

Compound heterozygosity for p.F508del mutation and deletion of exons 4-11 in CFTR in an infant with cystic fibrosis- Limitations of ARMS-PCR and Sanger sequencing

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Summary

Cystic fibrosis (CF) is a life threatening genetic condition caused due to mutations in the CFTR gene. This is an autosomal recessive condition and usually parents are carriers of a heterozygous mutation in the CFTR gene. We describe an infant diagnosed with the severe phenotype of CF and detected to be homozygous for p.F508del by ARMS-PCR. However, parental studies confirmed carrier state in mother but the father showed a wild allele by ARMS-PCR and sequencing. This prompted us to look for uniparental disomy (UPD) and large deletions. UPD was ruled out by studying both intragenic and extragenic markers for chromosome 7. The proband and the father were found to have a large deletion detected by MLPA involving exons 4-11 in the CFTR gene which includes the region coding for phenylalanine at position 508. This case highlights the need of caution when interpreting results of molecular genetic testing during genetic counseling. It is the first documented case from India with point mutation and large deletion of the CFTR gene giving rise to apparent homozygosity for p.F508del.

Case Report

A 5 month old boy diagnosed with CF was referred to the Clinical Genetics Unit of Christian Medical College, Vellore by the Department of Child Health for molecular confirmation and genetic counselling. He was the first offspring of non-consanguineous parents born through LSCS.

He weighed 2.5 kgs at birth and was admitted in the nursery for one week with respiratory distress probably secondary to meconium aspiration and managed conservatively.

Previous history of recurrent hospital admissions for pneumonia, passage of oily loose stools and failure to thrive was obtained from the parents. The current admission was for severe pneumonia leading to unstable vital signs and probable hypoxic seizures requiring ventilation.

He had achieved partial head control and had been immunised till the age of 6 weeks. The family history was unremarkable.

His length at 55cms and weight at 2.5kgs, were <3SDs below the age related mean; the head circumference at 38 cms was normal. There were no dysmorphic features. Chest examination revealed crepitations and conducted sounds with decreased bilateral air entry. The liver was palpable 1 cm below the right costal margin and rest of the physical examination was normal. The child was treated with infusion of antibiotics and antiepileptics. Stool examination showed numerous fat globules. He was started on nasogastric feeds on day 4 of hospitalization. His high grade fever persisted and he deteriorated despite all supportive measures and succumbed to his illness.

Molecular analysis was done for confirmation of diagnosis and genetic counseling. Preliminary genetic counseling was given and parents agreed for carrier testing as they were considering prenatal diagnosis for future pregnancies.

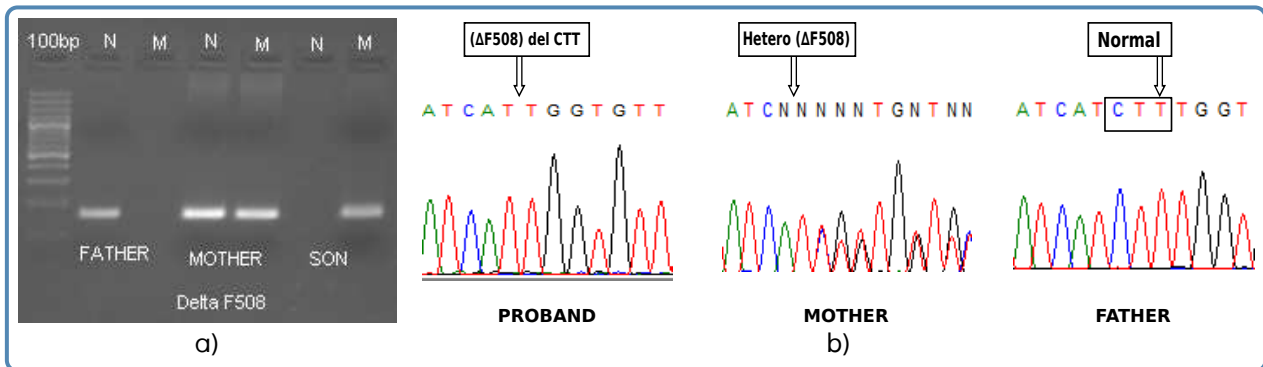


Figure 1 a). Gel picture of ARMS-PCR of proband and parents showing normal father, heterozygosity of del F508 for mother and homozygosity of del F508 for proband. N- normal M- Mutant. b). Sequencing chromatogram showing homozygosity for p.F508del in proband, heterozygosity in mother but normal result in father.

Material and Methods

Genomic DNA was extracted from leukocytes using QIAGEN Midi Kit. ARMS-PCR was done for the p.F508del mutation.¹ Sequencing was done (Applied Biosystems Genetic Analyzer 3500) to confirm the mutations detected by ARMS-PCR. Further molecular analysis was done using four intragenic (T854T, M470V, TUB9, TUB 20) and two extragenic (XV 2C, KM19) markers for chromosome 7. Specific primers were used according to previously described methods and the amplified products were restriction digested using *TaqI*, *PstI*, *MnII*, *HphI*, *Avall*, *PvuII* for XV2C, KM19, TUB9, M470V, T854T and TUB20 respectively.² The products of XV2C, KM19, M470V, T854T were separated using 2% agarose gel and TUB 9 and TUB 20 were separated using 8% native polyacrylamide gel electrophoresis (PAGE). Six VNTR markers were studied to rule out false paternity. To identify larger deletions affecting whole exons of the CFTR gene, the sample was sent to the Institute of Human Genetics, University Hospital, Technical University RWTH Aachen, Aachen, Germany where commercially available MLPA (multiplex ligation dependent probe amplification) assay for CF (P091; MRC Holland/Amsterdam/NL) was used according to the manufacturer's instructions.

Results

The mutation analysis for CFTR by ARMS-PCR³ showed a pattern consistent with homozygous

mutation for p.F508del. Genetic testing of the parents showed only mother as carrier and father had the wild type allele (Fig 1a). Sequencing results confirmed the pattern seen by ARMS-PCR (Fig 1b). Six VNTR markers confirmed the paternity. Intragenic and extragenic markers for chromosome 7 showed presence of both parental alleles for XV2C, KM19 T854T and TUB20. However the results for markers TUB9 and M470V were not compatible with paternal inheritance as only maternal alleles were detected. (Figure 2a,2b) The interpretation is as follows: the digested product of M470V (g.1540 A>G) using *HphI* yields one fragments at 492bp for homozygous allele 1, three fragments at 492bp, 301bp and 191bp for heterozygous allele 1 and allele 2, and two fragments at 301bp and 191bp for (homozygous allele 2). The digested product of TUB 9 (g.1525-61 A or G) using *MnII* yields four fragments 199bp,147bp, 130bp and 15bp for homozygous allele 1, four fragments at 199bp, 147bp,130bp and 95bp (for heterozygous allele 1 and 2) and five fragments at 147bp,130bp,104bp,95bp and 15bp in the case of homozygous allele 2. Thus UPD was ruled out but deletion/ chromosomal rearrangement was suspected due to presence of only maternal alleles located near exon 10 of CFTR. DNA analysis by MLPA at the Institute of Human Genetics in Aachen revealed that the proband carried a large scale heterozygous deletion spanning exons 4-11. The same deletion was detected in the father.

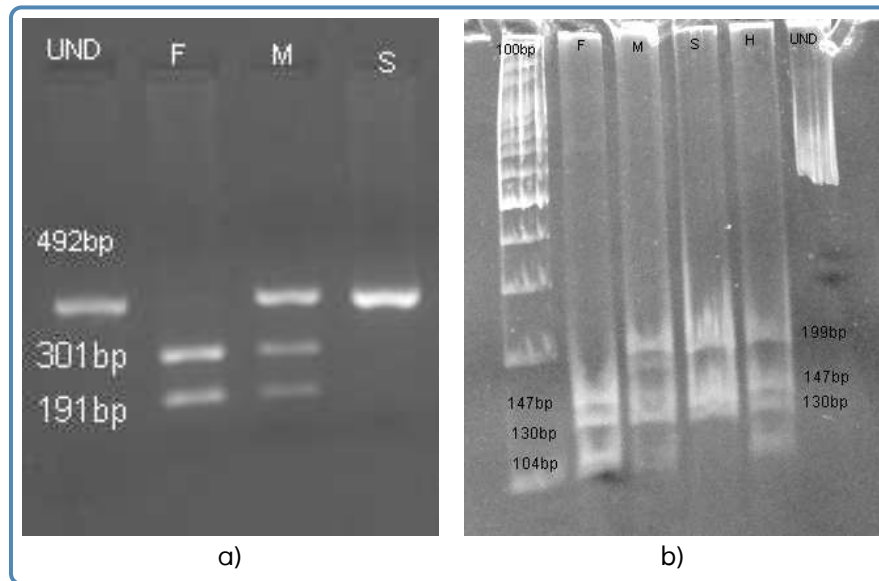


Figure 2 a). Markers status for M470V (HphI digest) F- Father's alleles 2/2 M- Mother's alleles 1/2 S- Son's alleles 1/1 UND- Undigested Product.
 b). TUB 9 marker study (MnlI digest) by PAGE Well 1–100bp ladder. The digested product of TUB 9 (g.1525-61 A or G) using MnlI yields four fragments 199bp, 147bp, 130bp and 15bp for allele 1 and five fragments at 147bp, 130bp, 104bp, 95bp and 15bp for allele 2. F- Father has allele 2 which has 5 fragments - 147, 130, 104 bp are seen and the smaller fragments (95 and 15 bp) which are not seen. M- Mother has 1 & 2 alleles (total 6 fragments) -199, 147, 130, 104 bp are seen and the two smaller fragments are not seen. S- Son has allele 1/1 showing 199, 147, 130 bp; the 104 bp is not seen. H- Healthy.

Discussion

Over 1900 different mutations have been identified in the CFTR gene (www.genet.sickkids.on.ca/). The most common mutations are c.1521_1523delCTT (p.F508del), c.1652G>A, c.1641G>T and c.489+1G>T.³ The mutation p.F508del (c.1521_1523delCTT), a deletion of three nucleotides resulting in removal of phenylalanine at position 508, is the cause of CF in majority of cases. It has a frequency of 66% in cases of CF worldwide.⁴ One study showed that affected individuals of Indian origin have a frequency of p.F508del mutation ranging from 19% to 44%, while another Indian study showed 20 CF cases out of 100 to be homozygous for p.F508del and 13 cases to be heterozygous for this mutation.^{5,6} Cases with the p.F508del mutation had a more severe phenotype. Single or multi exonic large deletion may be present in combination with point mutations in CFTR on rare occasions.⁷ These can account for 1% to 3% of affected individuals.⁸ In

the present case either maternal UPD or a large gene rearrangement was suspected when routine molecular methods showed apparent homozygosity for p.F508del, which was incompatible with the parental genetic status.

A recent study showed that the frequency of large scale gene deletions differs from population to population. The actual frequency of large scale deletion of the CFTR gene is still not clear since routine diagnostic analysis involves detection of point mutations and small indels only and large scale deletions can be missed out. Advanced techniques like MLPA, quantitative PCR and array CGH (comparative genomic hybridization) can be used to rule out the apparent homozygosity of point mutations and indels.⁹ Chevalier-Porst et al. reported 24% large deletions in a cohort of 1600 CF alleles tested. This French study reported two patients, a boy diagnosed with classical CF at one year harbouring a deletion of 95.738 kb size which removed the coding sequence from exon 2 to exon 10, and a girl with reported deletion of exon 4–10. Both the cases had the p.F508 mutation in the second allele

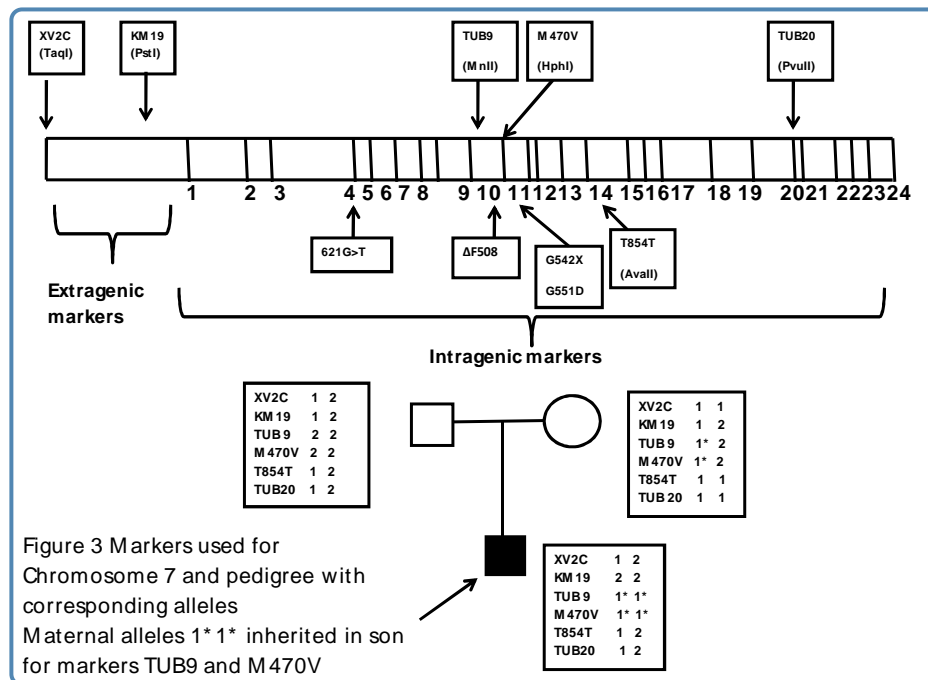


Figure 3 Markers used for Chromosome 7 and pedigree with corresponding alleles. Maternal alleles 1*1* inherited in son for markers TUB9 and M470V.

inherited from the mother while the large deletion was inherited from the father, as in the present case.¹⁰ In this case two extragenic markers and four intragenic markers ruled out UPD. Two intragenic markers near exon 10 showed both alleles to be maternal further increasing the suspicion of deletion. MLPA confirmed a large scale heterozygous deletion involving exons 4–11. This genotype detected was associated with a very severe phenotype in the proband leading to death in infancy. This is the first such case from India described in literature proving the importance of rarer CFTR rearrangements which can have implications for genetic counseling and prenatal diagnosis. This also highlights the limitations of ARMS-PCR and Sanger sequencing in detecting large genomic deletions in many single gene disorders. Hence, interpretation of molecular genetic results has to be carried out with caution while counseling a family.

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