Prenatal diagnosis of steroid 21-hydroxylase-deficient congenital adrenal hyperplasia: Experience from a tertiary care centre in India

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Background & objectives: Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder with a wide range of clinical manifestations. The disease is attributed to mutations in *CYP21A2* gene encoding 21-hydroxylase enzyme. In view of severe phenotype in salt-losing cases, issues related to genital ambiguity in girls and precocity in boys, most families opt for prenatal testing and termination of affected foetus. CAH can be diagnosed *in utero* through direct molecular analysis of *CYP21A2* gene, using DNA extracted from foetal tissues or cells obtained from chorionic villus sampling or amniocentesis. The objective of this study was to evaluate the feasibility and accuracy of prenatal diagnosis (PND) using sequencing and multiplex ligation probe amplification (MLPA) methods in families at risk for CAH.

Methods: Fifteen pregnant women at risk of having an affected offspring with CAH were included in this study. Ten families had previous affected children with salt-wasting/simple virilising form of CAH and five families did not have live children but had a high index of suspicion for CAH in previous children based on history or records. Mutation analysis was carried out by Sanger sequencing and MLPA method.

Results: Seven different mutations were identified in 15 families. Deletions and I2g mutation were the most common. Of the 15 foetuses analyzed, nine were unaffected while six were affected. Unaffected foetuses were delivered, they were clinically normal and their genotype was found to be concordant to the prenatal report. All except two families reported in the second trimester. None of the couples opted for prenatal treatment.

Interpretation & conclusions: Our preliminary findings show that PND by direct mutation analysis along with MLPA is a feasible strategy that can be offered to families at risk.

Congenital adrenal hyperplasia (CAH), an autosomal recessive disorder is caused due to deficiency of steroid 21-hydroxylase enzyme involved in the cortisol biosynthesis. Steroid 21-hydroxylase deficiency (21OHD) is very common and accounts for about 90-95 per cent of all CAH cases[1]. The classical

forms of CAH comprising salt wasting (SW) and simple virilizing (SV) have excess androgen due to 21OHD that leads to ambiguous genitalia in newborn females and progressive postnatal virilization in males and females. SW form also impairs aldosterone biosynthesis resulting in severe renal salt loss and shock, unless treated during the neonatal period. The SV form has normal aldosterone levels and thus salt loss is not present. The non-classical form is asymptomatic at birth and presents with various degrees of late-onset hyperandrogenism[2].

The overall incidence of classical CAH in the general population is between 1 in 13,000 and 1 in 15,000 live births[3]. The incidence of CAH in India has been found to be 1:2575 from a small sample survey[4]. In the absence of a national newborn screening programme for CAH in India, the exact incidence is not known. In view of the fact that severe forms of CAH can be life threatening and life-saving treatment is available, newborn screening of CAH has been recommended in urban areas of India[5].

Steroid 21-hydroxylase enzyme is encoded by *CYP21A2* gene which is located on human leucocyte antigen (HLA) class III locus. It is part of a complicated structure, referred to as the RCCX-module located on chromosome 6p21.3[6]. About 30 kb upstream of active gene *CYP21A2*, a highly homologous pseudogene *CYP21A1P,* is present that makes molecular analysis of CAH challenging. About 95 per cent of the mutations in patients with 21OHD are generated by transfer of DNA sequences from *CYP21A1P* to *CYP21A2* by gene conversion events[7]. The remaining five per cent of disease-causing mutations are new/rare and unique for single families or considered as population specific. Unequal crossing over during meiosis results in large gene deletions/duplications commonly found in *CYP21A2* locus[8].

The first successful prenatal diagnosis (PND) of CAH was carried out in 1965 by measuring elevated levels of 17-ketosteroids and pregnanetriol in amniotic fluid (AF)[9]. Later, 17 hydroxy progesterone (17OHP) became the ideal hormone to be measured in AF for PND of CAH. The main disadvantage of this method is that values of 17OHP cannot be relied upon if the mother is already on dexamethasone treatment to suppress foetal adrenal androgen oversecretion for prevention of genital ambiguity. Therefore, dexamethasone is to be stopped prior to the amniocentesis. Moreover, 17OHP values are not reliable when the foetus is mildly affected or has non-salt-wasting CAH. Later

on, HLA genetic linkage markers were used for PND after HLA was found linked to CAH[10]. This method is widely used but can result in diagnostic errors due to recombination or haplotype sharing. Direct molecular methods are considered ideal for diagnosing 21OHD in the foetal DNA. Various methods employed for molecular diagnosis of 21OHD are not foolproof due to complexity of *CYP21A2* locus[11]. This study was planned to evaluate the feasibility of PND of CAH using direct molecular methods in 15 CAH families using sequencing and multiplex ligation-dependent probe amplification (MLPA).

Material & Methods

Fifteen pregnant mothers in their first and second trimester with a history of confirmed or suspected diagnosis of CAH in previous children were referred to Genetics Clinic in the department of Pediatrics at All India Institute of Medical Sciences (AIIMS), New Delhi, India, for post-conception counselling during 2011-2014. This study was approved by the Ethics Committee of the Institute, and written consent was obtained from all families enrolled for this study.

All families enrolled were counselled about procedural risk, the possibility of allele dropout and *de novo* mutations prior to the PND. Chorionic villus sampling (CVS)/placental biopsies were performed in all families except F2 wherein amniocentesis was performed. Invasive procedures were performed between 11 and 21 wk of gestation. Amniotic fluid (20 ml) and 20-25 mg of chorionic villi (CV) tissue were aspirated. DNA was extracted from CV tissue by the phenol chloroform method[12] and from AF by Qiagen kit (QIAamp DNA extraction Blood Mini Kit catalogue No. 51104, Qiagen GmbH, Hilden, Germany).

Genetic testing was first performed in the affected child before analysis of foetal DNA using both Sanger sequencing and MLPA. In the absence of the index case, both parents were analyzed, and if found negative for point mutation(s) by Sanger sequencing, MLPA was carried out to detect heterozygous deletions. Subsequently, the foetal DNA was investigated for the same mutations found in the parents or the affected child. Maternal cell contamination was ruled out in each case using microsatellite markers (Igjh, DS180, ApoB, IVS17bTA and IVS8CA)[13-15].

Using 200 ng of genomic DNA was subjected to selective amplification of *CYP21A2* gene into two fragments of 1.1 and 2.1 kb, respectively (Fig. 1), with two sets of primers highly specific to active or
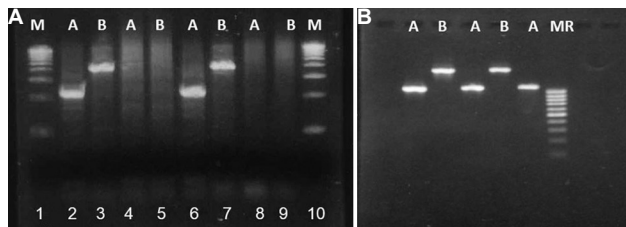
Fig. 1. (**A**) Polymerase chain reaction amplification of *CYP21A2* gene into two fragments; fragment A (1130 bp) and fragment B (2127 bp). M, DNA ladder; Lanes 2 and 6, fragment A; Lanes 3 and 7, fragment B; Lanes 4, 5, 8 and 9, absence of bands or amplification indicating gene deletion. (**B**) Purified polymerase chain reaction products of fragments A and B with mass ruler (MR).

CYP21A2 gene as described elsewhere[16]. Polymerase chain reaction (PCR) was carried out[17] and products were resolved on 1 per cent agarose gel stained with ethidium bromide and visualized under ultraviolet transilluminator (Fig. 1A). These fragments were purified using Qiagen kit (QIAamp PCR Clean-up, Qiagen GmbH, Hilden, Germany) and quantified with MassRuler (Fermentas Life Sciences, Thermo Fisher Scientific, Waltham MA, USA) (Fig. 1B). Purified products were subjected to direct sequencing with 11 different primers[17] using ABI PRISM 3130 Genetic Analyser (PE Applied Biosystems, Thermo Fisher Scientific, Waltham MA, USA). Mutations were screened using freely available Chromas v2.4 and SeqScape v2.1.1 (Applied Biosystems) against the NCBI reference sequence NM_000500.7 and transcript ID ENST00000448314. Patients showing absence of PCR amplification indicated homozygous deletion in *CYP21A2* gene and were subjected to MLPA (Salsa MLPA Kit P050-B2, MRC-Holland, Amsterdam, The Netherlands) to detect the extent of deletion. DNAs showing non-amplification or homozygous deletion were also subjected to another round of PCR with random primers (non CAH primers) to rule out failure of PCR amplification.

Results

Consanguinity was present only in one family. Within these 15 families, 10 had previous live children diagnosed with 21OHD and a history of infantile deaths, and in the remaining five families, index case was not available for genetic evaluation, however, CAH was suspected based on suggestive history in previous children. All index children that comprised four males and six females were in the age range from 1 to 5 yr. Eight index children had SW and two had SV phenotype and all were born full-term following uncomplicated pregnancies. Clinical presentation

of previous affected child/children is summarized in the Table. The data have been compiled based on history and clinical records provided by the family and evaluation of the affected child.

All five families without index cases had a history of infantile deaths in the early days of life ranging from 20 days to three and half months. Two families had some records of the clinical symptoms indicating 21OHD, and in the remaining three families, there was a high index of suspicion based on the verbal information provided by the parents. Due to absence of diagnosis of the previous affected children and high index of suspicion, these couples were screened for *CYP21A2* mutations to identify CAH due to 21OHD in previous children. All were found to carry the *CYP21A2* mutations.

Seven different mutations were detected in the affected children/parents and foetal DNA extracted from 15 CVS and one AF sample. These were I2g (c.290-13A/C>G) mutations - a splice site mutation in intron 2; c.515T>A (p.I172N) in exon 4 and c.952C>T (p.Q318*), c.1066C>T (p.R356W), c.1061G>C (p.R354P) mutation, all in exon 8, E6 or cluster mutation comprising three mutations [c.707T>A (p.I236N); c.710T>A (p.V237E); c.716T>A (p.M239K)] in exon 6 and deletions ranging from exon 1 to 8. Mutation c.1061G>C (p.R354P) has not been reported and is novel (NCBI GenBank Accession no. KR261930).

Of the 15 foetuses analyzed, nine were unaffected and six were affected. Of the nine unaffected foetuses, six were carriers and three were normal carrying both wild-type alleles. Of the six affected foetuses, three were compound heterozygous with point mutation on one allele and deletion on other allele *i.e.*, c.290-13A/C>G/exon 1-3 deletion in F4; c.290-13A/C>G/exon 1-6 deletion in F9 and c.1061G>C (p.R354P)/exon 1-8 deletion in F1 (Table). Three other affected foetuses were homozygous (F3, F5 & F11). One homozygous foetus (F5) carried additional c.1066C>T (p.R356W) mutation in one of the alleles (Table).

Deletions and I2g mutation were predominant and were found collectively in 60 per cent (9 out of 15) cases, either on one or both copies of *CYP21A2* gene. In isolation, however, six (40%) foetuses had deletions and five (33%) had I2g mutation. These deletions either involved single exon *i.e.,* exon 3 or multiple exons ranging from 1 to 8 (Fig. 2). The third common mutation was c.952C>T p.Q318* found in 20 per cent of foetuses. These mutations could not be associated

Family ID	Gestational age (wk)	Previous history	Clinical features of previous affected child	Genotype of affected child	Genotype of mother and father	Genotype of foetus	Outcome
F1	11[+4]	2 male infantile deaths; 1 ter, 1 abortion, 1 aff, 2 normal	SC	p.R354P/del ex 1-8	N/del ex 1-8 N/p.R354P	p.R354P/del ex1-8 (aff)	Terminated
F2	19[+2]	1 infantile death-45 days, 1 aff child	SC, HP	I2g/p. Q318*dup/ex 1-8/del ex 1-3	N/I2g/p. Q318*/dup ex 1-8 N/del ex 1-3	N/del ex 1-3	Delivered, normal male child-FUD
F3	20	1 aff child	SC	I2g/I2g	N/I2g N/I2g	I2g/I2g (aff)	Delivered aff male child, FUD
F4	15	1 infantile death at four months, 1 aff child, SV	AG, no EI	I2g/del ex 1-3	N/I2g N/del ex 1-3	I2g/del ex 1-3 (aff)	Terminated
F5	15	1 infantile death, 1 aff child	SC	I2g/I2g+p. R356W	N/I2g N/I2g	I2g/I2g + p.R356W (aff)	Terminated
F6	19[+6]	1 neonatal death-20 days, no live child	EI	NA	N/p.Q318* N/p.Q318*	N/p.Q318*	Delivered, normal male child, FUD
F7	19	1 previous live child	AG, C, EI	p.Q318*/del ex 1-3	N/p.Q318* N/del ex 1-3	N/p.Q318*	Delivered, normal male child, FUD
F8	21	Previous 2 children died at 2.5 and 3.5 months, respectively, No live child	AG, C, V, FTT	NA	N/del ex 1-3 N/del ex 1-8	N/del ex 1-8	Delivered, normal female child, FUD
F9	19	2 infantile deaths-60 and 44 days, respectively	V, FTT, EI, HP, AG	NA	N/I2g N/del ex 1-6	I2g/del ex 1-6 (aff)	Terminated
F10	16	1 female child died at six months, 1 live aff child, SV	Elevated 17OHP, C, no EI	p.I172N/p. R356W	N/p.I172N N/p.R356W	N/N	Delivered, normal female child, FUD
F11	18	2 infantile deaths at 40 and 50 days, respectively	Elevated 17OHP, V, EI	del ex 3/del ex 3	N/del ex 3 N/del ex 3	del ex 3/del ex 3 (aff)	Terminated
F12	12	1st female died at 20 days, 2nd live, SW	HP, C, AG	I2g/p.Q318*	N/I2g N/p.Q318*	N/p.Q318*	Delivered, normal male child, FUD
F13	19	1st child normal, 2nd SW male died at two months	AG, FTT	NA	N/p.Q318* N/p.Q318*	N/N	Delivered, normal female child, FUD

Table. Clinical presentation and genotype description of each family and foetus

Contd...

Family ID	Gestational age (wk)	Previous history	Clinical features of previous affected child	Genotype of affected child	Genotype of mother and father	Genotype of foetus	Outcome
F14	16	1st child died, SW with EI, no live child	SW, EI	NA	N/del ex 1-3 N/I2g	N/I2g	Delivered, normal male child, FUD
F15	17	2 abortions, 1 live aff male child	Elevated 17OHP	E6/del ex 1-8	N/E6 N/del ex 1-8	N/N	Delivered, normal female child, FUD

FUD, follow up done; N, normal allele; del, deletion; AG, Ambiguous genitalia; ter, termination; FTT, failure to thrive; HP, hyperpigmentation; C, cliteromegaly; aff, affected; EI, electrolyte imbalance; V, vomiting; SC, salt crisis; ex, exon; 17OHP, 17 hydroxy progesterone; SW, salt wasting; NA, not available. Superscript numeral denotes additional days
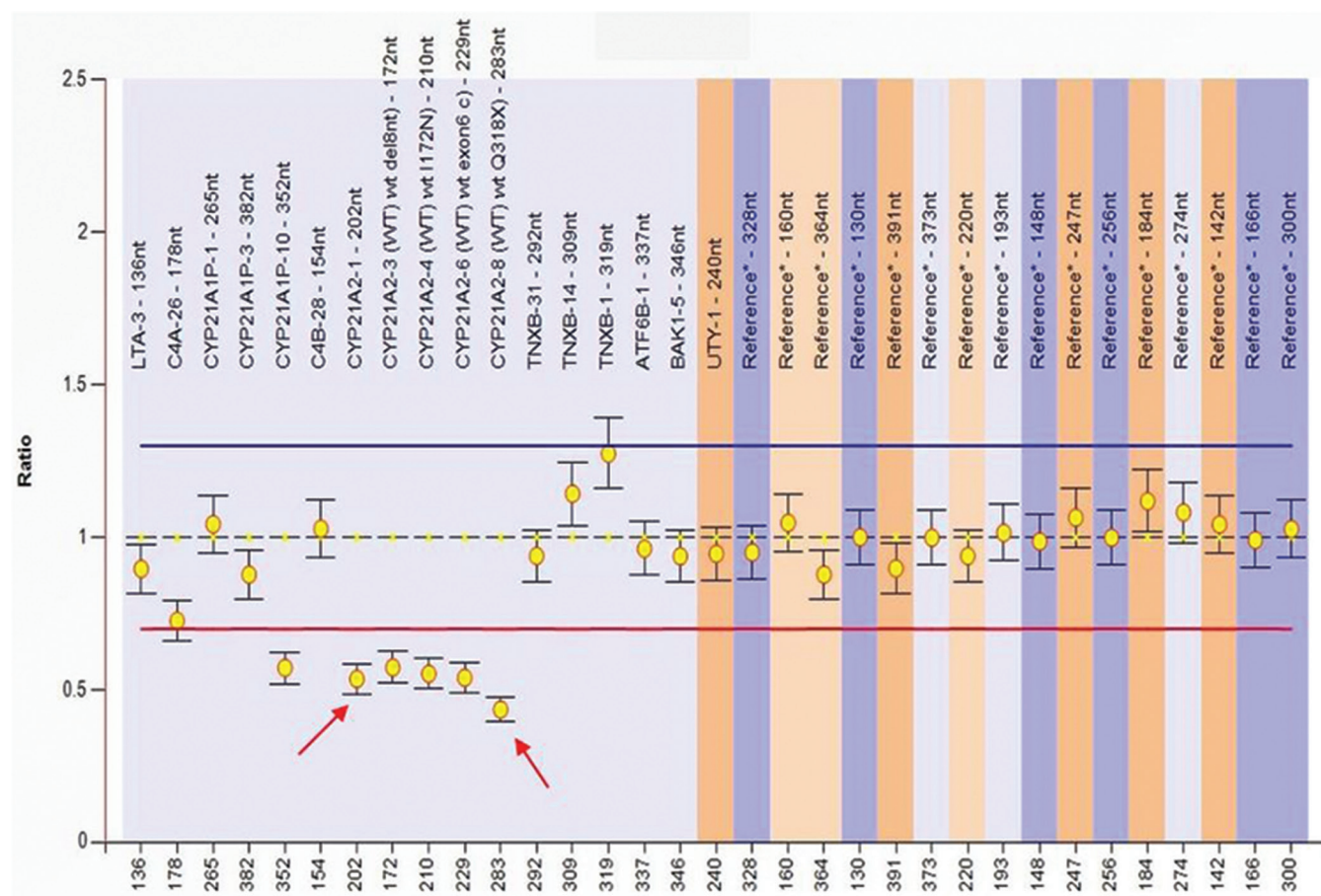

Fig. 2. Multiplex ligation probe amplification (MLPA) analysis showing heterozygous deletion of exons 1-8 of *CYP21A2* gene in foetal DNA in family F1. Deletions of exons 1 and 8 are indicated by red arrows. Normalized peak height ratio between 0.7 and 1.3 was considered as normal in patient DNA with respect to control DNA. The X-axis labels denote the length (bp) of each probe fragment generated during MPLA PCR.

with the ethnic origin of the families due to small sample size. There was uniform distribution of mutations among all the families.

These pregnancies were followed up for the outcome. Among the six affected families, five families decided to terminate the pregnancy and one

family (F3) decided to continue with the pregnancy after undergoing PND in view of religious grounds. A male child was born who later on manifested symptoms of 21OHD. His post-natal mutation analysis confirmed the affected status. All the nine unaffected foetuses have been delivered and
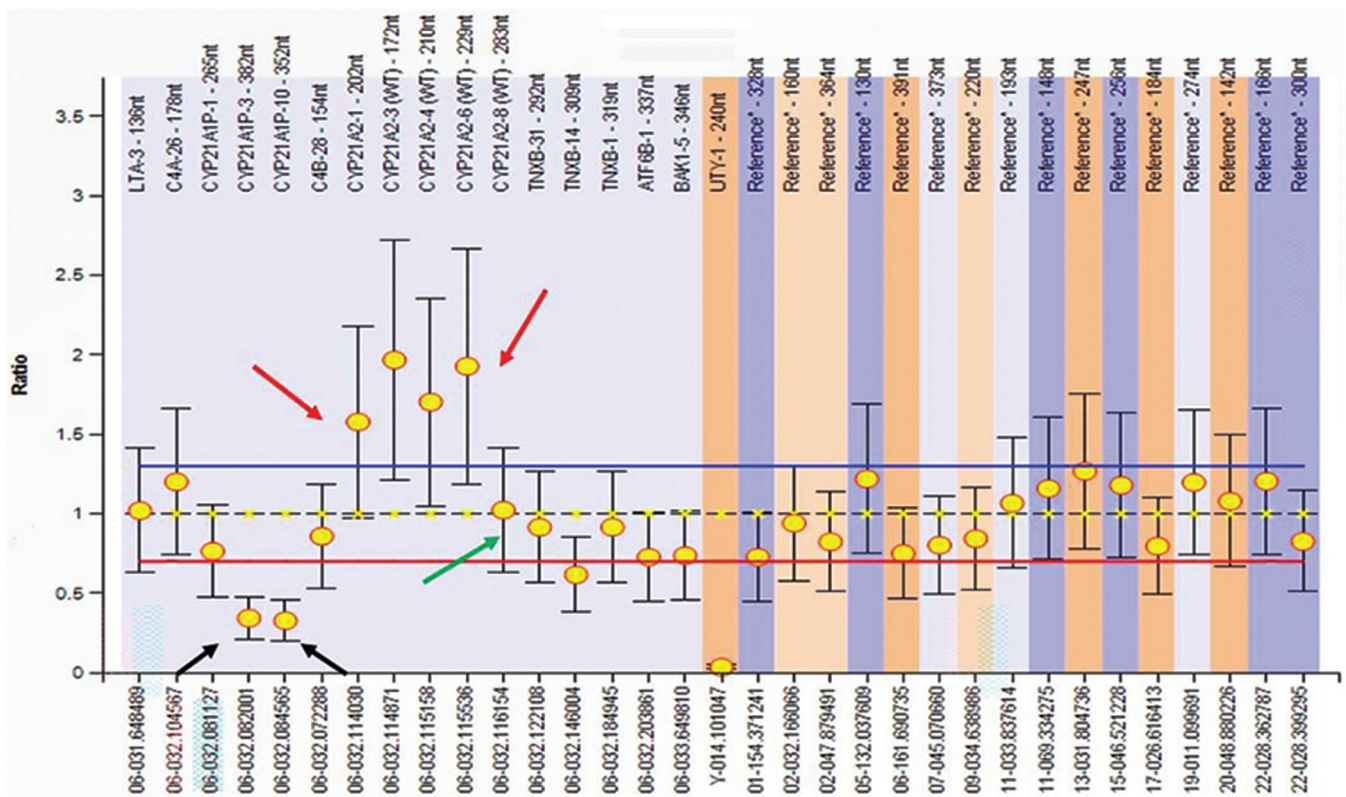
Fig. 3. Family F2 - mother showing duplication of exons 1-8 indicated by red arrows by MLPA analysis. Exon 8 illustrated by green arrow shows normal ratio as one of the *CYP21A2* copies has c.955C>T (p.Q318*) mutation and hence the normal probe could not bind to the third copy resulting in normal ratio of exon 8. Heterozygous deletion of exons 1 and 10 (indicated by black arrows) of pseudogene indicates conversion of its one of the copies to active gene. Normalized peak height ratio between 0.7 and 1.3 was considered as normal in patient DNA with respect to control DNA.

were found to be unaffected on follow up. Genetic analysis was performed in these unaffected infants to reconfirm the carrier status or absence of mutation in *CYP21A2* gene. However, in six families wherein the foetuses were found affected, termination was performed in their native places and thus sample could not be obtained for retesting.

In family F2, the index case was compound heterozygous for I2g and p.Q318* mutations. Mother had similar genotype but was asymptomatic. No mutation was detected in father by sequencing. Hence, MLPA in parents was done to look for deletions and duplications. Mother was found to carry a duplication of *CYP21A2* gene (Fig. 3) and father was carrier of deletion of exon 1-3 that was not picked up by sequencing. We assumed mother to carry these mutations on the chromosome with duplicated allele and a single normal allele on the other chromosome but genotyping could not be performed on her parents to confirm the segregation of alleles. However this assumption was proved right after the index case and the foetus were analysed using both MLPA and sequencing. The index case had inherited

the maternal chromosome with duplicated gene and the paternal chromosome with deletion (Fig. 4) and hence was compound heterozygous for I2g and p.Q318* mutations. Foetus had inherited normal chromosome from the mother and deleted chromosome from the father and hence was carrier *i.e.*, unaffected.

In family F5, the index case was homozygous for IVS2-13A/C>G mutation and also carried c.1066C>T (p.R356W) mutation in heterozygous state. Parents were carriers of IVS2-13A/C>G mutation but did not carry c.1066C>T (p.R356W) mutation. Foetus had the similar genotype as the index child.

Discussion

Extensive study of *CYP21A2* locus and its close association with phenotype made it possible to predict the clinical outcome in the affected child and hence facilitated early and accurate genotyping of the foetal DNA in the first trimester. Various direct methods such as amplified created restriction site or allele-specific oligohybridization, Southern blot, allele-specific amplification and sequencing have been used for foetal
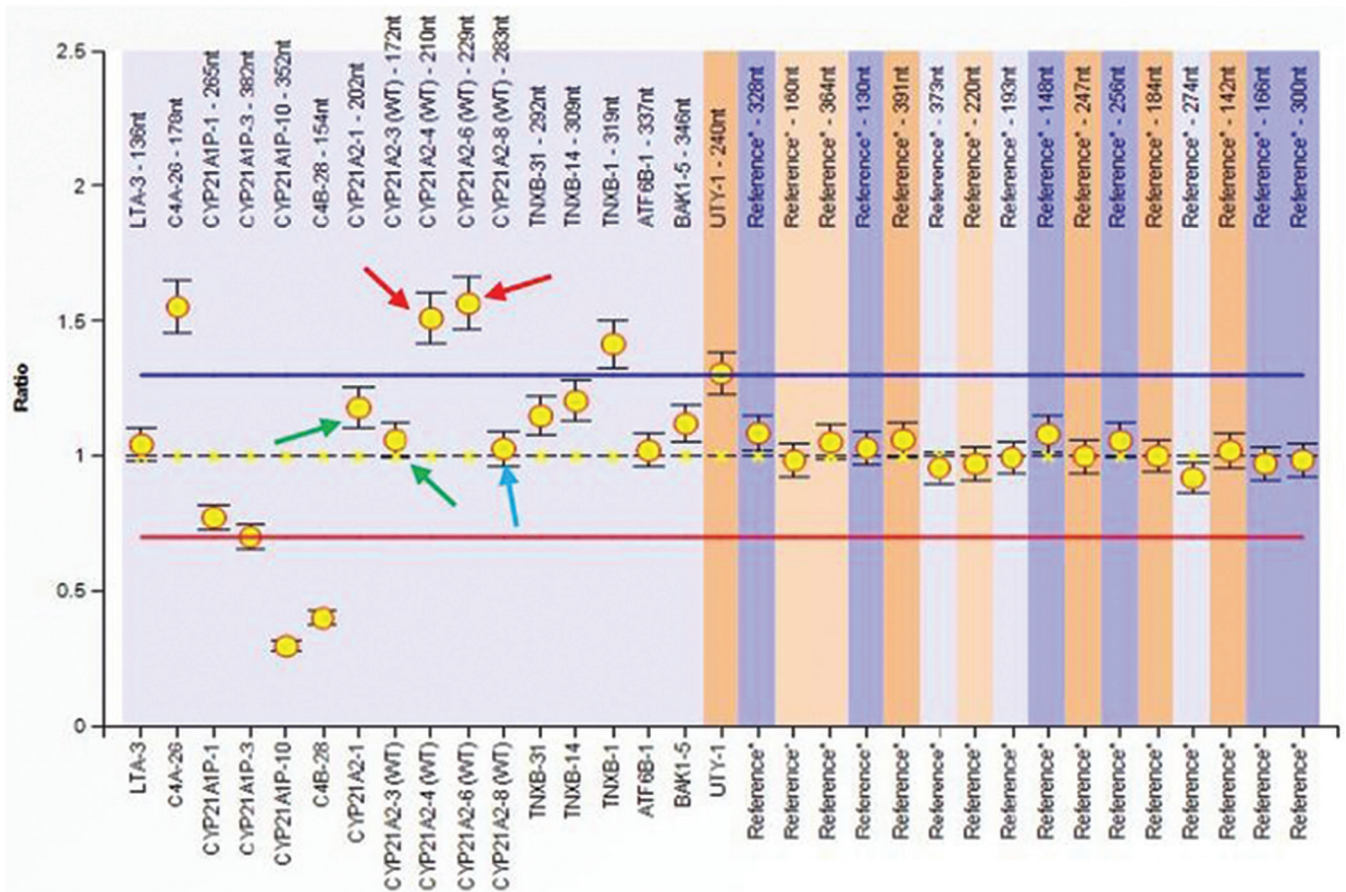
Fig. 4. Family F2 - index child inherits a chromosome harbouring duplication of exons 1-8 from the mother and a chromosome harbouring deletion of exons 1-3 (indicated by green arrows) from the father. MLPA shows duplication of exons 4 and 6 (shown by red arrows) but not exon 8 (shown by blue arrow) as one of the duplicated copies has c.955C>T (p.Q318*) mutation in exon 8 and therefore, the probe did not bind to the third copy and hence normal (two copies) ratio is seen for exon 8. Similarly, exons 1 and 3 also show normal ratios as the third copy inherited from the father has deletion of exons 1 and 3.

evaluation. However, single direct method may not provide complete information due to gene conversions and rearrangements frequently observed in *CYP21A2* gene[18-20].

The most common mutation observed in this study was deletion ranging from single to multiple exons. About 40 per cent of the foetuses carried deletions either on one or both the chromosomes. High frequency of deletions has also been reported in other populations[21,22] but not in Indian patients[23-25]. The reason for detection of high frequency of deletions could be MLPA, a highly sensitive method used in this study. The second most common mutation was I2g or IVS2-13A/C>G observed in 33 per cent of the foetuses either in homozygous or heterozygous state. One of the PND studies carried out on 24 pregnancies reported I2g mutation in 52 per cent and deletions in 22 per cent of the alleles[26]. Overall, I2g is the single most prevalent

mutation reported from other ethnic populations as well as from Indian studies[24,27,28].

MLPA was useful for accurate genotyping of index case in family F2. It has been previously reported that individuals with c.955C>T (p.Q318*) mutation frequently have duplication of *CYP21A2* gene[29], and simply sequencing such cases can lead to erroneous results[30]. An individual with c.955C>T (p.Q318*) mutation can be a normal individual and not a carrier if he/she carries this mutation on the duplicated allele. Similarly, an individual homozygous for this mutation can be a carrier and not affected if he/she has duplication on one of the chromosomes. Hence, MLPA should be performed to rule out duplications as well as the carrier status in individuals harbouring this particular mutation. Considering a high rate of polymorphism and gene conversion events in *CYP21A2* gene, a comprehensive knowledge of *CYP21A2* genetics is

required while considering MLPA for the deletion and duplication in *CYP21A2* gene[31].

In family F5, the foetus had IVS2-13A/C>G mutation in homozygous state and also carried c.1066C>T (p.R356W) mutation in heterozygous state similar to the index case. As both parents did not carry c.1066C>T (p.R356W) mutation, its presence in proband and again in the foetus could be attributed to the gonadal mosaicism/germline mutation in one of the parents. This was not a *de novo* mutation since previous child also had the same genotype. This emphasizes the need to study the index cases prior to offering PND. In families, where index case is not available, the entire *CYP21A2* gene in foetal DNA should be screened by sequencing.

In Western countries, PND of CAH is done mainly to prevent virilization of classically affected female foetuses by prenatally treating them with dexamethasone. Since dexamethasone must be administered before or at nine weeks of gestation, presently the focus is on the development of non-invasive PND for early detection of mutations[32,33].

In conclusion, the strategy used on small sample size in this study seemed reliable for the evaluation of foetal status. However, direct mutation detection should be complemented with linkage for comprehensive evaluation, particularly in cases where parents are carriers of large gene deletions/duplications and gene conversions.

Conflicts of Interest: None.

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