table I. These validations were determined with bisulfite sequencing, which showed that CGI nos. 41, 109, 129 and 134 were incomplete methylation, CGI nos. 14, 38 and 75 were null methylation and CGI nos. 133 and 146 were complete methylation in Chinese patients, as shown in Fig. 3; these data validate the HM-PCR results. The methylation status of CGI no. 103 was detected by bisulfite sequencing, as it was short of the *Hpa*II and *Hha*I recognition sites. The composite methylation CGI no. 103 in Japanese patients was complete methylation in Chinese patients, as shown in Table I and Fig. 3. Furthermore, the various methylation statuses of CpG dinucleotides in 5 CGIs between trisomy 21 and the control were found, including 5 sites in CGI no. 14, 6 sites in CGI no. 75, 14 sites in CGI no. 109, 1 site in CGI no. 134 and 3 sites in CGI no. 146. A total of 29 CpG dinucleotides presented allele-specific DNA methylation, as shown in Fig. 3.

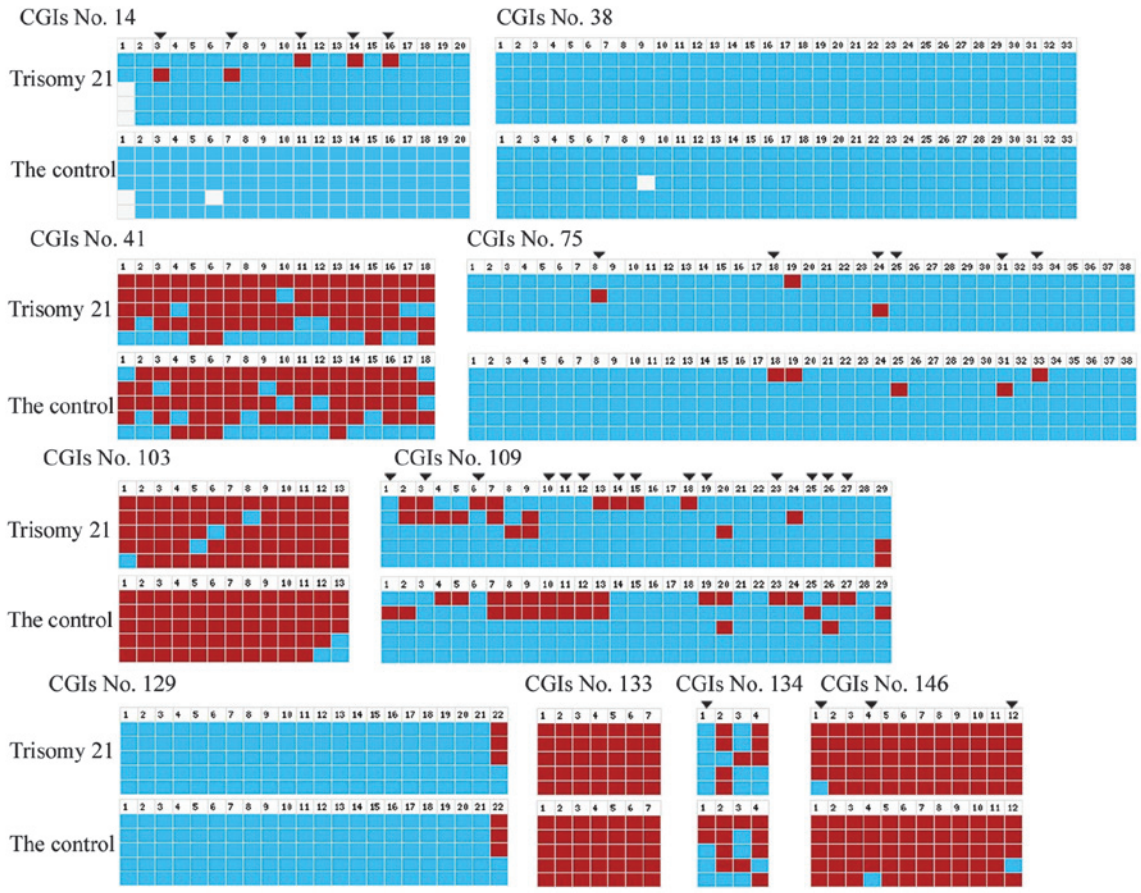

Figure 3. Methylation status of ten CGIs on chromosome 21q as determined by bisulfite genomic sequencing, defined as methylated (red), unmethylated (blue) and unknown (white). The different methylation statuses in CpG dinucleotide sites between trisomy 21 and the controls are indicated by downward arrows. Image depicts the various methylation CpG dinucleotide sites in CGI no. 14 (nos. 3, 7, 11, 14 and 16), 75 (nos. 8, 18, 24, 25, 31 and 33), 109 (nos. 1, 3, 6, 10, 11, 12, 14, 15, 18, 19, 23, 25, 26 and 27), 134 (no. 1) and 146 (no. 1, 4 and 12). CGI, CpG island.

Discussion

Individuals with DS have an additional chromosome 21, which is associated with the gene-dosage effect and a wide spectrum of phenotypic consequences, including life-threatening complications, clinically significant alteration of life course (e.g., mental retardation) and dysmorphic physical features (12). The mechanisms of gene regulation, include function of conserved nongenic regions, microRNA activities, RNA editing and DNA methylation. DS with a CHD is associated with a global hypomethylation status (13). DNA methylation is a possible mechanism of gene expression alteration, which may contribute to various abnormalities. Chango *et al* (14) used a combination of methylation-sensitive arbitrarily primed PCR and quantitation of DNA fragments to find six fragments that were hypermethylated in PBLs from eight individuals with DS, compared with eight normal controls. Kerkel *et al* (10) observed that 8 genes had different methylation status between the DS patients and normal controls. One of the 8 genes is named SUMO3 and is located on chromosome 21. The current observations are consistent with this data. There were differences in the DNA methylation status of CpG dinucleotide sites in 21q CGIs (nos. 14, 75, 109, 134 and 146) among individuals with trisomy 21 and the control. Molecular analysis reveals that the 21q22.1-q22.3 region, also known as the DS critical region (DSCR), appears to contain the gene or genes responsible for the CHD observed in DS (15-17). Altered DNA methylation in 21q may be constitutively silenced over-expressed genes in DS (10). Noteworthy gene candidates for specific dysfunctions in DS are already emerging from these research data. In the present study, CGI no. 75 was linked to *PRDM15*, which is a candidate gene for a particular phenotype of DS or bipolar affective disorder (18). CGI no. 14 was associated with *ADAMTS5*, which is a protease involved in regulating aggrecanase activity in cartilage; deletion of active ADAMTS5 prevents cartilage degradation (19,20). CGI no. 109 was associated with *C21orf2*, which is involved in the regulation of cell morphology and cytoskeletal organization (21). CGI no. 134 was associated with *PCBP3*, which is associated with frontotemporal dementia and hypothesized to participate in mRNA metabolic processes (22,23). CGI no. 146 was associated with *PCNT*, which may be important in preventing premature centrosome splitting during interphase by inhibition of NEK2 kinase activity at the centrosome (24). The methylation alteration may be associated with the DS phenotype, as insights from investigating DS as a model system have shed light on potential epigenetic biomarkers for noninvasive prenatal diagnosis in the general population.

The fetal-specific DNA methylation ratio permits noninvasive prenatal diagnosis of trisomy 21 by analyzing fetal-specific DMRs in free fetal DNA of the maternal circulation during pregnancy (25-28). A few differentially methylated sequences

are fetal DNA methylation markers, including *HLCS* and *DSCR4* located at chromosome 21, *RASSF1A* located at chromosome 3 and *ZFY* located at chromosome Y (29-31). However, these markers were not specific for individuals with DS. In the present study, methylation alteration in DS may represent a specific epigenetic biomarker for future prenatal diagnosis; however, DNA methylation must be further verified in fetuses with DS.

DNA methylation in DS individuals did not change significantly in comparison with the controls (14). In the present study, there was no significant difference in 21q CGIs in Chinese patients, as determined using HM-PCR; this result was in accordance with observations from Kerkel *et al* (10) of no significant difference between normal and DS samples. While the different methylation statuses of CpG dinucleotide sites between the normal and DS do not cause the difference in the CGIs' methylation status as screened by HM PCR assays, because CGIs comprise numerous CpG dinucleotide sites only a few altered methylation levels are likely to not affect HM PCR results (11). Varying DNA methylation statuses of CGIs in 21q existed among Japanese and Chinese patients. According to the results of alignment in the BGI YH database, data showed that 87% of CGIs (130 of 149) were >80% matched and 149 CGIs were feasible for the analysis of Chinese DNA sequences in the HM-PCR assay (32). In total, 149 CGIs in 21q were screened, including 102 null methylation, 26 complete methylation, 7 composite methylation and 13 incomplete methylation. There were 11 DNA methylation statuses of CGIs, no. 1, 12, 41, 55, 68, 75, 103, 109, 129, 134 and 137, among Japanese and Chinese patients. The racial disparities in DNA methylation patterns of differing ethnic groups implicate the probable role of molecular markers in determining an individual's susceptibility to disease. Racial disparities in DNA methylation patterns have been found in prostate cancer, endometrial carcinoma, breast cancer and laryngeal cancer and, in addition, are associated with racial difference in the cancer prognosis and survival rate (33-37). CGI no. 12 is associated with *ADAMTS1*, which is a protease involved in extracellular matrix proteolysis and antiangiogenesis and is involved in ischemia-induced retinal neovascularization, overexpressed in neurodegenerative disorders and downregulated in breast carcinomas (38-41). CGI no. 41 is associated with *RUNX1*, which is linked to a poor outcome of acute lymphoblastic leukemia and susceptibility to autoimmune disease; emergence of the *RUNX1* mutations was detected in advanced chronic myelogenous leukemias with acquired trisomy 21 (42-46). CGI no. 55 is associated with *HLCS* and a lack of *HLCS* may cause multiple carboxylase deficiency (47). CGI no. 68 is associated with *WRB*, which is a conserved tryptophan-rich motif in the membrane-proximal region of the HIV-1 gp41 ectodomain and is important for Env-mediated fusion and virus infectivity (46,48). CGI no. 129 is associated with *SLC19A1*, a transporter for the intake of folate (49). CGI no. 137 is associated with *COL6A1*, depletion of which is a cause of Bethlem myopathy and Ullrich congenital muscular dystrophy (50).

In conclusion, the different DNA methylation status of 21q CGIs status between Chinese and Japanese individuals, and the same DNA methylation status detected by HM-PCR between Chinese individuals with DS and the control indicates that DNA methylation is likely to be an epigenetic marker for distinguishing ethnicities. The different DNA methylation status of CpG dinucleotides between individuals with DS and the control may contribute to the DS complex phenotypes and be a potential epigenetic marker for diagnosing trisomy 21.

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