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Molecular Identification of Intron 2 Splice Mutation and 8bp Deletion in CYP21 Gene for Congenital Adrenal Hyperplasia (CAH) Patients in Kashmir (North India)

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Abstract

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder caused by alteration in CYP21 gene which ultimately leads to 21-hydroxylase deficiency. The present study aimed at evaluation of 2 common mutations viz, Intron 2 Splice (INT2S) mutation and 8 bp deletions in exon 3 of CYP21 gene and to establish their frequencies in Kashmir population (North India). The mutations were tested by Amplification Refractory Mutation System-PCR (ARMS-PCR) in 50 cases of CAH, proven by clinical features and raised 17-hydroxy progesterone (17OHP) levels.

The results revealed that 15(30%) cases had INT2S mutation while as 8 bp deletion was not detected in any patient. In INT2S mutation, 7 cases were homozygous with I2-G genotype and 8 cases were heterozygous. The frequency of AG heterozygotes was found in 5 cases and CG heterozygote genotype was found in 3 cases. CAH patients with ambiguous genitalia were seen to harbor most of the INT2S mutations with I2-G in 3 cases and CG heterozygotes in 2 cases. In non-consanguineous group of patients, 4 homozygous I2-G mutations and 4 were I2-GC heterozygotes were detected in comparison to 3 and 1 in consanguineous patients respectively. Our study confirms that INT2S mutations but not 8 bp deletions exist in CYP21 gene in CAH patients in Kashmir population.

Keywords: Congenital adrenal hyperplasias; Mutation; Intron 2 splice mutation; Heterozygote

Introduction

Congenital adrenal hyperplasia (CAH) is also referred to Adrenogenital syndrome. CAH is a group of autosomal recessive disorders resulting from the deficiency of one of the five enzymes required for the synthesis of cortisol in the adrenal cortex. The most frequent is steroid 21-hydroxylase deficiency, accounting for more than 90 percent of cases [1-3]. Decreased 21-OH activity results in impaired aldosterone and cortisol biosynthesis, leading to increased androgen production and renal salt loss. Three different clinical phenotypes have been described which relate to the degree of compromised 21-OH activity: the salt wasting (SW), the simple virilizing (SV) and the non-classical (NC) form. Severe phenotypes (SW and SV), associated with different degrees of virilization in affected fetuses, are classified as classic forms and occur in about 1:14,000 live births, while the NC variant occurs in about 1:100 births [4].

Diagnosis of CAH due to 21-hydroxylase deficiency is usually established by serum measurements of 17-OH progesterone (17-OHP), the metabolite immediately preceding the 21-hydroxylation step in steroidogenesis. Neonatally, the diagnosis can be made by measuring 17-OHP in dried blood spots on filter paper [3,5].

The gene encoding the 21-OH enzyme, CYP21, and the highly homologous pseudogene CYP21P are tandemly arranged with the complement C4A and C4B genes in the major histocompatibility complex class III region on chromosome 6p21.3 [6]. CAH due to 21-OH deficiency has been related with deletions of the gene [7] and gene conversions where point mutations which are normally present in the inactive CYP21P are transferred to the CYP21 gene. CYP21P gene is 98 % homologous to CYP21 gene, but it contains a number of deleterious sequences which result in a prematurely truncated protein. Thus, CYP21P is an inactive pseudogene, whereas CYP21 encodes the active 21-OHase [3]. There was observed variability in number of C4-CYP21P and C4-CYP21 repeat units [3]. If no CYP21 gene is present in the individual, it will result in salt-wasting form of CAH. Majority of CAH causing mutations are sequences that are normally present in the pseudogene. These mutations can be transformed from CYP21P to CYP21 gene by the process of gene conversion and thus inactivate the gene [8]. Mutations derived from the pseudogene can be divided into several groups: 1. frame shifting deletion/insertion mutations; 2. splice site mutation; 3. nonsense mutations; 4. Missense mutations. These mutations account for approximately 95 % of all mutated CYP21 alleles. The remaining 5% of mutations are population specific and are not derived from the pseudogene [3].

One of the mutations transferred from the pseudogene is 8 bp deletions in exon 3. Deletion of 8bp shifts the reading frame, thus producing inactive enzyme [9]. This mutation is associated with SW phenotype [10]. Very frequent mutation is the splice mutation in intron 2, in which an A or C at 659 nucleotide is replaced by G. This mutation alters the pre-mRNA splicing by activating another acceptor site in the splicing process and thus shifting the reading frame, creating a premature termination codon in exon 3 [11]. Splicing mutation is associated with SW and less severe SV phenotypes [12].

A number of CAH cases are being treated at our tertiary care hospital but the data regarding the molecular evaluation of CYP21 gene in Kashmiri patients is not available in the literature. No study has been carried out so far in this part of the world regarding the mutational analysis of CYP21 gene. Keeping in view the ethnic nature of Kashmiri population (North India) and prevalence of CAH here, this study was carried out to identify the spectrum of mutations in CYP21A2 gene, and to correlate genotype with the phenotype in a randomly selected cohort of CAH patients.

Methodology

Fifty highly suspected cases of CAH patients affected from related/unrelated families were referred to department of Advanced Centre for Human Genetics (ACHG), Sheri-Kashmir Institute of Medical Sciences (SKIMS) Srinagar (J&K, India). All the details, including clinical and family history, were collected through a detailed questionnaire. The patients particularly belonged to ethnic Kashmiri from at least four generations and represented all major ethnic groups. Patients considered were highly suspected for CAH on particular diagnostic criteria with clinical findings like Ambiguous genitalia, Clitoromegaly or Hirsutism and biochemical evaluation (elevated 17-OHP in blood).

DNA extraction

5 ml blood from each patient was collected in EDTA vials. Genomic DNA was isolated using standard proteinase-K digestion, phenol/chloroform extraction, and ethanol precipitation method from whole-blood samples.

PCR for detecting 8 bp deletion in exon 3 of CYP21

The 8 bp deletion in exon 3 was used to distinguish between CYP21 and CYP21P. Analysis of CYP21 gene, without contamination by CYP21P pseudogene sequences, is performed by using PCR primers discriminating between these two genes. For this purpose 8bp deletion in exon 3 of CYP21P can be used and was done using two round of PCR.

Primers CYP1 and CYP48 were used for the amplification of the first part of the gene (fragment 1/48) and CYP12 and CYP55 for the second part of the gene (fragment 12/55). 0.5 ul from both the fragments generated were used to detect the 8 bp deletion and PCR was carried out in a final volume of 25 mL containing 50 ng genomic DNA template, 1x PCR buffer (Biotools, B & M Labs, Madrid, Spain) with 2 mmol/L $MgCl_2$, 0.4 mmol/L of each primer (Genscript, Piscataway, NJ), 50 mmol/L dNTPs (Biotools, B & M Labs), and 0.5 U Taq polymerase (Biotools, B &MLabs). The primers used were for producing a control band of 900 bp were CYP5 as sense (located in exon 2) and CYP16 as antisense (located in exon 5). CYP55 sense primer corresponds to the region in exon 3, which in pseudogene, contains the 8 bp deletion [3].

PCR with CYP55 and CYP16 amplifies 507 bp long fragment only in the absence of the deletion. If the deletion is present in exon 3, the 507 bp fragment is not amplified because of no annealing of primer CYP55. Thus PCR amplification in patients without deletion is produces two bands on agarose gel (900 bp and 507bp), while patients with the deletion have only one band 900 bp (control fragment). For PCR amplification, the standard protocol was used as follows: one initial denaturation step at 94°C for 7 minutes, followed by 35 denaturation cycles of 30 seconds at 94°C, 30 seconds of annealing at 54°C, and 30 seconds of extension at 72°C, followed by a final elongation cycle at 72°C for 5 minutes.

Allele specific PCR for Intron 2 splice mutation

Intron 2 splice genotyping was performed on the CYP21 specific amplification products by an ARMS based approach. Like other point mutations in CYP21, INT2S is detected by PCR amplified in two parts, from the beginning of the gene to exon 3 and from exon 3 to the end of the gene. Primers CYP1 and CYP48 were used for the amplification of the first part of the gene (fragment 1/48) and CYP12 and CYP55 for the second part of the gene (fragment 12/55). For the amplification of fragments specific for intron 2 splice mutation 0.5 ml of 1/48 fragment was used in the reaction with specific primers [3] for the identification of either two wild type genotypes or mutant G genotype.

PCR was carried out in a final volume of 25 mL containing 50 ng genomic DNA template, 1x PCR buffer (Biotools, B & M Labs, Madrid, Spain) with 2 mmol/L $MgCl_2$, 0.4 mmol/L of each primer (Genscript, Piscataway, NJ), 50 mmol/L dNTPs (Biotools, B & M Labs), and 0.5 U Taq polymerase (Biotools, B &MLabs). Samples were subjected to denaturation at 94°C for 1 min followed by 35 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 2 min (for amplification of 55/12, 1/48 and deletion specific fragments) or 35 cycles of 94°C for 30 sec, 64°C for 30 sec, 72°C for 2 min (for amplification of intron 2 splice mutation specific fragments) or 30 cycles of 94°C for 30 sec, 54°C for 30 sec, 72°C for 2 min ending by extension at 72°C for 8 min. The reactions were then directly electrophoresed on 1 % agarose gel in the presence of ethidium bromide stain. For Intron 2 splice mutation three lines on agarose are necessary to detect mutation each displaying a band of 85 bp, one line each for the wild-type as it contains polymorphic variant either genotype A or C while as third line detects the mutant G genotype. The control band produced for the identification of Int2Splice mutation was 325 bp.

Results

50 highly suspected cases of CAH patients belonging to related/unrelated families between 01 months and 30 years were included where 64% belonged to age group of ≤15 years and 36% were >15 years. The most frequent clinical problem in patients with suspected CAH were Ambiguous genitalia in 20 (40.0%), followed by Hirsutism in 12 (24%), Precocious puberty in 8 cases (16%), Clitoromegaly in 5(10%) and Oligomennorhoea in 5 cases (10%) (Table 1). Few cases presented with two or more overlapped clinical conditions. We found 20 of 50 cases (40%) of patients were as a result of consanguine marriage and the degree of relationship between parents of these CAH patients was mostly second and third degree. 17-OH progesterone was found to be raised in every case.

Two prominent mutations, Intron 2 splice mutation (INT2S) and 8 bp deletion were evaluated in 21-hydroxylase gene (CYP21A2 gene) primarily in each case of CAH having 21-hydroxylase deficiency. 15 (30%) cases had INT2S mutation while as 8 bp deletion was not detected in any patient. In INT2S mutation, out of 15 mutations 07 cases were homozygous with I2G genotype (46.7%) and 08 cases were heterozygous (53.4%).

Wild type genotype of INT2S exist in two polymorphic genotypes which is either A or C. The frequency of AG heterozygotes was found to be 33.4% (5 of 15) and CG heterozygote genotype was found to be 20% (3 of 15). The frequency of wild type AA and CC genotype were found to be 48% (24 of 50) and 12% (6 of 50) respectively while as

heterozygous condition of INT2S in wild AC was observed in 5 cases (10%) (Table 2).

Variable	Cases (n=50)	Percentage (%)
Age group ≤15 >15	32 18	64.0 36.0
Gender Female Male	30 20	60.0 40.0
Dwelling Rural Urban	28 22	56.0 44.0
Consanguinity Yes No	20 30	40.0 60.0
17-OH Progesterone Raised Normal	47 03	94.0 06.0
Virilization Present Absent	- -	- -

Table 1: Demographic detailing of study subjects suspected for CAH.

Type of mutation	No. of patients	Frequency %	Wild type AA	Wild type CC	Het AC	Hetero-zygous AG CG	Homo-zygous GG
I2splice mutation	50	30(15)	24	6	5	5 3	7
8bp deletion	50	(0)0	0	0	0	0	0

Table 2: Frequency of mutations CYP2A1 studied in CAH.

While further stratification into the various parameters studied, we found I2-G homozygous mutation was found only in females (07) (Table 2). In patients presenting with different clinical conditions, Ambiguous genitalia was observed to harbor most of the INT2S mutations with I2-G in 3 cases (20%) and CG heterozygotes in 2 cases(13.4%) (Tables 3 and 4). This was followed by Hirsutism with 1 homozygous I2-G mutations (%) and two heterozygous mutations in I2-AG (6.7%). Precocious puberty was observed to contain I2-G homozygous mutation in 02 cases (13.4%) and one heterozygous mutation I2-CG (6.7%) (Table 2).

In non-consanguineous group of patients, 4 (26.7%) homozygous I2-G mutations and 4 (26.7%) I2-GC heterozygotes were detected in comparison to 3 (20%) and 1 (6.7%) in consanguineous patients respectively. Among 47 patients whose 17-OH progesterone was highly raised had all the 7 homozygous I2-G mutations. Further, 3 (20%) I2-CG and 5 (20%) I2-AG heterozygotes were found among this group but no mutation was detected in those CAH patients whose 17-OH progesterone was almost normal after few repeats. The clinical conditions and the spectrum of the INT2S mutation for all those CAH patients who tested positive for it is given in Table 4.

Total mutations	15	Frequency %	Wild type AA%	Wild type CC %	Het AC%	Heterozygous AG% CG %	Homozygous GG%
I2splice mutation	15	100%	48	12	10	10 6	14
8bp deletion	0	0%	0	0	0	0	0

Table 3: Distribution of mutations in CYP2A1 studied in CAH.

Variable	Cases n=50	Mutation type Intron 2 splice mutation						Mutation 8bp deletion
		Wild AA	Wild CC	Het AC	Het AG	Het CG	Hom GG	
Age group ≤15 >15	32 18	16 8	3 3	3 2	2 3	201 1	6 1	0
Gender Female Male	30 20	12 12	2 4	2 3	5 0	2 1	7 0	0
Consanguinity Yes No	30 20	16 8	3 3	3 2	4 1	0 3	4 3	0
17-OH Progesterone Raised Normal	47 2	22 1	6 0	4 1	5 0	3 0	7 0	0

Table 4: Distribution of Intron 2 splice mutation and 8bp deletion in various clinic-pathological characteristics.

Discussion

Genetic impairment of the CYP21A2 gene leads to steroid 21-hydroxylase deficiency and is a major cause of CAH. Majority of mutations causing CAH are sequences that are normally present in the pseudogene (CYP21P). These mutations can be transformed from CYP21P to CYP21 gene by the process of gene conversion and thus inactivate the gene [8]. Mutations derived from the pseudogene frame shifting deletion/insertion mutations; splice site mutation; missense/nonsense mutations. These mutations account for approximately 95% of all mutated CYP21 alleles [3]. 8 bp deletion in exon 3 is a mutation transferred from the pseudogene which shifts the reading frame, thus producing inactive enzyme [9]. This mutation is associated with SW phenotype [10]. The splice mutation in intron 2 is a frequent mutation, in which an A or C at 659 nucleotide is replaced by G and occurs in around 30% CAH patients. This mutation alters the pre-mRNA splicing by activating another acceptor site in the splicing process and thus shifting the reading frame, creating a premature termination codon in exon 3 [11]. Splicing mutation is associated with SW and less severe SV phenotypes [12].

Detection of CYP21A2 gene mutations is now vital to early diagnosis, management, treatment of CAH pathogenesis. A lot of variations in CYP21A2 gene mutations are reported in CAH in several ethnic groups and geographic areas. Population under study (Kashmir) remains conserved through generations as we do not prefer to marry outside our ethnic group and majority of the population are consanguine marriage. Identification of mutations in CYP21 gene in Kashmiri populations, however, remains poorly defined as there has been no study till this date. Keeping this in view, we conducted a preliminary CYP21 gene analysis in a group of patients suspected with CAH for two common mutations splice mutation in intron 2 is a frequent mutation, in which an A or C at 659 and 8bp deletion in exon 3 by ARMS PCR that can discriminate between mutation and wild type sequences, whereas pseudo gene was excluded by selective amplification [13].

In our study ARMS PCR was applied to detect INT2S mutation. We observed INT2S mutation present with a frequency of 30% (15 of 50) including both mutant homozygotes as well as heterozygotes. The pattern of frequency is consistent the report on INT2S mutation from Turkish population [14]. The frequency of INT2S mutation in Turkish population is in complete agreement with population under study (22% Turkey versus 22% population under study) but in slight agreement with few other population (28% Iranian, 27% Pakistani versus 22% ours population [15,16]. Intron 2 Splice mutation is the most frequent mutation of CYP21 in SW patients, the frequency of which in the studies including the patients with all types of CAH phenotypes ranges between 19 to 42% [17-19] and our results which showed a frequency of around 22% in INT2S mutation falls in the list of the studies conducted. The frequency of the allele with 8 bp deletion studied in our study varies between 1 and 10 % in classic patients [20,21]. The frequency of this mutation in our group including all classic patients was ironically 0% in our study of CAH which is contrasting to previous study also which to some extent resemble us like Iranian population [15].

The frequency of wild type AA and CC genotype were found in 24 and 6 cases respectively while as heterozygous condition of INT2S in wild AC was observed in 5 cases. The A or C to G substitution in intron 2 is also the most frequently found among Spanish patients, as well as described for other populations [22-24].

We found patients with Ambiguous genitalia to harbor most of the INT2S mutations with I2-G in 2 cases and CG heterozygotes in 02 cases. We found that newborn with Ambiguous Genitalia exist in our region in a good number and unfolding the sex help in proper assignment of gender in intersex disorders. It is commonly observed that Children with CAH are usually brought to medical attention late in life [25,26] as observed in our report where 36% of the suspected CAH cases were attended at >15 years o age at the time of diagnosis which was comparatively high as reported from other regions [23,27]. Establishing the cause of Ambiguous genitalia where CAH is a prime reason especially when CYP2A1 gene is altered will certainly help in proper management and treatment of the newborn.

In this study, the mutations were evaluated for inter related patients where in non-consanguineous group of patients, 04homozygous I2-G mutations and 04 heterozygotes were I2-GC detected in comparison to 03 and 01 in consanguineous patients respectively. The nature of the mutation determined as either homozygous or heterozygous assist in identifying the consanguine couple to decide for prenatal genetic testing for the fetus. Thus with genetic testing and availability of genotypes, counseling for CAH patients becomes easier and better in translating message and intervention modalities to help consanguineous couples for making informed and intelligent reproductive decisions.

Almost all the CAH cases had markedly elevated 17OHP levels. All the homozygous G -INT2S mutations were detected in cases with raised 17-OH P levels. Further CAH patients identified with homozygous G-INT2S were all salt wasters (SW) and these include some heterozygous patients (I2-G/A or G/C) whose possible cause for this condition would have been compound heterozygosity in other mutations which could not be ascertained in this report. Keeping in view the 40% consanguine couples, patients detected as simple virilizing nature in this report could be due to compound heterozygosity.

Conclusion

Our study showed INT2S mutations but not 8 bp deletions exist in CYP21 gene in CAH in our population. The present study depicted a good relation between genotype and phenotype specifically when INT2S mutation is evaluated in CAH cases.

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