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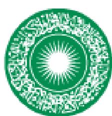

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Characterization of Pathogenic Mutations in 21-Hydroxylase Gene of Pakistani Patients with Congenital Adrenal Hyperplasia and their Family Members - a Preliminary Report

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Abstract

Objective: To characterize specific mutations within the 21-hydroxylase gene (CYP21-B) using ARMS-PCR assay in patients with congenital adrenal hyperplasia (CAH) and to compare it with that reported in other populations.
Subjects and Methods: Five families, having an index case with CAH diagnosed on the basis of clinical and biochemical findings volunteered to give blood samples for analysis. A strategy, based on ARMS-PCR (Amplified Refractory Mutation System) was employed for the detection of mutations in 21 -hydroxylase gene. The products of ARMS-PCR were resolved on agarose gels and the PCR products were visualized over ultra violet illumination.
Results: Twenty-six specimens were analyzed for common point mutations in the 21-hydroxlase genes at the nucleotide positions 659, 1004 and 1688. Seven samples belonged to index cases with CAH. Of these 7, the assigned sex was male in 5 and female in 2 cases. However, genotypic sex was 3 males and 4 females. The mean age was 8 months in 5 cases while the median 17-OH Progesterone levels was 273.2 ng/ml. Vomiting, precocious puberty and ambiguous genitalia were the presenting features in 2, 1 and 4 cases respectively. Analysis for mutation at 659, 100 and 1688 was performed on 7 index cases and the family members of 5 index cases. The mutation analysis for the family members of index case 6 and 7 was not performed due to non-availability of their blood specimens. Index case No. 1, 4 and 7 showed homozygosity for splice mutations at nucleotide position 659, intron 2 with a sequence change of A to G, while the index case No. 2 and 6 showed heterozygosity for the same mutation. No mutation was found at 659, 1004 or 1688 in index case No. 3 and 4 at the analyzed nucleotide position. Nineteen family members of Case Nos. 1-5 were also analyzed for the same mutations. (Family No. 1, 2, 3, 4 and 5 included 3, 2, 7, 4 and 5 members respectively). These included 8 males and 11 females. All were asymptomatic. Both the parents of index case I and 4 were heterozygous at 659 while the father of index case No. 2 was heterozygous at 659 and mother was normal.
Conclusion: Our results demonstrated the A to G transition at nucleotide 659 causing aberrant splicing, reported for some other populations as the most commonly identified point mutations. All cases were appropriately assigned to paternal or maternal chromosomes (JPMA 52:287;2002).

Abbreviations: CAH = Congenital adrenal hyperplasia; 17-OHP = 1 7-Hydroxy **Progesterone;** SW = Salt
Wasting; SV = Simple Virilizing; NC = Non-Classical; CYP21 B or CYP21A2 = **21-hydroxylase gene;** CYP21 P or
CYP21P = 21-hydroxylase pseudogene; ARMS = Amplified Refractory Mutation System.

Introduction

The newborn with abnormal development of genitalia presents a difficult diagnostic and treatment challenge. It is important that a defmitive diagnosis be determined as quickly as possible so that an appropriate treatment plan can be established to minimize medical, psychological and social complications. Ever since corticosteroids substitution therapy became available and it's beneficial effects on Congenital Adrenal Hyperplasia (CAH) were discovered, the outcome of CAH has been a target ot mterest. Keports on long-term prognosis have mostly been focused upon growth, fertility and also during recent years on bone mineral density[1-3].

Steroid 21-hydroxylase deficiency is the major cause of CAH, which exists in three forms: Salt wasting, Simple Virilizing and nonclassical, which reflect different degrees of clinical severity of the disease[4,5]. All three forms of CAH are inherited as recessive disorders and the gene defectscausing the enzyme deficiencies are all believed to be found in the 21-hydroxylase gene known as CYP21, located on the short arm of chromosome Two highly homologous and closely linked steroid 21-hydroxylase genes are found in humans; the CYP21 gene (CYP21B or CYP21A2) is active, while the CYP21P (CYP21A) gene is a pseudogene and is inactive due to small insertions, deletions and point mutations in the gene[4-7]. CYP21 gene deletion and gene conversion to the inactive CYP2IP form are common in CAH subjects, but less than 35% of all CYP21 gene mutations fall into these categories. Likewise amino acid substitutions have been described in CYP21; however these mutations account for only a relatively small percentage of CAH cases[8-10].

In general, a close correlation exists between genotype and phenotype, with certain mutations associated closely with salt wasting, others with simple virilizing and others with non-classical disease[8]. However, phenotypes have been reported that were more or less severely affected than would have been predicted by genotype. The most common point mutation in CYP21B is the intron 2 splice mutation at nucleotide Variable clinical manifestations have been reported in patients who have this mutation. Leakiness of splice mutations has been postulated as one source of the genetic variability. A small amount of mRNA is spliced normally, resulting in the synthesis of a small amount of functioning enzyme and sufficient enzyme activity to ameliorate the severity of the phenotype[4,5].

So far no data exists in our population about the incidence of this disease though a number of cases have been presented at the cytogenetics division of the Department of Pathology with ambiguous genitalia and with clinical and biochemical fmdings of CAH. This study is an attempt to find mutations in the CYP21 gene of patients with CAH and to characterize the specific mutations in our population. The initial database produced from this study will be utilized for prospective studies in future.

Subjects and Methods

Subjects

Five families, each having an index case with CAH diagnosed on clinical and biochemical grounds volunteered to give blood samples for analysis. Blood samples were also collected from two other index cases, but the samples from their family members were not collected. The index case No.1 was phenotypically and genotypically a male child, who presented at 5 months of age with severe vomiting, diarrhea and failure to thrive and very high 17-OHP levels (273.2 ng/ml). Index case No.2 presented with genital ambiguity including clitoromegaly and persistence of urogenital sinus at 40 days of life. The child was assigned a male sex however, karyotyping showed 46XX chromosomes and uterus and ovaries were seen on ultrasound examination. The 17-OHP level was >400 ng/ml. Index case No.3 and 6 presented with features of virilization. Index cases No. 5 and 7 also presented with ambiguous genitalia with kaiyotyping assigned appropriately to sex.

Methods

A strategy, based on the use of ARMS-PCR was employed for detection of mutation as previously described by Wedell and Luthman[9]. A preamplification step was performed to amplify specifically the 21B sequence in preference to the 21A. A second round of ARMS was then performed to identify mutations in using the amplified 21B sequence as a template.

Protocol for detection of mutation

The 21B gene was amplified in two fragments using standard PCR reactions. Fragment A was used for analyzing point mutation at position 659 whereas fragment B was tested for mutations at 1004 and 1688 bp. Briefly, 500 ng of DNA was amplified in the presence of primers P1 and P48 (100 ng each) for fragment A and primers P4 and P55 (100 ng each for fragment B in 1 X PCR buffer, 0.2 mM deoxyribonucleotides, 1.5 mM MgCi2, 1.25 U Taq polymerase (Advanced Biotechnologies, USA) in a fmal volume of 25 1. The amplication was performed in a thermal cycler model 9700 (Perkin Elmer, USA) using the following cycling conditions; 93°C for 1 min, 54°C for and at 72°C for 1 min for a total of 20 cycles. The primer sequences of P1, P48, P4 and P55 were described previously by Wedell and Luthman[9]. The products of this reaction were diluted to seed the second round of mutation specific PCR using primer sequences described previously[9].The products of the second round of amplification were resolved on agarose gels (3% for the 659 mutation and 1% for the others) and bands were visualized using ethidium bromide.

Results

Twenty-six specimens were analyzed for common point mutations in the 2l-hydroxlase genes at nucleotide positions 659, 1004 and 1688 (Table 1).

Table 1. Common Point Mutations in the 21-Hydroxylase Gene.

Nucleotide position	Position Gene	Sequence Change	Protein Change	Clinical phenotype
659	Intron 2	A or C → G	Splice mutation	Salt wasting
1004	Exon 4	ATC → AAC	Ile 173 → Asn	Simple virilizing
1688	Exon 7	GTG → TTG	Val 282 → Leu	Non-classical
2113	Exon 8	CGC → TGG	Arg 357 → Trp	Salt wasting

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Of the total, 7 samples belonged to index cases with congenital adrenal hyperplasia. Of these 7, the assigned sex was male in 5 cases and female in 2 cases. However, genotypic sex was 3 males and 4 females. The mean age was 8 months in 5 cases and the median 17-OHP level found in these cases was 273.2 ng/ml. Two cases presented with vomiting, 1 with precocious puberty and 4 with ambiguous genitalia.

Mutations in CAH subjects

Analysis for 659, 1004 and 1688 was performed on 7 index cases (Table 2).

Table 2. Study Subjects and Genotypes.

Index cases	Genotypes		
	659 (A/C/G)	1004 (A/T)	1688 (G/T)
1	GG	TT	-
2	AG	AT	GG
3	AC	AT	GG
4	GG	AT	*
5	AC	TT	GG
6	AG	TT	GG
7	GG	TT	GG

* PCR failure

Index case No. 1, 4 and 7 showed homozygosity for splice mutations at nucleotide position 659, intron 2 with a sequence change of A or C to G, while the index case No. 2 and 6 showed heterozygosity for the same mutation. No mutation was found in nucleotide position 659, 1004, 1688 in index case No. 3 and 4.

Mutations in Family members

A total of 19 family members of Case Nos. 1-5 were also analyzed for the same mutations. (Family No. 1,2, 3, 4 and 5 included 3, 2, 7, 4, and 5 members respectively). These included 8 males and 11 females. All were asymptomatic. The mutation analysis for the family members of index case 6 and 7 was not performed due to non-availability of their blood specimens.

Mutations in Parents

Both the parents of index case I were heterozygous

at 659 (father CG, mother AG). However they showed normal genotypes at nucleotide position 1004 and 1688. The parents of index case 4 also showed heterozygosity at 659 (father AG, mother AG). The father of index case 2 was heterozygous at 659 (AG) and mother was normal. The mutational analysis at 1004 and 1688 demonstrated normal genotypes.

The parents of index case 3 and 5 did not show any of the analyzed mutation. However instances of PCR failure were also noted for index case 3.

Mutations in Unaffected sibling pairs

The brother of index case 1 was heterozygous for 659 (AG). No mutation was identified at the examined nucleotide positions in the sisters of index case 4 and the unaffected siblings of index case 3 and 5

Table 3. Study Subjects and Genotypes.

S. No.	Study Subjects	Genotypes		
		659 (A/C/G)	1004 (A/T)	1688 (G/T)
Families with homozygous index case				
1	Index Case	GG	TT	-
	Father	CG	AT	GG
	Mother	AG	AT	GG
	Brother	AG	AT	GG
4	Index Case	GG	AT	*
	Father	AG	AT	GG
	Mother	AG	AT	GG
	Sister	AA	TT	GG
	Sister	AA	TT	*
Families with heterozygous index case				
2	Index case	AG	AT	GG
	Father	AG	AT	GG
	Mother	AA	AT	GG
5	Index Case	AC	TT	GG
	Father	CC	TT	GG
	Mother	AC	TT	GG
	Sister	CC	TT	GG
Families with no identified mutation in index case				
3	Index Case	AC	AT	GG
	Father	*	AT	GG
	Mother	*	AT	GG
	Sister	*	TT	*
	Sister	AC	TT	*
	Sister	CC	AT	GG
	Brother	AC	AT	GG
	Brother	AC	AT	GG

* PCR failure

Discussion

Our results demonstrated A to G transition at nucleotide 659 causing aberrant splicing, reported for some other populations as the most commonly identified point mutations[11-15]. All cases were appropriately assigned to paternal or maternal chromosome.

At least 16 different mutations have been described within the 21B gene. Most have been derived from

the 21A pseudogene. However, novel mutation will always be a possibility[4,16]. Consequently, it will not be feasible to screen exhaustively for all mutations in a service environment. It has been reported that by screening for the common point mutations at positions 659, 1004, 1688 and 2113 in addition to the short-range mapping strategy described for identifying deletions and gene conversion events, majority of pathogenic mutations are identified4. Causative mutations can be identified in 95% of patients using this system. The point mutation at 2113 in our cases could not be performed due to technical reasons.

Given the diversity of clinical presentation and the relationship of severe disease with specific genotypes it is important to clearly characterize the CAH genotypes among Asian population[17]. Also given the high rates of consanguinity in Pakistan, genotyping of chorionic villous sampling may be the only practical option for antenatal diagnosis. It is important and interesting to investigate in this area, which has been suggested to be the most common autosomal recessive disorder in humans, with prevalence in Ashkenazi Jews of 3.7% and in a diverse Caucasian population of 0.1%[5]. Studies have indicated a variable incidence of the disease in children with early onset of pubic hair as well as in adolescents and adult females who have hirsutism[5]. Moreover prenatal diagnosis is also possible by use of a number of modalities like amniotic fluid hormonal levels, HLA typing of fetal cells obtained by amniocentesis.

If a couple has had a child with CAH, only 1in 4 subsequent pregnancies will be affected, and only 1 in 8 will be an affected female. Because androgens, irrespective of source, masculinize the genitalia between 6 and 14 weeks of gestation, treatment with dexamethasone must be instituted as early as possible in pregnancy. Thus, these groups have advocated that all fetuses that are potentially at risk, both affected and unaffected, should receive dexamethasone from 4 to 6 weeks gestation until about 12 weeks, when chorionic villous biopsy and DNA analysis can disclose the fetal sex and presence of P450c21 gene defects. If chorionic villous biopsy is unavailable, treatment is continued until 15 to 18 weeks, when amniocentesis can be performed for DNA analysis. At this point treatment is continued only in affected homozygous fetuses, i.e., only in 1 of 8 pregnancies initially treated. The ethics of needlessly subjecting 7 of 8 fetuses not at risk for CAH to an experimental therapy with unknown long-term consequences remain unresolved and the long term safety and outcomes have not been established. Prenatal diagnosis reduces the risk of treatment to the mother if the fetus is a male or unaffected female[18,19].

Because of the wide range of mutations in the 21- hydroxylase gene, unraveling the genotype in CAH among Asians is a daunting task. There is a clear need to setup national or regional gene banks and it may be cost effective to pool resources to do so.

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