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A parent-of-origin detectable polymorphism in the hypermethylated region upstream of the human *H19* gene

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Abstract The *H19* gene is a paternally imprinted gene located on chromosome 11p15.5. In this study the H19FR haplotype polymorphism including three SNPs upstream of the *H19* gene was investigated. Six genotypes derived from three alleles were detected in the Japanese population by means of PCR and subsequent constant denaturing gel electrophoresis. Based on the methylation status of the genomic DNA from blood samples, selective detection of the parental allele for H19FR was examined by using two types of enzyme, the methylation-sensitive restriction enzymes *Hpa*II or *Hha*I and *Mcr*BC. Genomic DNA digested by either *Hpa*II or *Hha*I, revealed a single band derived from the paternal allele, as a result of cleavage of unmethylated recognition sites on the maternal allele. On the contrary, the use of *Mcr*BC, which can digest a methylated paternal sequence, resulted in exclusively amplifying the maternal allele. This method could be one of the useful techniques for discriminating the parental origin of alleles.

Keywords Parental discrimination · *H19* gene · Methylation · Methylation-sensitive restriction enzyme

Introduction

Identification of human parental origin sometimes provides significant information for forensic examinations. Polymorphic markers on the Y chromosome having paternal traits and those on mitochondrial DNA for maternal lines have been utilized for personal identification [1, 2], parentage testing and phylogenetic studies [3, 4, 5]. However, analyses of parental alleles on autosomal markers are rarely

examined, since there is no suitable method to discriminate either of the parent-derived alleles.

Genomic imprinting is an epigenetic phenomenon of parent-of-origin-specific expression of genes. There is a multi-step process to achieve the mono-allelic expression of imprinted genes, including a differential methylation of parental DNA, which is thought to play a major role in causing genomic imprinting. There are techniques using methylation-sensitive restriction endonucleases (msREs), which digest an unmethylated recognition site, and/or bisulfite treatment converting unmethylated cytosine to uracil, which have been developed to elucidate the methylation status of imprinted genes. The *H19* gene is located in a cluster of imprinted genes on human chromosome 11p15.5, where the *TH* gene is also involved but not imprinted (Fig. 1), and maternally transcribed. Several studies have shown that CpGs (CG dinucleotides) in the paternal sequence upstream of the *H19* gene are highly methylated [6, 7, 8]. In the forensic field, not so many studies concerning DNA methylation have been investigated [9, 10].

In this paper, we describe three closely located single nucleotide polymorphisms (SNPs) upstream of the imprinted *H19* gene, designated as the H19FR haplotype, and a selective discrimination of either parental allele by using an enzymatic digestion on differentially methylated genomic DNA.

Materials and methods

Genomic DNA

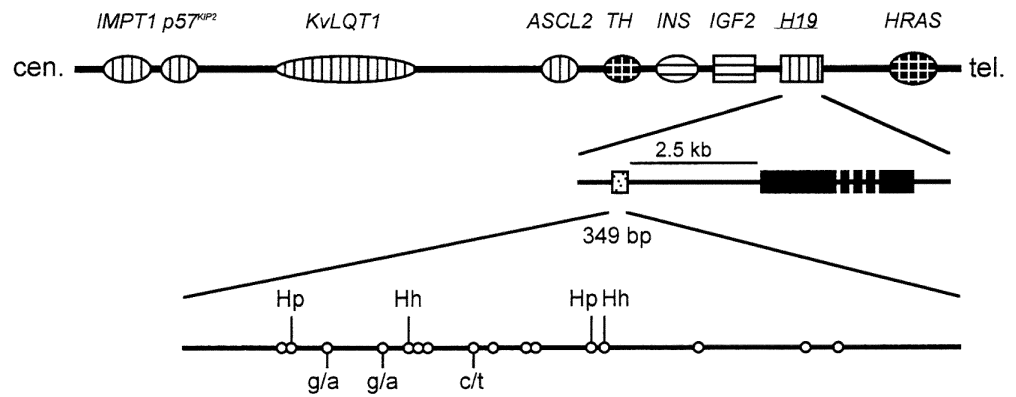
Blood samples were taken from healthy Japanese volunteers who gave written consent, and genomic DNA extracted by the phenol/chloroform method was analyzed.

Sequence data

The upstream sequence of the *H19* gene used in this study is available from GenBank accession number AF125183.

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Fig. 1 Maps of imprinting genes on human chromosome 11p15.5 and H19FR region: paternally (*horizontal lines*), maternally (*vertical lines*), and biallelically (*cross-hatching*) expressed genes. Recognition sites of methylation-sensitive restriction enzymes (*HpaI*, *HpaII*; *Hh*, *HhaI*) and three SNPs (*g/a*, *g7523a*; *g/a*, *g7547a*; *c/t*, *c7591t*; accession no. AF125183) are denoted by long and short vertical lines, respectively, 16 CpG sites (in type 1 allele) are indicated by open circles



PCR

The composition of the PCR cocktail (25 μ l) was as follows: 1 \times buffer (Qiagen, Hilden, Germany), 2.5 mM MgCl₂, 200 μ M of each dNTP, 20 pM of each pair of primers (H19F 5'-ggctcttgca tagcacatgt-3', and H19R-GC, GC clamp, cgcccgcgcg ccccgcgcg cgtcccgcgcg ccccgcgcg, attached to the 5' end of H19R primer, 5'-tcccacatc ccatggaact-3'), 0.5 U of HotStarTaq DNA polymerase (Qiagen) and 10–20 ng of genomic DNA. After heating the cocktail at 94°C for 15 min, PCR was carried out for 32 cycles of 94°C for 30 s, 61°C for 30 s and 72°C for 1 min, followed by post-extension at 72°C for 4 min.

Denaturing gradient gel electrophoresis (DGGE) and constant denaturing gel electrophoresis (CDGE)

DGGE and CDGE [11] were carried out using the DCode Universal Mutation Detection System (BIO-RAD, Hercules, CA). The gel (160 \times 100 \times 1 mm in size) was composed of 6% acrylamide (2.6% C), 1 \times TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0), and an appropriate concentration of denaturants (100% solution containing 7 M urea and 40% formamide). After heating the running buffer, 1 \times TAE at 60°C, the PCR product was mixed with the same volume of 2 \times dye (70% glycerol, 0.05% bromophenol blue and 0.05% xylene cyanol), applied on the gel, and electrophoresis was carried out at 120 V for 2–3 h. After the run, the bands were stained with SYBR Gold Nucleic Acid Gel stain (Molecular Probes, Eugene, OR) or silver nitrate.

Enzymes

Based on the sequence around CpGs in the H19FR fragment (Fig. 1), either of the msREs, *HhaI* (recognition site of G^mCGC) or *HpaII* (C^mCGG) was used. As an enzyme having an inverse effect to msREs, *McrBC* [5'...Pu^mC(N_{40–3000})Pu^mC...3'] was applied to cleave a cytosine-methylated sequence [12]. Treatment was performed in a 10 μ l reaction mixture containing 5 U of enzyme, 1 \times test reagents, and 10–40 ng of genomic DNA, at 37°C for 2 h followed by 90°C for 5 min to stop the reaction.

After the enzymatic treatment, digested DNA without a purification step was subjected to PCR amplification and CDGE analysis.

Results and discussion

H19FR polymorphism

Three closely situated SNPs, nucleotide positions 7523 (dbSNP rs2735971), 7547 (not registered) and 7591 (rs2735972) [13] located 2.5 kb upstream of the *H19* gene

were examined (Fig. 1). To detect as a haplotypic polymorphism, designated H19FR, we designed a pair of primers (H19F and H19R-GC) to amplify the target region of 349 bp (Fig. 1) by attaching a 40 bp GC clamp. DGGE and CDGE, a modified method of DGGE, are highly sensitive and easy methods to discriminate point mutations among PCR products amplified with a primer having a GC clamp [11]. On an amplified fragment, a suitable concentration of denaturant at 60°C was examined using a perpendicular DGGE analysis with a 20–70% denaturing gradient (data not shown), so that a 45% denaturant (3.15 M urea and 18% formamide) could be applied to a CDGE analysis for H19FR, which resulted in distinctive bands and reproducible effects. Of six band patterns observed in the Japanese population, three allelic bands, named types 1, 2 and 3, were clearly separated (Fig. 2, lanes 1–3), and each of three heterozygous genotypes showed characteristic

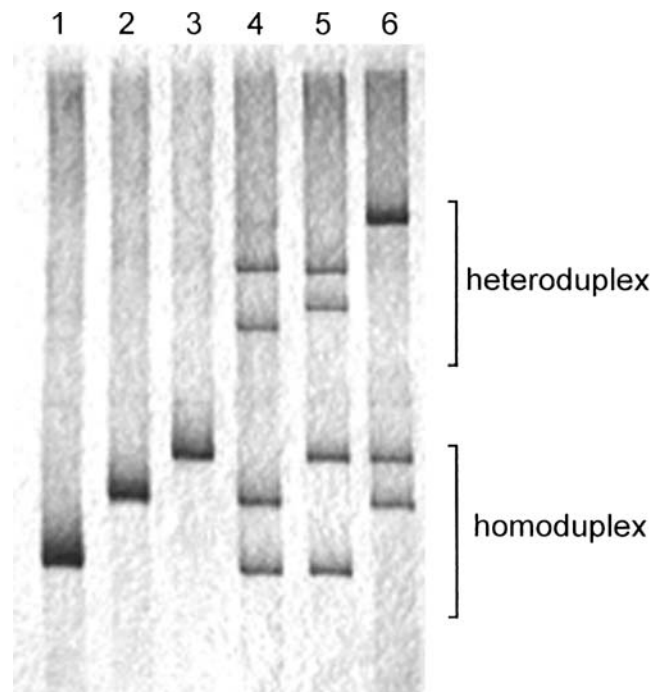


Fig. 2 CDGE band patterns of H19FR polymorphism. Lane 1 type 1-1, lane 2 2-2, lane 3 3-3, lane 4 2-1, lane 5 3-1, lane 6 3-2

Table 1 Distribution of H19FR genotypes in a Japanese population sample

Genotype	Observed	Expected
1-1	47	42.0
2-1	44	56.5
2-2	26	19.0
3-1	27	24.4
3-2	15	16.4
3-3	3	3.6
Total	162	162.0

$H19FR^*1=0.5093$

$H19FR^*2=0.3426$

$H19FR^*3=0.1481$

$\nu=3, \chi^2=6.42, 0.1 > P > 0.05$

EH Expected heterozygosity 0.6013.

PIC Polymorphism information content 0.5238.

PE Probability of paternity exclusion 0.3161.

heteroduplex band(s) migrating slower than homoduplex ones (Fig. 2, lanes 4–6).

The nucleotide composition of the three SNPs in each allele was revealed by direct sequencing as follows: positions 7523, 7547 and 7591 are G, G and C, respectively, for type 1 allele, G, A and C for type 2, A, G and T for type 3. In 162 Japanese samples, the frequencies of $H19FR^*1$, $H19FR^*2$ and $H19FR^*3$ were 0.5093, 0.3426 and 0.1481, respectively. The frequency data of the SNP at position 7591 (C/T) was similar to that described for the *AvaI* RFLP [13]. Furthermore, statistical values of heterozygosity (HZ), polymorphism information content (PIC) and the probability of paternity exclusion (PE) were estimated to be 0.6013, 0.5238 and 0.3161, respectively (Table 1).

Selective detection of parental alleles in the H19FR system

In imprinted genes, the cytosines of CpGs, especially around the promoter region, are differentially methylated between parental alleles, and it is thought that the methylation accompanying various tissue-dependent states could play a suppressive role in the gene expression. Many methods using msREs have been developed to detect parental origin in the study of genomic imprinting, while *Mcr*BC has not been so commonly used. *Mcr*BC has an inverse effect compared to msREs and can cut the DNA sequence between methylated CpGs [9]. Investigations using both msREs and *Mcr*BC were only achieved to diagnose Prader-Willi (PWS) and Angelman syndromes (AS) with uniparental disomy on 15q11–q13 [14]. Several studies on the *H19* gene have revealed that the paternal CpGs 5' upstream of the transcription site were heavily methylated but maternal ones were almost unmethylated [6, 7], even in blood DNA [8]. Therefore, it is expected that there is the possibility of selective parental discrimination of the H19FR alleles from blood samples, i.e. a suitable msRE would cleave the maternal H19FR allele, while the *Mcr*BC would digest the other.

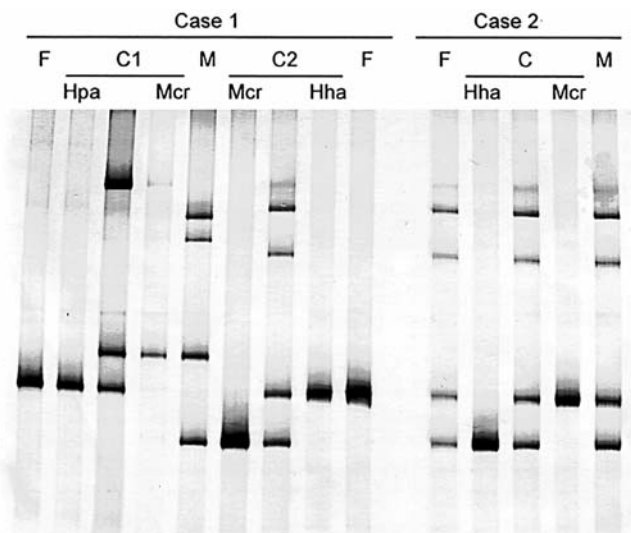


Fig. 3 Selective detection of parental alleles in two families. CDGE analysis was carried out after amplification of H19FR region for genomic DNA with and without digestion by methylation-sensitive restriction enzyme (*Hpa*, *Hpa* II; *Hha*, *Hha* I) or *Mcr*, *Mcr* BC. Case 1: F (father, type 2-2), C1 (child 1, 3-2), C2 (child 2, 2-1), M (mother, 3-1). Case 2: F (2-1), C (2-1), M (2-1)

Genomic DNA showing a heterozygous H19FR genotype was digested with msRE (*Hha*I or *Hpa*II) or *Mcr*BC followed by PCR and CDGE (Fig. 3). All of the msRE-digested samples tested, exclusively showed a single band and the *Mcr*BC-digestion revealed the other band. Figure 3 shows a CDGE analysis from two typical families. In case 1, the paternal allele (H19FR2) of two children (C1 and C2) was detected from msRE-digested DNA, while maternal alleles of C1 (H19FR3) and C2 (H19FR1) were amplified from *Mcr*BC-digested DNA. In case 2 (Table 2, #7), it is genetically impossible to know the parent-of-origin of the child where the parents and their child have the same genotype, however, this method could clearly solve the problem. No inconsistency between parent-derived alleles of children as expected from Mendelian inheritance and those presumed from this method was confirmed in 17 family studies (Table 2). To estimate the methylation status in the paternal allele, a bisulfite treatment and methylation-specific PCR [15] was carried out on several samples. By sequencing of the bisulfite-treated DNA, no conversion of cytosine to uracil in CpGs was observed, indicating that they were fully methylated (data not shown). In our investigation, DNA samples (ages of healthy personnel ranged from several months to over 70 years old) stored at 4°C for many years showed a reproducible result for parental detection in the H19FR system, which suggests that the methylation status in genomic DNA extracted from blood samples might be stable. However, further investigations on DNAs from forensic samples, such as blood aged under various conditions, bloodstains and other biological sources, will be required to use this method for practical investigations. In general, disruption of imprinting would lead to developmental dysregulation resulting in malformations and malignancies [16]. Although

Table 2 Allelic distribution of H19FR in 17 family studies

Family no.	Father	<i>Hha</i> I	Child	<i>Mcr</i> BC	Mother
1	1-1	1	2-1	2	2-2
2	1-1	1	2-1	2	2-2
3	1-1	1	2-1	2	2-2
4	1-1	1	2-1	2	2-1
5	2-1	2	2-1	1	1-1
6	2-1	2	2-1	1	1-1
7*	2-1	1	2-1	2	2-1
8	2-1	1	2-1	2	2-2
9	2-2	2	2-1	1	1-1
10	2-2	2	2-1	1	1-1
11	2-2	2	2-1	1	2-1
12	3-2	2	2-1	1	1-1
13	1-1	1	3-1	3	3-1
14	3-1	3	3-1	1	1-1
15	2-1	2	3-2	3	3-1
16	3-2	3	3-2	2	2-2
17*	2-2	2	2-1	1	3-1
			3-2	3	

Pre-digestion of child DNA by *Hha*I or *Mcr*BC was examined for selective parental discrimination.

*CDGE results of families 7 and 17 are shown in case 2 and case 1 in Fig. 3, respectively.

the reversal of methylation status between parental alleles has never observed, the possibility of an imprinting failure caused by epimutation onto methylated or unmethylated CpGs owing to an epigenetic modification cannot be excluded, which might lead to confusion of the methylation status and also of the parental discrimination.

Recently, a VNTR [17] located 7.6 kb upstream of the *H19* gene has been demonstrated to be detectable in the paternal allele by msRE digestion and nested PCR within an approximately 1.7 kb region [10]. We tried to detect the VNTR by our simple method using a new primer pair which can just amplify the VNTR, however, msRE-treated DNA showed the original biparental alleles, while the *Mcr*BC-digested one was difficult to amplify (data not shown). These results suggest that the maternal CpGs around/in the VNTR might be somewhat methylated, although the paternal ones in the restricted region within 1.7 kb surrounding the VNTR (possibly nearer to the *H19* gene) would be hypermethylated. On the other hand, the H19FR region 2.5 kb upstream of the *H19* gene was fully methylated in the paternal allele. Therefore, the 5' boundary of methylation relating to the mechanism of imprinting could exist between 2.5 and 7.6 kb upstream of the *H19* gene, which is consistent with the previously defined range of -4.4 and -5.6 kb [7] and many more SNPs applicable for parental discrimination are expected to be found in the region up to the boundary.

Many genetic markers on Y chromosome or mitochondrial DNA have greatly contributed to forensic examinations and paternity testing. However, a feature of the transmission form of these markers is limited to the same genetic information (haplogroup) from father to son or mother to child, respectively. On the other hand, selective

detection of the parental allele from autosomal polymorphisms can provide biparental characters and might bring more useful information in cases where, for example a paternity case with the same heterozygous genotype in a parents-child trio (Fig. 3, case 2), a paternity case without parent(s) or relatives, and a rape case involving multiple putative fathers having the same paternal information, such as brothers and their father. Detection of parental H19FR alleles is easy and accurate, so that it could be used as a polymorphic marker having a possibility of parental discrimination. At present, a low percentage of human genes have been found to be imprinted genes [18], and it is assumed that some imprinted genes might have differentially methylated regions between parental alleles, such as H19FR. If many SNPs in such regions including H19FR could be combined, this enzymatic technique should be a more powerful tool for the discrimination of parental allele.

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