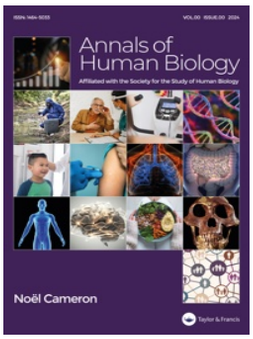

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Identification of a novel SNP mutation causing drop-out alleles in a paternity test using combined nest and touch-down PCR with Sanger sequencing

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ABSTRACT

Background: Short tandem repeat (STR) markers are widely used in forensic DNA analysis due to their ability to provide automated and standardised typing. However, incorrect STR typing can have a significant impact on forensic outcomes.

Aim: In this study, we detected drop-out alleles at the SE33 locus in a putative father-son pair using the Microreader™ 28 A ID System. This result could lead to a false conclusion of non-paternity.

Subjects and methods: To investigate the cause of the drop-out alleles, we developed a nest and touch-down PCR program for Sanger sequencing of the SE33 locus. Subsequently, we investigated the mutation frequency in 300 unrelated individuals and reviewed the results of 429 paternity tests.

Results: The results showed that the frequency of the G>T mutation at this locus was less than 0.01, which is a novel and rare mutation. Our analysis revealed a novel G>T mutation in the primer-binding region of both samples, which was a rare single-nucleotide mutation site in the Chinese population. This variation was found to be responsible for the drop-out alleles observed in the samples.

Conclusion: Our findings have important implications for optimising primer design and constructing DNA databases for forensic analysis.

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KEYWORDS
Forensic genetics; SE33 locus; SNP mutation; Drop-out alleles

1. Introduction

Short tandem repeat (STR) markers are highly polymorphic and widely distributed in the human genome, making them a primary tool for personal identification and paternity testing. Commercial multiplex PCR amplification kits typically use primers designed to avoid SNPs or to use degenerate primers, which may reduce specificity (Li et al. 2015). However, unpredictable mutations in the primer-binding region can still occur, resulting in amplification failure and the appearance of pseudogenotypes, such as drop-out alleles or silent alleles (Abdelmanova et al. 2020; Cho et al. 2021; Grgicak et al. 2021). In forensic practice, this situation can lead to low likelihood ratios (LRS), which can affect the determination of personal identification and paternity test if the results are not handled properly. There have been case reports of null alleles found in common STR loci associated with variations in the primer-binding region (Budowle et al. 2008; Lane 2013).

The SE33 (ACTBP8) locus is a STR locus located on the long arm of human chromosome 6 and is a pseudogene related to the β-actin gene, with the heterozygous index ranging from 0.94 to 0.966 (Wenda et al. 2005). SE33 was applied to forensic individual identification and paternity tests in the early 1990s (Polymeropoulos et al. 1992). Recently, we found drop-out alleles at SE33 locus in an assumed father-son pair using Microreader™ 28 A ID System (Microreader Genetics, Beijing, China), which is commonly used in China (Yin et al. 2021).

Paternity cannot be excluded in this case because only one locus does not follow the genetic rules. In such cases, additional testing of the mother's sample can be performed, another widely-used kit can be used for re-analysis to verify the results by increasing the number of STR markers, or new primers can be designed to amplify loci and sequence the product (Heinrich et al. 2004). Forensic blood stain samples are often relatively old and the template content cannot fully meet the requirements of Sanger sequencing after a single PCR amplification. Therefore, the nest PCR program combined with touch-down procedure can ensure the specificity of amplification and produce enough amplification products for subsequent sequencing.

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In this study, we reported a paternity case with an allelic dropout at SE33 locus and developed a nest and touch-down PCR program for Sanger sequencing of the SE33 locus. Subsequently, we investigated the mutation frequency in Chinese unrelated individuals and reviewed the results of 429 paternity tests.

2. Material and methods

2.1. Case sample collection

For a paternity test, a putative father and his son provided fingertip blood stain samples for DNA analysis. The mother did not attend. Meanwhile, we collected venous blood samples from 300 unrelated Chinese individuals and reviewed the genotyping profiles of 429 paternity tests, in which parental relationships were confirmed, using Microreader™ 28 A ID System from the China Medical University Centre of Forensic Investigation from 2021 January to 2022 December. The ethics approval was approved by the ethics committee of China Medical University (approval number [2016] 063) and written informed consents were obtained.

2.2. DNA extraction, amplification, and capillary electrophoresis

Genomic DNA was extracted using Chelex-100 method (Walsh et al. 1991). Autosomal STR genotyping was performed using the Microreader™ 28 A ID System (Microreader Genetics) and GlobalFiler™ PCR Amplification Kit (Applied Biosystems) in a GeneAmp® PCR 9700 thermal cycler (Thermo-Fisher, USA) according to the manufacturer's instructions. PCR products were detected and separated using the Applied Biosystems™ 3500 Series Genetic Analyzer™ (Thermo-Fisher). Raw data were analysed using GeneMapper *ID-X* 1.4 software (Thermo-Fisher). Allelic nomenclatures were determined using an allelic ladder provided by the Microreader™ 28 A ID System and GlobalFiler™ PCR Amplification Kits.

2.3. Design and amplification of new primers for nest PCR and Sanger sequencing

In paternity testing, it is crucial to identify the molecular cause of the phenomenon occurring in commonly used STR loci. Due to the low DNA content in the blood stain samples and their susceptibility to degradation, a nested and touch-down PCR program was designed to ensure the high efficiency and specificity of the amplification reaction. The final amplification fragments included the SE33 amplification fragment from the kit and its upstream and downstream 300 ~ 500bp (Table 1), and the primer of SE33 locus was obtained with the support of reagent vendors (Table 2).

The primer-binding and sequencing regions are summarised in Figure 1. Two pairs of new primers were designed for nest PCR using Premier 5.0 software (Table 1), based on the NCBI reference sequence (accession no: NC_000006.12). The initial step for nest and touch-down PCR involved the following conditions: an initial denaturation at 94 °C for 5 min, followed by 20 cycles of denaturation at 94 °C for 30 sec, annealing at 56 °C (a subsequent drop of 0.5 °C per cycle) for 30 sec, and extension at 72 °C for 2 min 30 sec. This was followed by 10 cycles of denaturation at 94 °C for 30 sec, annealing at 46 °C for 30 sec, extension at 72 °C for 2 min and 30 sec, and a final extension at 72 °C for 7 min. The first step product was purified with Takara MiniBEST DNA Fragment Purification Kit Ver.4.0 (Takara, Dalian, China) and then 1 µl of the purified product was used as a template for the second step of nest and touch-down PCR. The amplification conditions for the second step were as follows: initial denaturation at 94 °C for 5 min, followed by 20 cycles of denaturation at 94 °C for 30 sec, annealing at 62 °C (a subsequent drop of 0.5 °C per cycle) for 30 sec, and extension at 72 °C for 1 min and 20 sec. This was followed by 10 cycles of denaturation at 94 °C for 30 sec, annealing at 46 °C for 30 sec, and extension at 72 °C for 1 min and 20 sec, and a final extension at 72 °C for 7 min. Sanger sequencing was performed on the purified product from the second step using the E.Z.N.A.® Gel Extraction Kit (Omega Bio-Tek, USA) (Cheong and Caramins 2014). The DNA samples extracted from 300 venous blood samples were amplified using S3 primers and genotyped by Sanger sequencing.

Table 1. Primers designed for nest and touch-down PCR and Sanger sequencing.

Primer	Sequence (5′ to 3′)	Position (GRCh38)	Product length (bp)
F-D1	ATCTTGATCTTCATCGTGC	88276024-88276042	2579
R-D1	ATTGATGAGTGTAAATCGGTA	88278582-88278602	
F-S3 (sequencing)	CAGTCACACGCACCTCGTT	88276719-88276737	1238
R-S3 (sequencing)	AAGGCATTTTAGTATTGGCTCTC	88277934-88277956	

Table 2. Primers for SE33 amplification in the Microreader™ 28 A ID System.

Primer	Sequence (5′ to 3′)	Position (GRCh38)	Product length (bp)
SE33p1-28A	CGCGGTGTAAGGAGGTTT	88277068-88277085	416
SE33f2p-28A	CGTGGTGTAAGGAGGTTT	88277068-88277085	
SE33n2-28A	TCTGTAATTCCAGCTCCTAGGG	88277462-88277483	

3. Results

3.1. STR genotyping of Microreader™ 28 A ID System

In the genotyping analysis of a putative father-son pair using the Microreader™ 28 A ID System (Microreader Genetics), we observed that all loci, except for SE33, exhibited a match (Figure 2). However, the assumed father displayed the "30.2" allele, whereas the son exhibited the "25.2" allele, the son and father did not show a match at SE33 locus.

3.2. STR genotyping of GlobalFiler™ PCR Amplification Kit

We employed the GlobalFiler™ PCR Amplification Kit (Life Technologies) to analyse the loci and compare the results with those obtained using the Microreader™ 28 A ID System. The loci were matched in the putative father-child pair, and

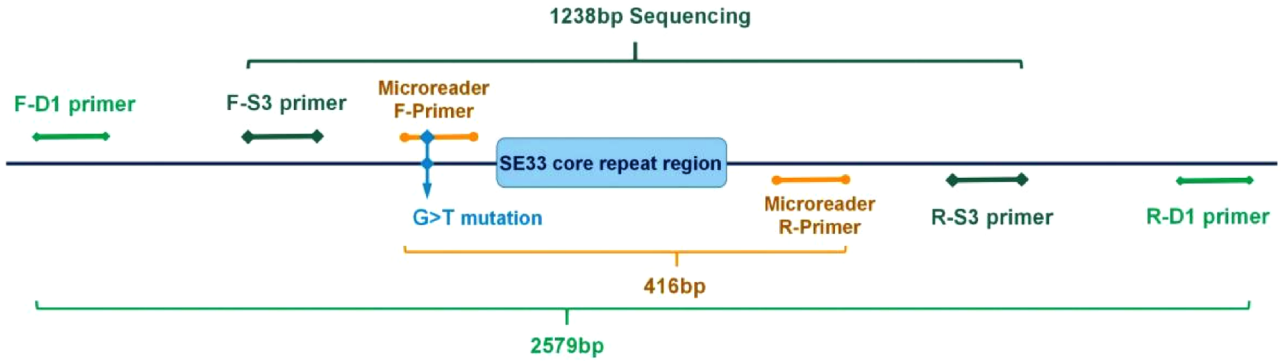

Figure 1. Scheme summarising all primer-binding and sequencing regions

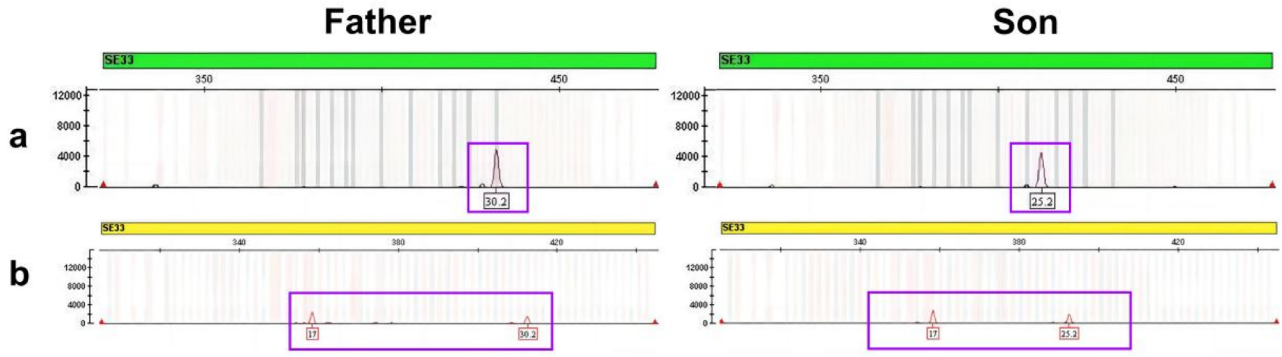

Figure 2. The STR genotyping results of SE33 locus of father and son.
a: the STR genotyping results of Microreader™ 28A ID System.
b: the STR genotyping results of GlobalFiler™ PCR Amplification Kit.

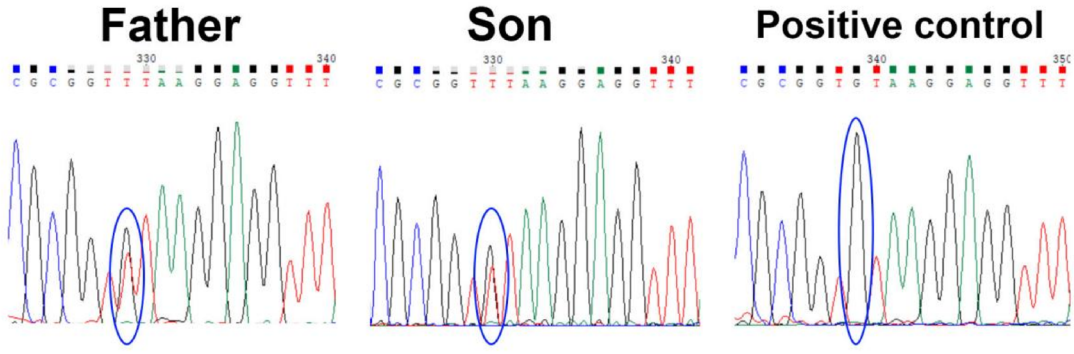

Figure 3. Sanger sequencing results of the newly found SNP mutation in both father and son, compared with the positive control.

the two kits produced identical results for the overlapping loci except for SE33. In particular, the GlobalFiler™ PCR Amplification Kit (Life Technologies) detected heterozygosity at the SE33 locus in which the assumed father was "17,30.2" and the son was "17,25.2" (Figure 2). Conversely, drop-out alleles were found at the SE33 locus within the Microreader™ 28 A ID System for both the father and son samples.

3.3. Validation of SNP mutation through Sanger sequencing

Sanger sequencing revealed bimodal peaks at the same location for both father and son, while the positive control had a single peak (Supplementary Figure.S1). Upon comparing the positive control, a G>T mutation was found which could perfectly explain the appearance of the drop-out alleles in this case (Figure 3).

3.4. Mutation frequency analysis

To investigate the mutation frequency, we performed Sanger sequencing on 300 unrelated individuals and reviewed the genotyping profiles of 429 paternity tests. The results showed that no G>T mutation was found in 300 Chinese individuals. Besides, there was no drop-out allele at SE33 locus in the 429 paternity cases. The results showed that the G>T mutation is novel and rare without the polymorphism in the population.

4. Discussion

In paternity case analysis, incorrect conclusions may arise if a heterozygote is mistaken for a pseudo-homozygote due to drop-out alleles, which can impact individual identification, familial searching, and data exchange (Takayama 2022). For instance, null alleles can lead to false negative results and erroneously rule out any relationship between two samples (Ma et al. 2012). Fortunately, with the advent of DNA databases, we can perform population comparisons in the database or retest with different STR kits to ensure further confirmation (Lohmueller et al. 2014).

The probability of one-step mutation caused by replication slippage is about 5.24×10^{-5} times per locus per generation (Steely et al. 2022), whereas the probability of SNP mutation in the primer-binding region is 10^{-10}-10^{-8} times per site per generation (Guo et al. 2023; Fu et al. 2014; Karmin et al. 2015; Trombetta et al. 2015). Therefore, when the difference in core sequence repeat number is less than 2, we prefer to infer STR mutation caused by replication slippage, otherwise SNP mutation in the primer-binding region should be considered. Additionally, in capillary electrophoresis (CE), the homozygous allele has a higher peak than the heterozygous allele. When drop-out alleles due to SNP mutations in the primer-binding region renders a heterozygote pseudo-homozygote, the peak height of the pseudo-homozygote is usually similar with those of heterozygotes at other loci, which can indicate the type and cause of drop-out alleles. Therefore, we speculated that the discrepancy in the SE33 locus was not due to a five-step mutation but a SNP mutation in the primer-binding region, which might result in the loss of allelic heterozygosity.

In cases where we encounter suspected drop-out alleles, triband type, or other issues in our casework, we often utilise an alternative kit for re-analysis and verify the initial results. In this study, we compared the STR genotyping results obtained with GlobalFiler™ PCR Amplification Kit and designed a nested and touch-down PCR program for Sanger sequencing of the SE33 locus in the Microreader™ 28A ID System to determine the underlying molecular cause. We searched the forward and reverse primer sequences of SE33 in the kit, as well as the known SNP sites contained within it, using the Ensembl Genome Browser (http://grch37.ensembl.org/index.html) and dsSNP database (https://www.ncbi.nlm.nih.gov/snp/). At the SNP loci, G>C mutation has been reported (rs1779427319, global frequency: $C=0.000004$), while the G>T mutation identified in this case has not been reported. We then investigated the mutation frequency in 300 unrelated individuals and reviewed the results of 429 paternity tests. The results showed that the frequency of the G>T mutation at this SNP loci was less than 0.01, which is a novel and rare mutation. Therefore, we have discovered a previously unreported mutation, which is near the STR marker applied for forensic individual identification and paternity tests.

In forensic practice, drop-out alleles can often be resolved by using different kits. However, elucidating the mechanism at the molecular level plays a unique role in improving the construction of DNA databases and optimising primer design (Heinrich et al. 2004). It is important to value every sample encountered in our daily work, as genetic mutations are random in nature and widespread in organisms. Therefore, samples that contain specific types of mutations are valuable for forensic genetics research.

5. Conclusion

Our analysis revealed a novel G>T mutation in the primer-binding region of both samples, which was a rare single-nucleotide mutation site in the Chinese population. This variation was found to be responsible for the drop-out alleles observed in the samples. Our findings have important implications for optimising primer design and constructing DNA databases for forensic analysis.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Ethics approval

The ethics approval was approved by the ethics committee of China Medical University (approval number [2016] 063) and written informed consents were obtained.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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