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CASE REPORT

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A Single Mutation in the FGA Locus Responsible for False Homozygosities and Discrepancies Between Commercial Kits in an Unusual Paternity Test Case

ABSTRACT: We report an unusual paternity test case showing multiple peculiarities. Using AmpFlSTR® Profiler Plus and AmpFlSTR® Identifiler PCR Amplification kits, the alleged father and the two children were apparently homozygous at the FGA locus, but using the PowerPlex® 16 kit the three individuals were found to be heterozygous. Drop-out was caused by a single mutation event in the presumptive binding site of the reverse primer. In addition, three inconsistencies were detected between the daughter and the alleged father among 18 STR markers. The occurrence of the rare null allele at the FGA locus and case history suggested that the true father was the brother of the alleged father. Furthermore, a single-step repeat maternal mutation was also detected at D16S539. This puzzling case was solved by using multiple analytical approaches, including the use of different primer pairs, the use of a high number of STR markers, and the characterization of the mutation causing the "null allele."

KEYWORDS: forensic science, paternity test, FGA mutation, null allele, maternal mutation

Paternity tests are ordinarily resolved by using STR informative markers, that are amplified and analyzed simultaneously in multiplex format, using commercial kits and semiautomatic systems. Rare typing discordance attributable to mutations in primer-binding regions has been reported for STR systems included in the commonly used kits (1–5). While the exact primer sequences for Powerplex® 16 kit are available (6), the primer sequences for AmpFlSTR® kits are not and the identification of these sequence variants in primer-binding sites between the two kits has only been performed occasionally. Nevertheless, it is important that these mutations and their frequencies be published (7). Hendrickson et al. (8) observed a strong disparity in amplitude peak height at the FGA locus in one heterozygous sample due to a mutation in the binding site of the forward PCR primer (2834G > A according to GenBank M64982). Budowle and Sprecher (9) found a case of discrepancy for FGA between different kits with a 0.2% frequency, but no further analyses were conducted to identify the underlying sequence change. Delamoye et al. (10) revealed two discrepancies between commercial kits caused by a 3116G > A mutation in the reverse primer (GenBank M64982) in one case while the reasons for discrepancy were not determined in the second case. Amorin et al. (11) reported one sample that was heterozygous with AmpFlSTR® Identifiler and homozygous with PowerPlex® 16, but the DNA sequence was not investigated.

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Here, we described a paternity test in which allelic drop-out occurred in the STR system FGA. In addition, a relative of the alleged father was the probable father. A final complication in the case was a single maternal mutation at the STR system D16S539. Moreover, we observed genotyping inconsistencies between AmpFlSTR® Profiler Plus, AmpFlSTR® Identifiler, and PowerPlex® 16 kits for the FGA locus. In this intriguing paternity test, the reasons for the discrepancies between kits detected in three individuals (alleged father, son, and daughter), and presumably also present in the untested true father, were analyzed by sequencing and restriction enzyme analysis.

Methods

DNA from saliva collected with buccal swabs was extracted using the Qiagen mini kit (Qiagen, Hilden, Germany), and the recovered DNA was quantitated with a spectrophotometer.

All samples were amplified with the commercially available kits AmpFlSTR® Profiler Plus PCR Amplification kit, AmpFlSTR® Identifiler PCR Amplification kit (Applied Biosystems, Foster City, CA) and PowerPlex® 16 System (Promega, Madison, WI), and analyzed with an ABI Prism 310 Genetic Analyzer (Applied Biosystems). FGA locus and the other CODIS' loci were also analyzed with four multiplex amplification systems in combination with an automated sequencer LICOR-4200 (LI-COR Biotechnology, Lincoln, NE), as previously reported (12). Moreover, the additional STR markers F13B, SE33, Penta D, and Penta E were analyzed as described (13). The FGA locus was also singleplex amplified with a previously described short primer pair and analyzed by capillary electrophoresis (14).

TABLE 1—*Primers and hybridization temperatures used to amplify the FGA locus.*

	Primer Sequences	Annealing Temperature (°C)	Size (Allele 24)
FGA (12)	F—5′-GCCCCATAGGTTTTGAACTC-3′ R—5′-TGATTTGTCTGTAATTGCCAGC-3′	60	204
FGA short (14)	F—5′-CCAAAATAAAATTAGGCTATAAATGTTCGA-3′ R—5′-GCTGAGTGATTTGTCTGTAATT-3′	60	174
FGA AmpFlSTR® (15)	Unknown	60	243
FGA PowerPlex®16 (6)	F—5′-GGCTGCAGGGCATAACATTA-3′ R—5′-TTTCTATGACTTTGCGCTTCAGGA-3′	60	354
FGA (this study)	F—5′-CTTTGGGATTACTAATTGC-3′ R—5′-TCTATGACTTTGCGCTTC-3′	50	402

For AmpFlSTR®, 243 bp is the predicted size for an allele 24 without the mutation observed in this case.

TABLE 2—*Cumulative results of the paternity test.*

System	Alleged Father	Mother	Son	Daughter
TPOX	9, 11	8	8, 11	8, 11
D3S1358	14, 17	14, 17	14	17
FGA	20, 24	22, 23	22, 24	23, 24
CSF1PO	10, 12	10, 12	10, 12	10, 12
D5S818	11, 13	11, 12	11, 12	11
D7S820	7, 8	10, 12	7, 12	7, 12
D8S1179	10, 15	11, 13	10, 11	10, 11
TH01	6	9, 9.3	6, 9	**9**
VWA	16, 17	14, 16	16	16, 17
D13S317	9, 11	12, 13	11, 12	11, 13
D16S539	8, 12	12	8, 12	8, *11*
D21S11	29, 31.2	29, 30	29, 30	29, 31.2
D18S51	13, 14	13, 16	13	16, **18**
D2S1538	19, 23	22, 25	19, 22	19, 25
D19S433	14, 15.2	16, 17	15.2, 17	14, 16
Penta D	8, 12	10, 13	10, 12	10, 12
Penta E	14, 15	10, 11	10, 14	11, 15
SE33	18, 19	18, 26.2	18, 19	18, **24.2**
F13B	8, 10	9, 10	9, 10	10
Paternity index	—	—	2.6×10^{10}	—

In bold: paternal alleles in the daughter that are absent in the alleged father's profile. Italicized: daughter allele resulting from a maternal mutation.

To investigate the presuntive mutation in the primer-binding region, PCR amplification was performed using a new primer pair external to the amplicons generated by the AmpFlSTR® kits (15). Table 1 shows the sequences for all the primers used. The amplicon generated by this new primer pair had a length of 402 bp for allele 24 compared with a length of 243 bp with the Profiler Plus and the Identifiler kits. After agarose gel analysis to detect PCR products, dNTPs were removed with exonuclease ExoSAP-IT® (USB Corporation, Cleveland, OH), following the manufacturer's directions. Sequence analysis was performed in both directions using the new long amplification primers and the Big Dye Terminator cycle sequencing kit (Applied Biosystems), in combination with an ABI Prism 310 Genetic Analyzer (Applied Biosystems). To confirm the nature of the point mutation, restriction enzyme digestion with TaqI (Promega) was performed at 37°C overnight.

Results and Discussion

Discrepancy at Locus FGA

Table 2 shows the results of paternity tests. Initially, apparent homozygosity at the FGA locus was revealed in three subjects

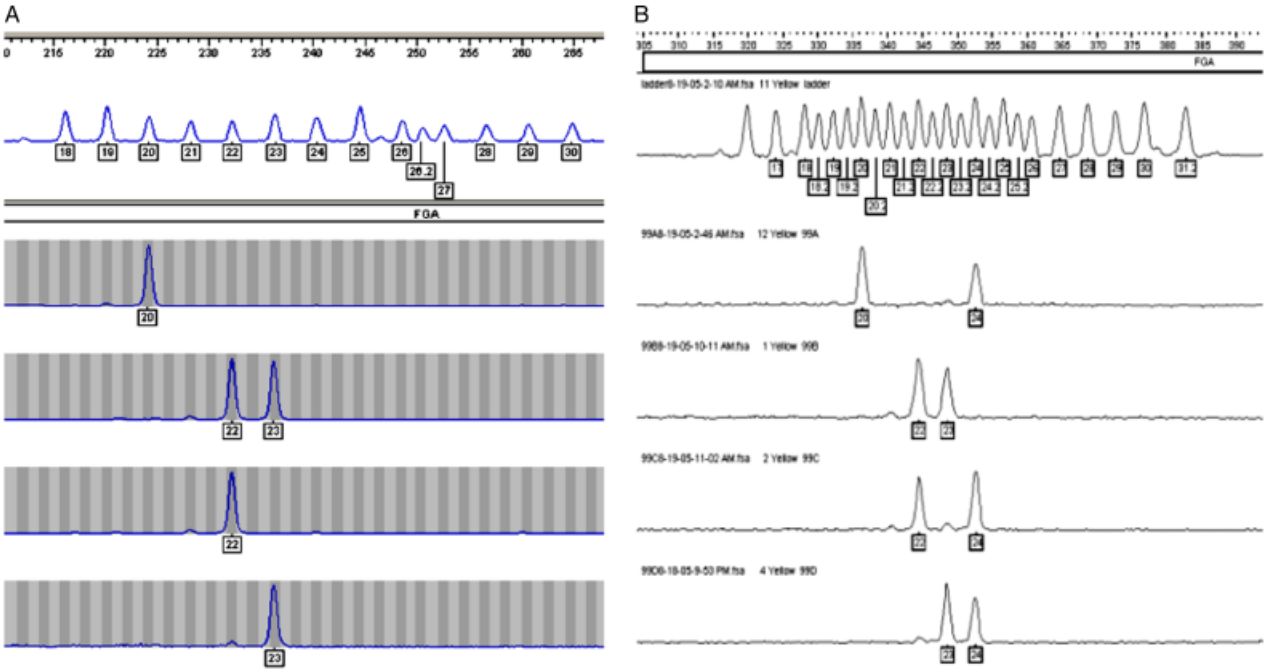

FIG. 1—*Electropherograms showing results of FGA typing. (A) Profile obtained with the AmpFlSTR® Identifiler; (B) the profile obtained with PowerPlex®16. From top to bottom: alleged father, mother, son, daughter.*

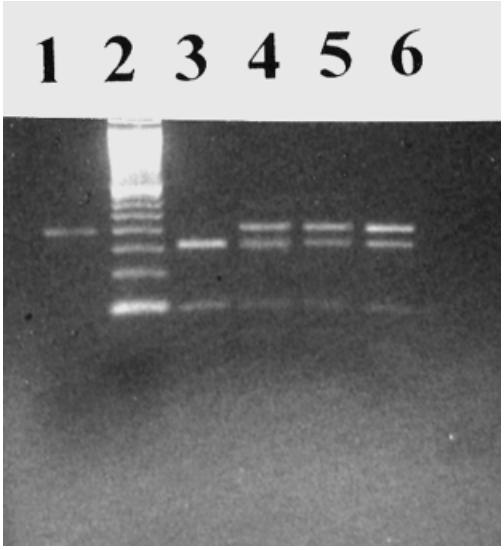

FIG. 2—*Restriction enzyme digestion analysis of the FGA PCR products with TaqI. Line 1: undigested PCR product from the mother; line 2: 100 bp allelic ladder; line 3: digested PCR product from the mother (wild type); line 4–6: digested PCR products from the alleged father, the son, and the daughter with a 402 bp undigested band for the larger 24 allele with G>T mutation.*

with Profiler Plus and Identifiler: the alleged father (20, 20), the son (22, 22), and the daughter (23, 23). The mother was heterozygous (alleles 22, 23). However, using the PowerPlex® 16 kit, the father and the children were heterozygous at the FGA locus, with allele 24 revealed (Fig. 1). With this kit, two inconsistencies between the alleles observed for the daughter and the alleged father at D18S51, TH01, and the maternal mutation at D16S539 were confirmed. Finally, the heterozygote profile for FGA in these three subjects was confirmed (data not shown) using two separate sets of alternative primers reported previously (12,14) and with a new primer pair described in this study (Table 1).

Mutation at the FGA Locus

Sequence analysis of the FGA products with external primers revealed a G–T single mutation at position 3047 (GenBank sequence M64982), corresponding to a region located downstream of the polymorphic repeat site in intron 3 of the FGA locus (data not shown). As the mutation changes a cleavage site (TCGA) of the TaqI restriction endonuclease, it could be confirmed by endonuclease restriction analysis. In this case, the wild-type alleles with the G base were digested by TaqI, while the alleles with the mutated site T were not sensible to digestion. Samples from the subjects heterozygous for the G>T variant (alleged father and the two children) showed the presence of a 402 bp band corresponding to the undigested PCR product, easily recognizable in the agarose gel (Fig. 2), which was not observed in the mother, who had a wild-type genotype.

The 3047G>T variation was associated with allele 24, and the primer pairs that correctly amplified this allele did not coincide with the mutation site (Fig. 3). Thus, we can speculate that the 3047G>T mutation is presumably located within the binding region of the reverse primer.

Rare mutations in FGA are responsible for some cases of congenital afibrinogenemia (OMIM: #202400). They were also reported in association with some cases of dysfibrogenemia α type (OMIM: #+134820) and hereditary renal amyloidosis (OMIM: #+176300). The mutation found in this study is localized in an intronic region and does not change the splice recognition site;

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2701  acacctttaa aattccaaag aaagttcttc ttctatattt ctttgggatt actaattgc
      tgtggaaatt ttaaggtttc tttcaagaag aagatataaa gaaaccctaa tgattaacg

2761  attaggacat cttaactggc attcatggaa ggctgcaggg cataacatta tccaaaagt
      taatcctgta gaattgaccg taagtacctt ccgacgtccc gtattgtaat aggttttca

2821  aaatgcccca taggttttga actcacagat taaactgtaa ccaaaataaa attaggcat
      tttacggggt atccaaaact tgagtgtcta atttgacatt ggttttattt taatccgta

2881  tttacaagct agtttctttc tttctttttt ctctttcttt ctttctttct ttctttctt
      aaatgttcga tcaaagaaag aaagaaaaaa gagaaagaaa gaaagaaaga aagaaagaa

2941  ctttctttct ttctttcttt ctttctcctt ccttcctttc ttcctttctt ttttgctgg
      gaaagaaaga aagaaagaaa gaaagaggaa ggaaggaaag aaggaaagaa aaaacgacc

3001  aattacagac aaatcactca gcagctactt caataaccat attttcGatt tcagaccgt
      ttaatgtctg tttagtgagt cgtcgatgaa gttattggta taaaagCtaa agtctggca

3061  ataataccta caaccgagtg tcagaggatc tgagaagcag aattgaagtc ctgaagcgc
      tattatggat gttggctcac agtctcctag actcttcgtc ttaacttcag gacttcgcg

3121  aagtcataga aaaagtacag catatccagc ttctgcagaa aaatgttaga cctcagttg
      ttcagtatct ttttcatgtc gtataggtcg aagacgtctt tttacaatct cgagtcaac
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FIG. 3—*FGA sequence (GenBank M64982) (allele 21): three repeat unit lengths should be added to obtain allele 24. The primer pairs used to confirm the presence of allele 24 in the alleged father, the son, and the daughter are indicated: the primer pair from the PowerPlex® 16 kit is underlined; the primer pair described in (12) is shadowed; the primer pair described in (14) is dashed. The new primer pair designed for sequencing in this study is in bold. The location of the mutation found in the presumptive primer-binding region of reverse primer in the AmpliType kits is in bold and in capital letters.*

thus, it may not have a pathological effect. In support of this, to our knowledge, the mutation has not been reported in association with pathological conditions.

Paternity Test

Using 19 STR markers, alleged father's paternity for the son was confirmed with a very high posterior probability (0.99999999996 W, *a priori* = 0.5). Paternity for the daughter could be excluded by the occurrence of three genetic inconsistencies between the alleged father and daughter at loci D18S51, TH01, and SE33 (16). Paternal mutation events could be excluded, because the simultaneous occurrence of three multi-step STR changes is highly unlikely. The presence of the 3047G > T mutation in FGA in the alleged father and in the daughter suggests that the biological father may be a relative of the alleged father. Case history and the low number of incompatibilities support this hypothesis. By using the Familias® program (17), the probability that the brother of the alleged father is the biological father of the daughter was 0.999 (avuncular index).

Interestingly, we also observed a single-step mutation at D16S539 in the maternal allele (alleles 12, 12) transmitted to the daughter (alleles 8, 11), probably due to loss of a repeat sequence in allele 12 producing 11. The one repeat unit change is the most common type of mutation seen with STRs (18). The occurrence of this maternal mutation is a very rare event (0.03), as reported by the AABB (19).

In conclusion, the present case shows the importance of the use of a large number of STRs to resolve *a priori* a paternity dispute when a close relative of the alleged father could be the biological father of the child. Moreover, the publication of primer sequences for AmpFlSTR® kits would assist in the accurate identification of mutations that may be the cause of erroneous homozygosity (null alleles) and discordant results between methods that use different primers.

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