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Short communication

Identification of new primer binding site mutations at TH01 and D13S317 loci and determination of their corresponding STR alleles by allele-specific PCR

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ABSTRACT

Several commercial multiplex PCR kits for the amplification of short tandem repeat (STR) loci have been extensively applied in forensic genetics. Consequently, large numbers of samples have been genotyped, and the number of discordant genotypes observed has also increased. We observed allele dropout with two novel alleles at the STR loci TH01 and D13S317 during paternity testing using the AmpFℓSTR® Identifiler® PCR Amplification Kit. The lost alleles reappeared when alternative PCR primer pairs were used. A sequence analysis revealed a G-to-A substitution 82 bases downstream of the last TCAT motif of the repeat region at the TH01 locus (GenBank accession: D00269) and a G-to-T substitution 90 bases upstream of the first TATC motif of the repeat region at the D13S317 locus (GenBank accession: G09017). The frequencies of these two point mutations were subsequently investigated in the Chinese population using sequence-specific primer PCR (SSP-PCR), but neither of these mutations was detected in any of the samples tested. In addition, the DNA samples in which the mutations were identified were amplified to type the point mutations by SSP-PCR to determine the corresponding STR alleles at the two loci. Subsequently, the amplified PCR products with different point mutations and STR repeat numbers were directly sequenced because this strategy overcomes the appearance overlapping peaks generated by different STR alleles and accurately characterizes genotypes. Thus, our findings not only provide useful information for DNA databases and forensic identification but also establish an effective strategy for typing STR alleles with primer binding site mutations.

1. Introduction

Short tandem repeat (STR) markers are the primary means used today for personal identification, paternity testing, and DNA database construction. Multiple loci may be simultaneously amplified and genotyped by commercially available kits from Applied Biosystems by Life Technologies (Carlsbad, CA), Promega Corporation (Madison, WI), or Biotype (Dresden, Germany) [1–3]. Importantly, because of higher heterozygosity and polymorphic information content, STRs are the most practically applicable genetic markers in forensic casework and have become the foundation for the development of DNA databases and investigative leads. However, with the wide application of STR, isolated genetic inconsistencies and discordant genotyping results generated from different commercial kits can occur; these inconsistencies are usually based on de novo mutations in one of the parental germlines [4,5].

Insertions or deletions of tandem repeat motifs in the nascent strand primarily occurs due to a polymerase slippage on STR-loci [6]. As a consequence, mismatches are often observed between parent and offspring in paternity cases. In addition, rare typing discordance attributable to mutations in primer-binding regions has been reported for STR systems included in the commonly used kits [7–9]. Primer binding site mutations of STR loci are of forensic relevance because they can be interpreted as potential allele dropouts. Allele dropout causes a heterozygote to be falsely recorded as homozygous, causing the result to be interpreted as non-concordant when searching data banks and incorrectly interpreted as an exclusion when allele dropout occurs in a paternity test [10]. Thus, finding mutations, accumulating data, and correctly interpreting discordant phenomena are quite important in the field of forensic genetics.

In the present study, we described two paternity cases analyzed with the AmpFℓSTR® Identifiler® PCR Amplification Kit, which caused allelic dropout at the TH01 and D13S317 STR loci. The

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reasons for these dropouts were evaluated by sequence analysis after redesigning the PCR primers. Furthermore, we also investigated the novel mutations' frequency in the Chinese population and determined their corresponding STR alleles by SSP-PCR and subsequent DNA sequencing.

2. Materials and methods

2.1. Samples and DNA extraction

The samples were collected from children and their parents in two paternity cases from our laboratory. In addition, 200 samples were obtained from unrelated randomly selected individuals collected from the Chinese population to investigate the novel mutations' frequency. All of the samples were collected with the participants' informed consent. The DNA was extracted from peripheral blood using the phenol-chloroform extraction method.

2.2. STR genotyping

STR-typing was carried out on the samples in two family trios for parentage testing. The 15 autosomal STR loci were simultaneously amplified by the multiplex PCR method using an AmpFℓSTR® Identifiler® PCR Amplification Kit (Applied Biosystems by Life Technologies, USA). Quality control was ensured by using internal laboratory control standards and kit controls.

2.3. PCR amplification and DNA sequencing

To identify potential primer binding site mutations, new primers outside the range of the kit primer sets at the TH01 and D13S317 loci were designed based on their NCBI sequences. The primer sequences for the amplification of a 342 bp TH01 locus product are 5′-TTC CCA GGC TCT AGC AGC AG-3′ (forward, F1) and 5′-CAC CAC ATT TCA ATC AAG GTC CAT A-3′ (reverse, R1). The primer sequences for amplification of a 371 bp D13S317 locus product are 5′-CAA ATG GTA ATT CTG CCT ACA GCC-3′ (forward, F2) and 5′-AAC AGT CTG AAA GTA CAA GTG GGG A-3′ (reverse, R2). The thermal cycling conditions were 94 °C for 1 min; 35 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 40 s; and 72 °C for 5 min. The PCR products were subsequently sequenced using the forward and reverse primers to determine the flanking sequences of repeat core units.

2.4. Nomenclature

Polymorphisms identified in this paper were designated by the locus name (TH01 or D13S317) and the number of base pairs upstream (−) of the first base of the STR or downstream (+) from the final base of the STR of interest. Orientation was based on the top (forward) strand sequences of GenBank # D00269 (TH01) and # G09017 (D13S317).

2.5. SSP-PCR and determination of corresponding STR allele

SSP-PCR was used to further confirm the mutations identified in this study and to investigate the mutants' frequency [11]. The specific primer sequences for amplification of wild type and mutant sequences at the TH01 locus are 5′-CTG TGG GCT GAA AAG CTC CC-3′ and 5′-CTG TGG GCT GAA AAG CTC CT-3′, and those at the D13S317 locus are 5′-TGG ACA TGG TAT CAC AGA AGT CTG G-3′ and 5′-TGG ACA TGG TAT CAC AGA AGT CTG T-3′. The common primers are F1 for TH01 and R2 for D13S317. The thermal cycling conditions were 94 °C for 1 min; 35 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 30 s; and 72 °C for 5 min. The PCR products were visualized on a 2% agarose gel. Using this SSP-PCR protocol, the mutants' frequency was investigated in a total of 200 unrelated Chinese individuals.

In addition, the SSP-PCR products from two STR loci were directly sequenced to determine the corresponding STR alleles.

3. Results and discussion

The parentage cases included in this study were from 2 father-mother-child triplets, in which parenthood was determined using 15 autosomal STR markers in an AmpFℓSTR® Identifiler® PCR Amplification Kit. The results showed that a genetic inconsistencies were observed at the TH01 and D13S317 loci in two separate families, while the remaining STR markers were consistent with a parentage relationship, with the probability of parenthood considering all consistent loci being >99.9999%. To evaluate the genetic inconsistencies, we further characterized the two STR loci.

3.1. Discrepancy at locus TH01 and point mutation screen

STR genotyping at locus TH01 displayed genotypes of (8, –), (9.3, –) and (9, 9.3) as putative father, child and mother, respectively (Fig. 1A). An opposite 'homozygosity' was observed between the putative father and child, which suggested the presence of a primer binding mutation causing an allele dropout. The occurrence of allele dropout was first confirmed by re-testing the locus using alternative primers as described above. DNA was amplified with alternative primers localized largely outside the common amplicon, and DNA sequencing was subsequently carried out with bidirectional primers. As shown in Fig. 1B, a G-to-A substitution 82 bases downstream of the last TCAT motif of the repeat region on TH01 locus (GenBank accession: D00269), designated as +82G/A, was detected in both the putative father and the child. This mutation is thought to overlap the primer binding site used in the commercial STR genotyping kit. To further validate the point mutation, we performed SSP-PCR and obtained a conclusive result (Fig. 1C–F).

Multiple concordance studies on the effect of allele dropout when using various STR multiplex kits have been performed, and allele dropout was predominantly observed with kits from Applied Biosystems, Promega as reported on STRBase (http://www.cstl.nist.gov/biotech/strbase/NullAlleles.htm) [12], as well as Biotype [13], but discordant results have been rarely observed with kits from QiaGen [14]. Although primer sequences from some commercial kits are available, information regarding the primer sequences from AmpFℓSTR® Identifiler® PCR Amplification Kit has not yet been made available. Thus, the point mutation reported here is hypothesized to be responsible for primer mismatch and for the discrepancy based on PCR products size and nonamplification. In all published data about genotyping inconsistencies at the TH01 locus, a single point mutation (G-to-A transition) located 37 bases upstream of the first TCAT motif of the repeat region was identified as the cause of allele dropout when using the AmpFℓSTR® Profiler Kit [15]. These results suggested that mutations at the TH01 locus may be rare relative to other STR loci [13,16].

3.2. Discrepancy at locus D13S317 and point mutation screen

In another case, we observed an inconsistency between mother and child with genotypes of (11, 13), (13, –) and (12, –) at the D13S317 locus for the putative father, child and mother, respectively (Fig. 2A), indicating that the child did not possess a maternal allele. Likewise, the mother/child study was performed with alternative primers. DNA from the mother and child was amplified and the PCR products were sequenced. As shown in Fig. 2B, sequencing analysis revealed a G-to-T substitution 90 bases upstream of the first TATC motif of the repeat region on D13S317