

Double Incompatibility at Human Alpha Fibrinogen and Penta E loci in Paternity Testing

Anna Lucia Nutini, Alessandro Mariottini, Laura Giunti¹, Francesca Torricelli, Ugo Ricci¹

U.O. Citogenetica e Genetica, Azienda Ospedaliera Careggi and ¹U.O. Genetica e Medicina Molecolare, A. Meyer Hospital, Florence, Italy

We present a paternity testing case in which a double incompatibility was found for two short tandem repeat (STR) markers, human fibrinogen alpha (FGA) and Penta E. Analysis of the trio (mother, father, and daughter) included the amplification with a battery of 15 autosomal short tandem repeats (STR) by using a commercially available PowerPlex[®] 16 System kit, and the detection with an ultraviolet-automatic sequencer. The biological paternity was confirmed with 12 additional markers. Reanalysis of the trio for the same markers with different primers was carried out by using an infrared automated sequencer and infrared-fluorescent primers. High paternity index confirmed that the observed inconsistencies were due to a double mutation, which was confirmed by sequence analysis at FGA and Penta E loci. Amplification and detection results obtained by the infrared-protocol showed consistent results with those obtained by ultraviolet-protocol and a commercially available kit. This has been our first case of double mutation at FGA and Penta E in a paternity testing. The use of our approach, based on two amplification and detection formats and on the sequence analysis, confirmed the observed meiotic paternal mutations.

Key words: genetics, medical; mutation; paternity; polymorphism (genetics); sequence analysis, DNA; tandem repeat sequences

It is well known that microsatellites are prone to mutation in maternal and paternal meiosis. Mutation rates are fairly high for many short tandem repeat (STR) loci and meiotic mutations can be detected in children (1). Moreover, the mutation value for paternal meioses is higher than that for maternal meiosis, with variations between loci (2). However, the presence of two mutations between a father and a child is not so frequent. Raskin et al (3) reported five double exclusionary cases in a dataset of 3,322 trios (a trio including the mother, father, and child) in which the paternity was confirmed by additional markers, with an observed frequency of 0.099%. Geada et al (4) reported a case with two paternal inconsistencies in D7S820 and CSF1PO in approximately 300 paternity tests. Brandt-Casadevall et al (5) found inconsistencies for D5S818 and vWA, with about 0.7% of all paternal meiosis, in two out of 294 cases of paternity testing. However, it is unclear how many of these inconsistencies may be null alleles (primer binding site variation preventing hybridization) rather than *de novo* mutations.

Case Report

We present a paternity testing case in which a double incompatibility was found for two STR mark-

ers, human fibrinogen alpha (FGA) and Penta E. Since the child involved in the investigation was female, the analysis of Y-chromosome polymorphisms we previously showed (6) could not be used to confirm the paternity. Thus, the biological paternity of the alleged father was confirmed by using six additional allele-specific oligonucleotide (ASO) markers, one minisatellite, and five STRs. We used a commercially available kit for the amplification, and an ultraviolet-automatic sequencer for the detection. The DNA samples of the trio were further examined for the same markers with different primers, in combination with an infrared automated sequencer. We also performed a sequence analysis of all alleles of the child and the alleged father.

DNA Extraction

Blood from the mother and the alleged father was obtained by venipuncture, and a saliva swab was taken from the daughter. DNA was obtained by the phenol-chloroform extraction method and final concentration was evaluated on gel agarose, followed by ethidium bromide staining.

Amplification and Detection with Ultraviolet Protocol

To perform the paternity test, we used the standard method based on an ABI PRISM[®] 377 DNA se-

quencer (Applied Biosystems, Foster City, CA, USA), with the PowerPlex® 16 System (Promega Corporation, Madison, WI, USA), according to the manufacturer's recommendations (7). The degenerate sequences of primers used for the amplification of the two STR markers, the FGA and Penta E, included in the kit were as follows (8):

FGA:

F-5' – TMR – GGCTGCAGGGCATAACATTA – 3'

R-5' – ATTCTATGACTTTGCGCTTCAGGA – 3'

Penta E:

F-5' – FL – ATTACCAACATGAAAGGGTACCAATA – 3'

R-5' – TGGGTTATTAATTGAGAAAACCTCTTACAATTT – 3'

Amplification and Detection with Infrared Protocol

To confirm the observed inconsistencies at FGA and Penta E loci, we used an alternative strategy based on a polymerase chain reaction (PCR) monoplex protocol and different primers for the amplifications. The characterization of the alleles was achieved with the IR-based Automated DNA Sequencer (LI-COR 4200, Lincoln, NE, USA), as described elsewhere (9). Briefly, the PCR was performed in a total volume of 25 µL by using 10 ng of genomic DNA in GeneAmp® PCR buffer II (Applied Biosystems) containing 100 mmol/L Tris-HCl (pH 8.3), 500 mmol/L KCl, 1.5 mmol/L MgCl₂, 200 mmol/L each of dNTP, and 0.3 U of AmpliTaq Gold® Polymerase (Applied Biosystems), added directly to the reaction mix.

We used the following labeled and unlabeled primers at a final concentration of 0.5 mmol/L:

FGA (10):

F-5'-IRDye™800 GCCCCATAGGTTTTGAACTCA - 3'

R-5' – TGATTTGTCTGTAATTGCCAGC – 3'

Penta E:

F-5'-IRDye™800 GATCAAGACCAGCCTGGGCA - 3'

R-5'-TGGGTTATTAATTGAGAAAACCTCTTACAATTT – 3'

These primers produced smaller amplicons than the primers included in the PowerPlex® 16 System (Table 1). The following amplification conditions were used in a PTC-100 Thermocycler (MJ Research Inc., Waltham, MA, USA): 95 °C for 10 min, then 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, for 30 cycles, with a final extension at 60 °C for 60 min. At the end of the amplification cycles, 6 µL of stop solution containing 95% formamide, 10 mmol/L EDTA, and 0.1% blue bromophenol were added. For the exact detection of the alleles, we used specific allelic ladders and positive controls.

Additional DNA Markers

The AmpliType® PM+ DQA1 PCR Amplification and Typing kits (Applied Biosystems) were used according to manufacturer's recommendations (11). Moreover, the minisatellite YNZ22 marker was analyzed, as reported previously (12). Finally, five additional STR markers (F13B, D19S433, D2S1338, D12S391, and SE33) were analyzed with monoplex amplification systems on the LI-COR's DNA automated sequencer.

Table 1. Sequence of human fibrinogen alpha (FGA) and Penta E loci to show the sequences of the primers used in the present study*

M64982. Human fibrinogen alpha chain gene
2701 acacctttaa aattccaag aaagtcttc ttctatatt cttgggact actaattgct
2761 attaggacat ctaactggc atcatggaa ggctgcagg cataacatta tccaaaagtc
2821 aaat gcccc taggtttga actca cagat taaactgtaa ccaaaataaa attaggcata
2881 ttacaagct agtttcttc ttcttttt ctctttctt cttctttt ttcttttt
2941 ctttcttt ttcttttt ctttctct cttctctt ttctttt tttctggc
3001 aattcacagc aatca ctca gcagctact caataaccat atttcgatt 15
3061 ataataccta caaccgagtg tcagaggatc tgagaagcag aattgaag tc ctgaagcgca
3121 aagtcataga aaa aagtagc catatccagc ttctcagaa aatgttaga gctcagttgg
3181 ttgatagaa acgactggag gtaagtatgt ggctgtgttc ccgagtgtcc ttgttttga
AC027004 Homo sapiens chromosome 15
84361 ttgataaga gaaaataaa tacatt ttac caacatgaaa gggtagcaat a acaagaaaa
84421 ttgggacag gtcgggatg tcacgcctgc aatcctagca cttggaggc ccgatgcagg
84481 tgtattacct gagctcagga gatcaagacc agcctgggca acatggtgaa acccctctc
84541 tactaaaata caaaaatta gctgggtgtg gttgtaggca cctgtaatcc cagctactc
84601 ggaggctgaa acaggagaat cactgaacc caggaggagg agattgaagt gagccgagat
84661 cagccattg cactccagcc tgccgagct agcaagactc agtctcaag aaaaagaaaag
84721 aaaagaaaag aaattgtaa ggagtttct caattaataa ccca aataag agaattctt
84781 ccatgatca atcatgatac taagcacttt acacacatgt atgttatga atcattat
84841 catcatgca agtgaatgag tattatttc ctcatttat aaaaagaggaa actgatgttt
84901 gaggcactt tgcttaagac cacagaacta gcaaaagaaa agagaagtag atgtatccct
84961 gatcccttt aacacttct acacagcctc cccacaatgt ccagatataa cttcataaat

*The PowerPlex® 16 System primers are shaded in grey and the alternative primers are underlined. For Penta E, the same reverse primer was used as in the PowerPlex® 16 System.

Sequence Analysis

The sequence analysis was also performed for FGA and Penta E alleles of the daughter and alleged father. After the monoplex amplification reactions, the amplicons were separated on a Nusieve GTG 2.3% agarose gel, excised, and extracted by means of the QIAquick Gel Extraction kit (Quiagen GmbH, Hilden, Germany). The alleles were reamplified and sequenced by using IRDye™800-labeled primers in combination with a Thermo Sequenase Fluorescent-labeled primer cycle Sequencing kit with 7-deaza-d-GTP (Amersham Biosciences, Piscataway, NJ, USA). The detection was performed on the LI-COR 4200 automatic DNA sequencer.

Data Analysis

The paternity index (PI) and the *a posteriori* probability (W50%) were calculated from a database of a Tuscan population (13), without considering mutations, as suggested by Evett et al (14). The mutation value of the paternal meioses for FGA (0.29%) was deduced from the report of the American Association of Blood Bank (2). The mutation rate for Penta E was 0.14% (Lotte Henke, Institut für Blutgruppenforschung, Cologne, Germany, personal communication). The calculation for the paternity index including mutations was performed by using two approaches, one suggested by Fimmers (15) and the other by Brenner (16).

Results

All but two loci showed compatibility among the child, the mother, and the alleged father (Table 2). Two genetic inconsistencies between the alleged father and daughter were detected at FGA and Penta E

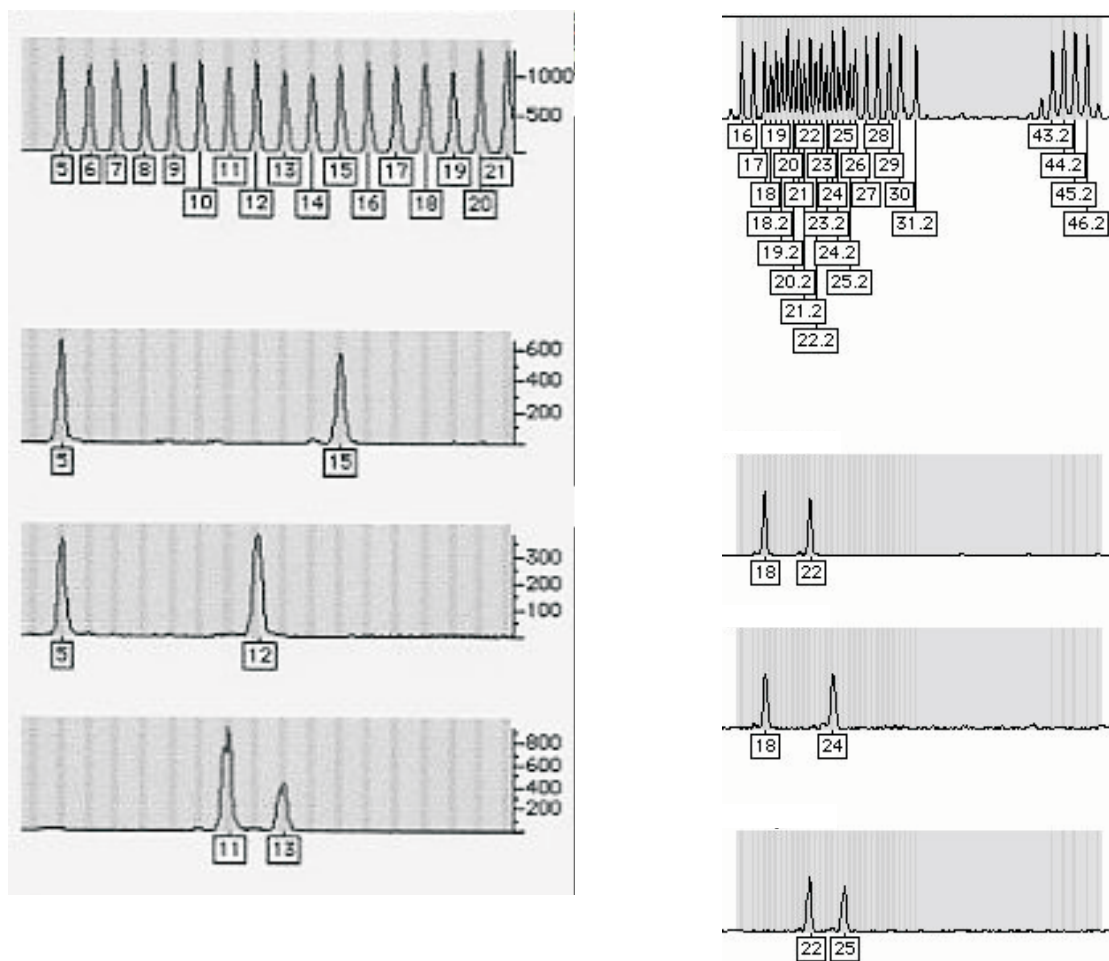


Figure 1. Electropherograms of Penta E (left) and human fibrinogen alpha (FGA, right) in the paternity investigation with a double incompatibility. From the top: allelic ladder, mother, daughter, and alleged father. The numbers indicate the alleles from the trios.

Table 2. Results of the paternity investigation with loci of the PowerPlex® 16 System, which showed double incompatibility between the daughter and alleged father

Locus	Mother	Daughter	Alleged father	PI†
TPOX	8, 8	8, 11	8, 11	2.05
D3S1358	19, 19	17, 19	17, 17	4.18
FGA	18, 22	18, 24*	22, 25	-
D5S818	11, 12	11, 13	12, 13	2.76
CSF1PO	10, 12	11, 12	11, 13	1.55
D7S820	9, 11	9, 10	8, 10	1.63
D8S1179	13, 14	14, 15	14, 15	3.16
TH01	6, 9.3	6, 7	7, 7	5.10
VWA	15, 17	14, 17	14, 17	4.58
D13S317	10, 12	12, 13	13, 13	12.2
Penta E	5, 15	5, 12*	11, 13	-
D16S539	11, 12	11, 11	11, 11	2.97
D18S51	12, 14	12, 14	12, 17	1.50
D21S11	29.2, 30	30, 31	31, 33.2	11.30
Penta D	10, 11	10, 12	12, 12	5.05
Cumulative PI				13,000,000

*Mutated alleles in the child.

†Paternity Index (PI) did not include the mutation values for the human fibrinogen alpha (FGA) and Penta E.

loci, as shown by electropherograms obtained with the ABI PRISM® 377 Genetic Analyser (Fig. 1).

We used an alternative amplification format and a detection infrared format to confirm the observed

haplotypes for the involved persons. The result of electrophoresis with the LI-COR 4200 DNA Sequencer appeared in an autoradiogram-like image (Fig. 2), automatically registered as a TIF image and filed in the hard disk of the computer. By using this approach, possible errors in genotyping were eliminated and Mendelian discrepancies could be ascribed to mutations or parenthood errors. The obtained haplotypes were completely in agreement and the inconsistencies between the daughter and the alleged father were confirmed. Confirmation of the biological paternity of the alleged father was performed with six ASO markers, one minisatellite, and five additional STR loci (Table 3).

Calculation of PI by the two different formulas produced substantial differences between the two methods (Table 4). However, high paternity index (> 300,000) in both cases confirmed the biological paternity and the presence of two STR mutations in the child.

The cumulative paternity index obtained by the Fimmers method (15) was 3.5 times smaller than that obtained by the Brenner method (16). However, these two calculation methods use different theoretical approaches. Since Fimmers calculation does not

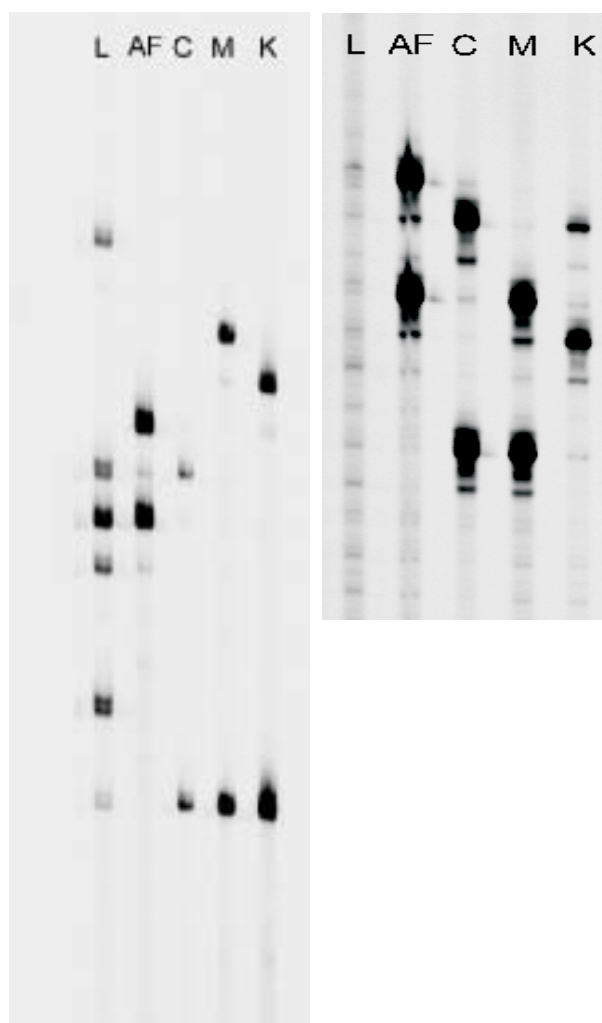


Figure 2. Autoradiogram-like images obtained with the LI-COR 4200 DNA sequencer in the paternity investigation with a double incompatibility. Left panel: Penta E locus, right panel: human fibrinogen alpha (FGA) locus. L – allelic ladder, AF – alleged father, C – child, M – mother, K – K562 DNA control.

take into account the frequencies of the alleles involved in a pattern, the resulting paternity indexes are the same for all mutations for a given system. On the other hand, Brenner's "STR formula" takes into account the mutated allele and the mechanism involved. In this case, the one-step model of mutation was the most probable one (17). Thus, the "STR formula" removed the tendency to underestimate the "true paternity" of the alleged father.

Sequence analysis confirmed the variation in the length of the repeats between the child and alleged father. However, no variations in the sequences (microvariants and point mutations) could indicate the exact paternal origin of the mutated child's alleles. According to the stepwise mutation model, the two mutations that were found can be explained by gain or loss of one repeat. For FGA, the inconsistent allele 24 of the girl was probably brought about by deletion of a single repeat from allele 25 of the alleged father. For Penta E, it was impossible to understand the exact pa-

Table 3. Results for 12 additionally investigated loci in the paternal testing, which all showed compatibility between the daughter and alleged father

Locus	Mother	Daughter	Alleged father	PI*
HLA-DQ1	1,2, 2	2, 4,1	3, 4,1	2.33
LDLR	BB	BB	AB	1.11
GYPA	BB	AB	AB	0.94
HBGG	AB	AA	AB	0.94
D7S8	BB	BB	AB	1.28
GC	AC	AC	BC	0.60
YNZ22	3, 4	2, 4	2, 3	3.12
F13B	9, 10	8, 8	8, 9	3.33
D19S433	13, 14	13, 13	13, 13	1.79
D2S1338	17, 17	17, 18	17, 17	5.00
D12S391	20, 21	18, 20	20, 20	5.40
SE33	19, 29,2	28,2, 27,2	27,2, 19	5.07
Cumulative PI [†]				4,468

*Paternity index (PI) for the single loci was calculated as suggested by Evett et al (14).

[†]Product of multiplication of all paternity indices.

Table 4. The variation of paternity index (PI) including the values for human fibrinogen alpha (FGA), Penta E, and 12 additional investigated markers, according to the Fimmers method (15) and approximation suggested by Brenner (16)

PI	Mutation rate	Method	
		Fimmers' X/Y	Brenner's X/Y
for the PowerPlex® 16 System loci without FGA and Penta E	–	13,000,000	13,000,000
for FGA	0.29%	0.0038	0.0065
for Penta E	0.14%	0.00177	0.00370
including mutations	–	87.4	330.1
for additional 12 loci	–	4,468	4,468
Cumulative	–	390,000	1,400,000
W (50%)*	–	0.999997	0.99999992

*The a posteriori probability of paternity (W50%) was very high, confirming the biological paternity of the alleged father.

ternal allele in which the mutation had occurred and the origin was declared unknown. In fact, the inconsistent allele 12 of the girl could derive from a contraction of allele 13 or from insertion of one repeat unit of allele 11 of the alleged father. Our data were in agreement with previously reported data in which 90% of STR mutation events occurred for a single repeat step (17).

Discussion

Out of 30 paternity tests we performed using the PowerPlex® 16 System, only a single one showed mutations. As far as we know, this is the first report of a double incompatibility in a paternity test in Italy. Due to the limited number of the examined cases, no statistical analysis for frequency mutation could be performed. However, this case will be useful for the evaluation of mutation frequency in STR loci in Italian as well as other populations (6). The use of shared polymorphic markers in the forensic community will allow the comparison of data between various Italian laboratories, as is already the case in other countries (2,17), with an accurate calculation of the mutation percentages.

In conclusion, our data suggest that we can expect to find paternity investigation with one or two inconsistencies when STRs are analyzed. In particular, if the two inconsistencies can be explained as single-step mutations or as null alleles, they are unlikely

to be a sufficient proof of non-paternity. Conversely, the use of a larger battery of highly polymorphic markers can be useful to reach a high value of paternity index. When no anecdotal reason exists to believe that another man, correlated to the alleged father, could be considered as possible father, and when the paternity index is over 10,000, which corresponds to $W(50\%) = 99.99\%$, we think that the biological paternity could be supported even in the presence of the incompatibility at two STR loci.

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Correspondence to:

Ugo Ricci
Genetics and Molecular Medicine Unit
University of Florence
Hospital "A. Meyer"
Via Luca Giordano 13
50132 Florence, Italy
ricciugo@tin.it