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Novel Paternity Testing by Distinguishing Parental Alleles at a VNTR Locus in the Differentially Methylated Region Upstream of the Human H19 Gene

ABSTRACT: Conventional PCR-based genotyping is useful for forensic testing but cannot be used to determine parental origins of alleles in DNA specimens. Here we describe a novel method of combined conventional genotyping and PIA typing (parentally imprinted allele typing) at a minisatellite region upstream from the H19 locus. The PIA typing uses two sets of primers and DNA digested with methylation-sensitive Hha I enzyme. The first amplification produces only the methylated fragment of paternal H19 allele, and the second detects polymorphism in the minisatellite. Hence, this distinguishes paternal and maternal alleles by difference in the DNA methylation. Furthermore, the polymorphism in this polymorphic locus was examined using 199 unrelated Japanese and 171 unrelated Germans, their polymorphism information content being 0.671 and 0.705, respectively. Feasibility of this typing is demonstrated for six families, and the usefulness is shown by application to paternity testing.

KEYWORDS: forensic science, paternity testing, human H19 gene, minisatellite, parentally imprinted allele typing, DNA methylation

There are numerous repeats such as VNTRs and STRs throughout the human genome (1). Some of them are polymorphic and have been widely employed as useful markers for the identification of disease genes and in population genetics and forensic examinations. PCR-based genotyping at these loci is routinely performed in most forensic laboratories and proves to be useful for forensic testing. However, further improvement is still possible in paternity testing. The ability to determine the parental origin of alleles would make this testing powerful. Especially in paternity cases where the mother or other relatives of the disputed child are unavailable, information that allows the determination of the allele of parental origin increases the probability of solving cases.

In mammals, some genes undergo genomic or parental imprinting and are preferentially expressed from either the maternal or paternal allele (2). Differential methylation in the CpG dinucleotides is found in the upstream region of imprinted genes and plays a key role in the regulation of gene expression and also in epigenetic inheritance of the imprinted genes (3–9). Since the methylated CpG dinucleotides are refractory to methylation-sensitive restriction enzymes, allelic difference in the methylation may be useful for the determination of the parental origin of alleles. Thirty-nine human imprinted genes have been identified in the current database (2),

among which the H19 imprinted gene (10,11) has been chosen as the locus for this study. Here we describe a novel method of combining conventional PCR-based genotyping and PIA typing (parentally imprinted allele typing) at a minisatellite or VNTR upstream from the H19 locus that distinguishes paternal and maternal alleles by the methylation difference. Also, we demonstrate applicability of this method to paternity analysis.

Materials and Methods

Samples

Genomic DNA was isolated by proteinase K digestion and phenol/chloroform extraction from blood samples. Blood samples were obtained from six Japanese families and a Japanese paternity case in PIA typing and from 199 unrelated Japanese and 171 unrelated Germans in population studies.

PIA Typing of a VNTR in the 5′ Flanking Region of H19

Designing Primer Pairs (Fig. 1)—Two primer pairs were designed based on the GenBank sequence data [accession number, AF043430 (12)]. One pair consisted of primers A (forward) 5′-CCATTACTTATATCTGGGTAGGTC-3′ and B (reverse) 5′-GTCATCTAGATAGACACATGAGC-3′, and the other consisted of primers C (forward) 5′-GGGTCATTATAGACGCAATCG-3′ and D (reverse) 5′-AGAACCTGTTGGGCGGTTAGA-3′. The A and B primers amplify the VNTR, and the C and D primers amplify the 1.7-kb region containing the VNTR and the four Hha I sites.

Digestion of Genomic DNA with the Enzyme Hha I, Which Cannot Cleave the Sequence GCGC if the Internal Cytosine is Methylated—Approximately 500 ng of genomic DNA was digested for 4 h at 37°C with Hha I (Takara) followed by heating for 3 min at 90°C.

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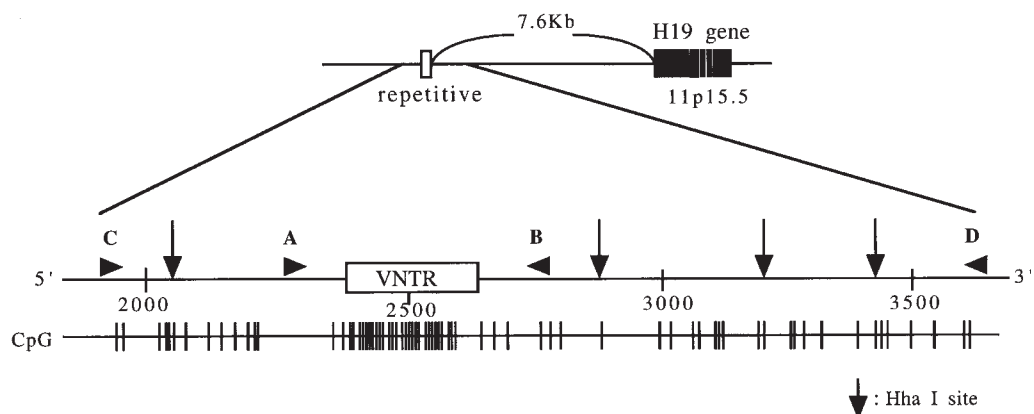
FIG. 1—*Sequence range in the 5′ upstream region of human H19 used in this study (AF043430, Bases 1910–3625). Hha I sites are indicated by arrows, and two sets of PCR primers (A and B, C and D) are shown by arrowheads. The second line shows the location of CpG dinucleotides on the basis of the published sequence. The target DNA contains 72 CpG (vertical lines) including 4 Hha I sites. The strategy for detecting paternal alleles is as follows. First, Hha I–digested DNA is subjected to the PCR with primers C and D. Then, the PCR product is given from methylated paternal allele and not from un-methylated maternal allele. Next, the VNTR is amplified using primers A and B from the first PCR product.*

Amplification of the 1.7- kb Region Using the C and D Primers and Purification of the Resulting PCR Fragment—The digested DNAs were diluted with sterilized water to 2.00, 0.40, 0.08, and 0.016 ng/μL. One microlitre of each dilution was amplified in a 10-μL reaction using Expand Long Template PCR System™ (Roche, Basel, Switzerland) and 10 pmol of each primer (C and D) according to the recommendation by the manufacturer. The cycling profile was 95°C for 2 min, twelve cycles of 98°C for 20 s, 63°C for 30 s, 68°C for 90 s, and 27 cycles of 98°C for 20 s, 63°C for 30 s, 68°C for 90 s, extending the elongation of 20 s for each cycle and then extension of 68°C for 7 min. The amplified products were separated by polyacrylamide gel electrophoresis (8% T, 2% C). The DNA band from the most diluted sample providing a PCR product was cut out from the gel and crushed into small pieces. The gel pieces were soaked in about 30 μL of High Salt Binding Solution in MERmaid Kit (Qbiogene, Carlsbad, CA) for 20 min at 60°C, centrifuged, the supernatant removed, avoiding gel pieces. After a repeat soak, the DNA of the pooled supernatants was recovered with EASYTRAP™ kit (Takara).

Amplification of the VNTR Region Contained within the Isolated Fragment Using the A and B Primers—One fifth of the recovered DNA was subjected to the PCR assay of the VNTR as a template as described below.

A VNTR Polymorphism in the 5′ Flanking Region of H19 and Population Studies

PCR and Electrophoresis—The repetitive region was amplified by modifying the PCR conditions for pMCT118 described by Kasai et al. (13). The reaction was carried out in 15 μL of PCR buffer containing 5-ng template DNA, 0.67 μM of each primer, 67-mM Tris-HCl (pH 8.3), 3-mM MgCl$_2$, 16.7-mM (NH$_4$)$_2$SO$_4$, 1-mM 2-mercaptoethanol, 0.017% bovin serum albumin, 10% dimethyl sulfoxide, 4 mM of each deoxy nucleotide triphosphates (dNTPs), and 0.75 U of Taq polymerase (Takara, Tokyo, Japan). The cycling conditions were 95°C for 7 min, 35 cycles of 95°C for 1 min, 52°C for 1 min, and 70°C for 6 min and an extension of 70°C for 10 min. Amplified products were separated by electrophoresis in 8% poly-acrylamide gels in a 0.5 × TBE buffer containing 5% glycerol and visualized by ethidium bromide staining or silver staining.

Allele Designation—The original determination of the repeat number of the VNTR leading to allele designation was performed by direct sequencing or sequencing after subcloning. Allele designation for the paternity cases and population samples was done using an allelic ladder on the gel.

Direct Sequencing—The PCR fragment of each allele was isolated from a 1 ~ 2% agarose gel with an EASYTRAP™ (Takara) kit containing glass powder after ethidium bromide staining and then sequenced directly with the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster, CA). The sequencing primers were A and B.

Subcloning—The two large alleles that were detected only in German samples were subcloned because of difficulties encountered in direct sequencing. Purified PCR fragments ([=]1.7 kb) were ligated into pGEM-easy T vector (Promega, Madison, USA) and electroporated into Escherichia coli host cells (DH5α). White colonies were isolated and plasmids carrying an insert of aimed size were subjected to sequencing described above.

Results

Strategy

Figure 1 shows positions of a VNTR and CpG dinucleotides in the 5′ flanking region of the human H19 gene. In this region, the paternal allele is methylated and the maternal allele is unmethylated (14). The map shows four Hha I sites in the vicinity of the VNTR and also the locations of two sets of PCR primers (A and B, C and D). Hha I–digested DNA being used as a template, PCR with the C and D primers can amplify about 1.7-kb sequence of the methylated paternal allele but not the unmethylated maternal allele, since digestion of the unmethylated maternal allele with Hha I destroys the intact 1.7-kb template. The second PCR with the A and B primers detects allele difference due to the VNTR polymorphism.

Parental Allele Determination by Combined Use of Genotyping and PIA Typing

Figure 2a shows genotyping with the A and B primers at the VNTR locus using total genomic DNA of a family with three generations. Two alleles detected are displayed by the number of

repeat units at the bottom of each lane. The child (C) had alleles 11/12 and inherited Allele 11 from her mother (M) and Allele 12 from her father (F) by comparison to the genotypes of her parents. In the same manner, the mother inherited Allele 9 from her mother (GM) and Allele 11 from her father (GF). Figure 2*b* shows PIA typing of the family. As explained above, DNA was digested with Hha I and then subjected to the first PCR with the C and D primer pair. The product was diluted, purified, and subjected to amplification with the A and B primers. As expected, one of the two alleles was amplified in each of the five individuals. The allele detected by PIA typing of the child's DNA was Allele 12. This was consistent with the determination for this trio based on the VNTR types that the child's maternal allele was an 11 and the child's paternal allele was the 12. A similar result was observed in the trio that consisted of the grandfather, grandmother, and mother. In this trio, the maternal allele was the 9, and the paternal allele was the 11. The PIA results

were consistent with the VNTR results, demonstrating that the paternal allele for the child was an 11. Figure 3 shows similar results of five trios. For each paternity trio, the paternal allele, demonstrated by VNTR typing, was consistent with determination of the paternal allele using the PIA procedure.

Figure 4 shows an application of the PIA typing to paternity testing. DNA samples from mother, child, and two alleged fathers (F1 and F2) were typed with PIA and VNTR analysis. The VNTR typing included both alleged fathers, since both alleged fathers had an Allele 9. Additionally, the child and mother shared both of their alleles, making it impossible to determine the child's paternal allele by analysis of the VNTR data. Hence, PIA typing was done for the child, which showed that Allele 10 from the child was inherited from the father. This excluded the F1 candidate from being the child's father and suggested that the F2 candidate was the biological father.
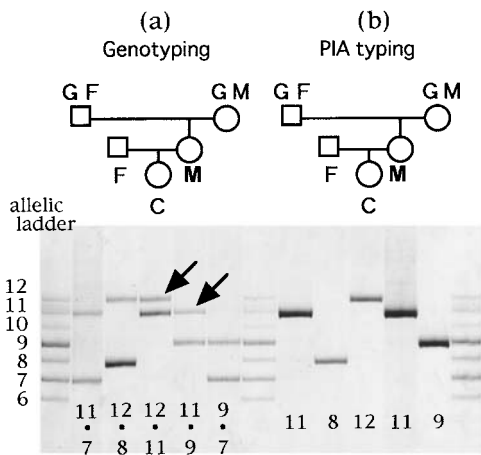

FIG. 2—*DNA typing of genotypes and parentally imprinted alleles for a family with three generations. The allele type detected for each sample is shown at the bottom of the sample lane. By the genotype analysis (a), child (C) and mother (M) inherited Allele 12 and Allele 11 from each father, respectively (indicated by arrows). By the PIA typing (b), Allele 12 was obtained from C and Allele 11 from M. This indicated that their paternal alleles were detected by the PIA typing method. C = child, F = father, M = mother, GF = grandfather, GM = grandmother.*
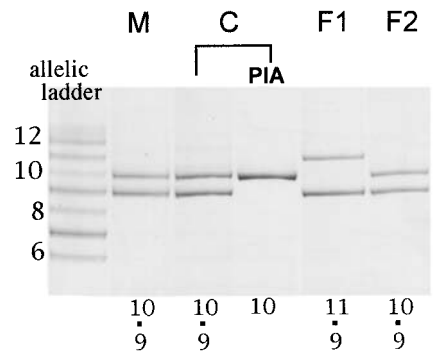

FIG. 4—*An application of the PIA typing to a paternity case. The silver-stained gel shows VNTR genotypes of the four individuals and paternal allele of the child (indicated by PIA). The PCR product of Allele 10 was obtained from child DNA by the PIA typing. The genotype of alleged father (F1) consisted of Alleles 9/11 and that of father living together (F2) Alleles 9/10. Therefore, F1 was excluded from being the biological father of the child. M = mother, C = child, F1 = alleged father, F2 = father living together, PIA = parentally imprinted allele.*
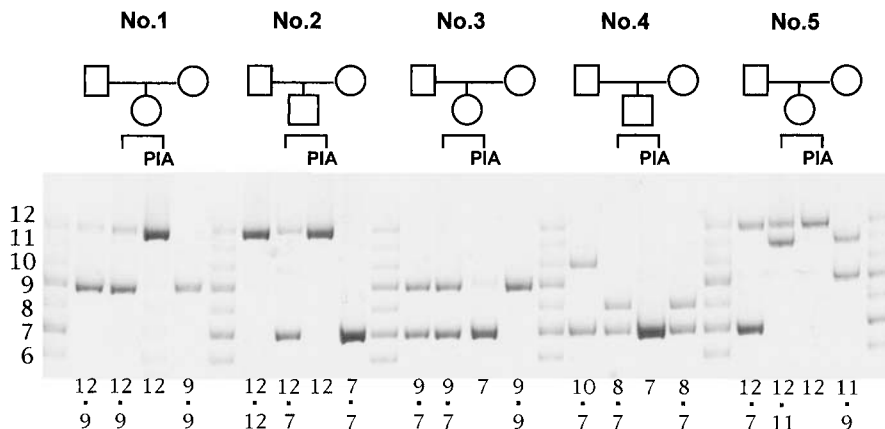

FIG. 3—*PIA typing of children in five families. In all trios, it was demonstrated that paternal allele type of the child (indicated by PIA on the top of the lane) was the same as that provided from genotyping of the family.*

TABLE 1—*Frequency distribution* (a) *and forensic efficiency values* (b) *of a VTNR polymorphism in the H19 5'flank. These were determined from 199 unrelated Japanese and 171 unrelated German samples*

(a) Frequency distribution

allele	Japanese n=199		German n=171	
6	23	(0.058)	20	(0.059)
7	88	(0.221)	149	(0.436)
8	6	(0.015)	7	(0.020)
9	188	(0.472)	63	(0.184)
10	36	(0.091)	8	(0.023)
11	33	(0.083)	57	(0.167)
12	24	(0.060)	29	(0.085)
13			7	(0.020)
14			2	(0.006)
Total	398	(1.000)	342	(1.000)

(b) Forensic efficiency values

	Japanese	German
Alleles identified	7	9
Heterozygosity	0.749	0.969
PIC	0.671	0.705
Genotypes identified	22	27
Discrimination power	0.870	0.917
χ^2	24.646 (p>0.100)	18.575 (p>0.100)

The VNTR Polymorphism in the 5' Flanking Region of H19

Table 1 summarizes the allele frequencies (Table 1a) and the forensic efficiency values of the VNTR located in the 5' flanking region of H19 (Table 1b). In the Japanese samples, seven alleles were identified, of which Allele 9 was the most frequent (0.472). The heterozygosity and polymorphism information content (PIC) were 0.749 and 0.671, respectively. On the other hand, nine different alleles were identified in German samples, and the frequency of Allele 7 (0.436) was highest. The heterozygosity and PIC were 0.969 and 0.705, respectively. Alleles 13 and 14 were found in the German samples but not in the Japanese individuals so far examined. Thus, the frequency distribution of this locus significantly differed in the two populations ($p < 0.01$).

Structural Variation in the VNTR Locus

A total of seven alleles were detected in the Japanese population samples, and nine alleles were detected in the German samples. Their fragments were sequenced and compared. Variation in the number of repeat units of the Japanese is shown in Fig. 5. The minisatellites consisted of five different kinds of repeat units (29 or 30 base pairs long; Fig. 5 II-VI). The polymorphism at this locus results from variation in the numbers of Repeat Unit III. Two SNPs of A or G and C or G were detected in the Unit III (indicated by arrows). In the former dimorphism, the nucleotide of Allele 10 was G and the other six alleles were A, while in the latter the nucleotide of Alleles 6, 7, and 8 was G and that of Alleles 9, 10, 11, and 12 was C. With the German samples, the repeat sequences of the Alleles 6 to 12 were identical with those of the Japanese ones. Alleles 13 and 14, which were not detected in the Japanese samples, had one and two more of the Repeat Unit III, respectively (data not shown).
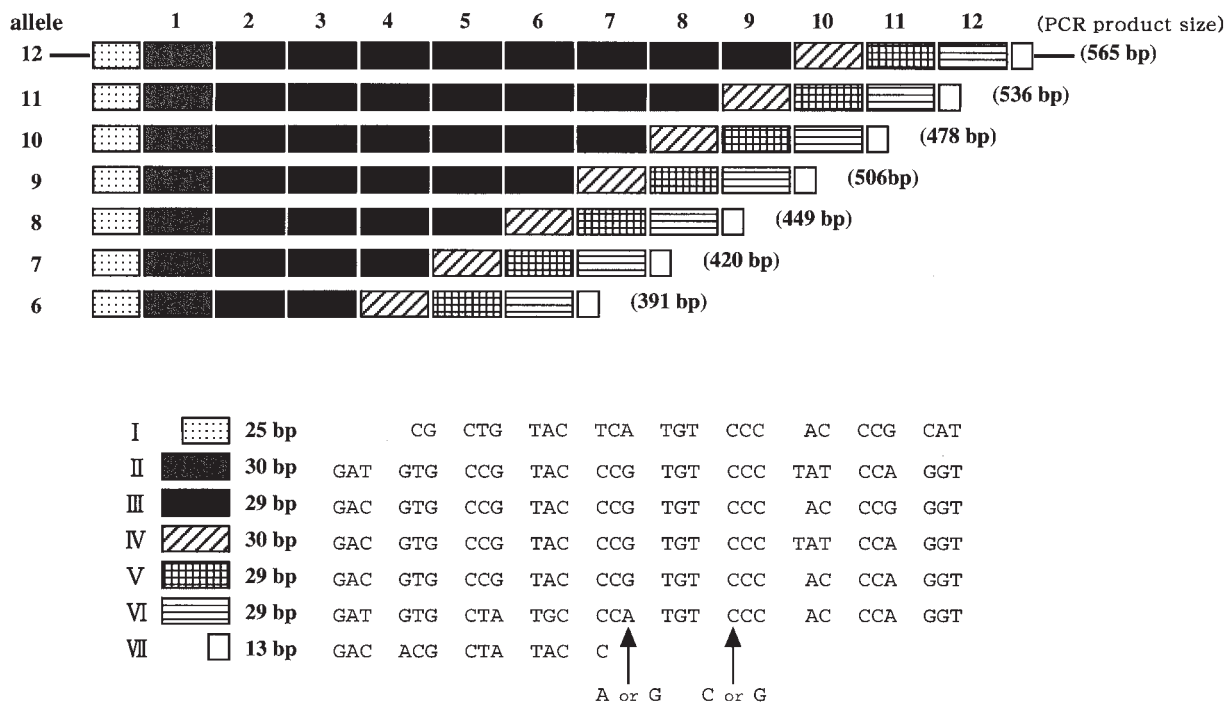

FIG. 5—*Structure of the repeat region analyzed in the present study. The VNTR was constructed with five repeat units (II-VI), and the number of Unit c was variable among alleles. The two arrows at the bottom of the figure indicate single nucleotide polymorphisms (SNPs). For the SNPs in Unit VI, three haplotypes (A-C, A-G, and G-C) were detected. The Haplotype A-C was for Alleles 9, 11, 12, the Haplotype A-G for Alleles 6, 7, 8 and the Haplotype G-C for Allele 10.*

Discussion

In the present study, we have established a novel method for paternity testing consisting of genotyping and PIA typing of a VNTR locus 5′ to the H19 imprinted gene. This procedure can be used to determine the parental origin of two alleles using differential DNA methylation. The PIA typing is a PCR-based DNA typing with two sets of primers and uses DNA digested with methylation-sensitive Hha I enzyme as a template. The first amplification produces only the paternal methylated fragment of about 1.7-kb long, and the second generates smaller fragments having a VNTR in the center of 1.7-kb fragment. Hence, the PIA typing detects only the paternal allele of the two alleles detected by genotyping. Incomplete digestion with Hha I may give a band of the maternally-derived allele, but 1000-fold dilution of the first PCR products can overcome this problem to reduce the amount of this maternal band.

Since methylation sites of the human H19 gene have been well studied, there are additional CpG sites other than the region used for the PIA typing. Jinno et al. (14) showed a CpG region −2.0-kb upstream that undergoes methylation on the paternal allele in somatic cells. The differentially methylated region (DMR) extends to approximately −5.5 kb, as was demonstrated by Frevel et al. (15) and Hamatani et al. (16). However, these regions were not suitable for PIA typing because there are no informative VNTRs in the vicinity.

We have examined the VNTR polymorphism in the 5′ flanking region of H19, which was identified by Standnick et al. (12) as human repetitive element type 3 and found that the repeat locus was highly polymorphic among both Japanese and German populations. Our study demonstrated that seven different alleles were found in the 199 Japanese and nine in the 171 German individuals (see Table 1). The allele distribution differed between Japanese and German samples. Among seven different repeat unit types detected in the VNTR, only Unit III was variable in number.

The most suitable application for the PIA typing is paternity testing. While identification of the child's paternal allele can usually be made in a paternity trio consisting of a mother, child, and alleged father, this is not possible when only the father is available for testing or when results depend on testing other second degree relatives such as grandparents or siblings of the alleged father. Alternatively, the PIA testing is also able to distinguish between two alleged fathers when the mother and the alleged father(s) share alleles such that both alleged fathers would be included. The method could also assist in resolving incest cases when multiple male relatives are suspected.

The result of a paternity test performed effectively by the method is shown in Fig. 4. In this case, the probability of paternity exclusion based on the PIA results was 82.6%, while the exclusion probability based on the genotyping results was 19.1%. This suggests that incorporation of the PIA testing into the conventional paternity test with STRs will give high resolution in paternity dispute cases.

For forensic application, we applied this method to postmortem tissue DNAs from kidney, liver, spleen, muscle, skin, cerebrum, cerebellum, uterus, ovary/testis, and thymus. The DNA samples were obtained from three autopsy cases in which death occurred within 2 to 11 h. All provided only the paternal allele (data not shown). The result suggests that the PIA typing provides useful information in forensic investigations.

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