

A CYP21A2 gene mutation in patients with congenital adrenal hyperplasia. *Molecular genetics report from Saudi Arabia*

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ABSTRACT

Objectives: The aim of this study is to determine congenital adrenal hyperplasia (CAH) with the pattern of CYP21A2 gene-mutations in Saudi children.

Methods: Between January 2011 and March 2014 at King Fahad Military Complex, Dhahran, Saudi Arabia, we thoroughly examined 11 patients with CAH and 2 asymptomatic individuals with a history of affected siblings. Additionally, we sequenced the full coding regions of the CYP21A2 gene and screened the gene for deletion(s)/duplication(s) using the multiplex ligation-dependent probe amplification (MLPA) technique.

Results: Nine patients had classic CAH and presented with ambiguous genitalia and/or salt losing crisis. Two patients had the non-classic form of CAH and presented with precocious puberty. The remaining 2 subjects were asymptomatic. Screening the CYP21A2 gene, we detected p.Gln318X mutation in 4 patients, c.290 -13 C>G (IVS2-13C>G) in another 4, and a common deletion, involving exons 6 and 8 in 3 patients.

Conclusion: Our strategy of Sanger sequencing followed by MLPA was very successful in detecting CYP21A2 mutations in all patients with CAH.

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Congenital adrenal hyperplasia (CAH) is an autosomal recessive condition caused by a deficiency of one of 5 enzymes involved in the steroidogenesis pathway.¹ The most common is 21-hydroxylase deficiency. This enzyme deficiency is caused by mutations in the CYP21A2 gene.² The level of residual enzyme activity determines the clinical phenotype that ranges from mild virilization to salt losing life threatening crisis.¹

Worldwide, the classic forms of 21-hydroxylase deficiency occur in one in 10,000 to 20,000 newborns.^{1,2}

While the prevalence of the non-classical form of 21-hydroxylase deficiency is estimated to be one in 1,000 individuals.¹ The recently introduced universal screening for CAH in Saudi Arabia reported an incidence of one in 6400 births.³ The prevalence of both classic and non-classic forms as well as their mutation pattern varies among different ethnic populations.⁴ Recent studies showed that an IVS2 AS -13 (A/C to G) mutation is prevalent in the Iranians while p.Q319X is common in Turkey, Tunisia, and East India.⁴⁻⁷ Different mutations result in variable deficiency of cortisol and aldosterone together with increased synthesis of androgen.⁸⁻¹⁰ The genotype/phenotype correlation of CAH has been reported in different populations and ethnic groups.⁴⁻⁷ Although the clinical presentations of CAH have been studied in Saudi children, literature review revealed no molecular report of 21-hydroxylase.³ Therefore, the aim of this study was to determine the pattern of CYP21A2 gene-mutations in Saudi children with CAH, and to describe the clinical phenotype of these patients.

Methods. This study was conducted at King Fahad Military Complex, Dhahran, Saudi Arabia between January 2011 and March 2014. Children diagnosed with CAH during this period were recruited. Subjects were included if they presented with salt losing crisis, ambiguous genitalia, precocious puberty or virilization, and their 17 hydroxyprogesterone was above 50 nmol/l. Siblings who had previously documented high 17 hydroxyprogesterone were also included. Identified subjects were systematically reviewed with an emphasis on demographic features, family history, consanguinity, age, gender, and clinical presentation were documented using a data report sheet. This study was approved by the ethics committee at King Fahad Military Complex. It adhered to the tenets of the declaration of Helsinki and all participants-guardians consented to participate in this study. Our strategy was to first sequence the CYP21A2 gene by Sanger sequencing in order to test for single base substitutions. If the patient was negative, we then proceed with the MLPA technique in order to search for deletion(s) and/or duplication(s).

Sample collection and DNA preparation. Blood samples (5 ml) were drawn in ethylenediaminetetraacetic acid (EDTA) tubes. Tubes were centrifuged at 5500 x g for 5 min and the Buffy layer was used for DNA extraction using the illustrated blood genomic Prep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK) and stored at -20°C in aliquots until further use.

Polymerase chain reaction (PCR) amplification and sequencing of the CYP21A2 gene. The full coding exons, exons-introns boundaries of the CYP21A2 gene were amplified utilizing primers described previously.⁷

Detection of deletion(s)/duplication(s) by multiple ligation dependent probe amplification (MLPA). We used SALSA MLPA Kit P050-B2 CAH (MRS, Holland, Amsterdam, the Netherlands) for quantitative analysis (detection of copy number alterations in the CYP21A2 gene). Probe hybridization and MLPA PCR were carried out according to the manufacturer's manual and as previously described.⁷ Results were analyzed with the free software Gene Marker 1.6 (Soft Genetics, State College, PA, USA).

Results. There were 6 males and 7 females. The age at presentation ranged from one day to 6 years. Consanguinity was documented in 10 patients while family history of CAH was observed in 9. All the 6 homozygous females presented on the first day of life with ambiguous genitalia and developed salt losing later (Table 1). Three males presented with salt losing crisis between the third and the fourth week of life while another 2 boys presented later with precocious puberty. So, the clinical features indicated that 9 patients had classic CAH, 2 were non-classic, and another 2 were carriers of CAH.

We sequenced the full coding exons, exons-introns boundaries of the CYP21A2 gene in 11 Saudi patients

Table 1 - Clinical characteristics of patients with congenital adrenal hyperplasia (CAH).

Patient identifier	Gender	Age at presentation	Consanguinity	Family history	Clinical phenotype
CAH-01	M	3 weeks	Yes	Yes	Salt losing
CAH-02	M	30 months	Yes	No	Precocious puberty / non-classic
CAH-03	F	At birth	Yes	Yes	Salt losing / ambiguous genitalia
CAH-04	F	At birth	Yes	Yes	Salt losing / ambiguous genitalia
CAH-05	F	At birth	Yes	Yes	Salt losing / ambiguous genitalia
CAH-06	M	3 weeks	Yes	Yes	Salt losing
CAH-07	F	At birth	Yes	Yes	Salt losing / ambiguous genitalia
CAH-08	F	At birth	No	No	Salt losing / ambiguous genitalia
CAH-09	M	4 weeks	Yes	Yes	Salt losing
CAH-10	M	4 years	No	No	Precocious puberty / non-classic
CAH-11	F	At birth	No	No	Salt losing / ambiguous genitalia
CAH-12	F	1 year	Yes	Yes	Asymptomatic / sibling of patient with CAH
CAH-13	M	6 years	Yes	Yes	Asymptomatic / sibling of patient with CAH

Table 2 - Mutations detected in the CYP21A2 gene among patients with congenital adrenal hyperplasia (CAH).

Patient identifier	Sequencing results	Homo/Hetero	MLPA	Homo/Hetero	Comments
CAH-01	c.952 C>T (p.Gln318X)	Homozygous	None	N / A	Involving exon 8 / reported mutation
CAH-02	c.290 -13 C>G (IVS2-13C>G)	Homozygous	None	N / A	Intron 2 / reported mutation
CAH-03	c.952 C>T (p.Gln318X)	Homozygous	None	N / A	Involving exon 8 / reported mutation
CAH-04	c.952 C>T (p.Gln318X)	Homozygous	None	N / A	Involving exon 8 / reported mutation
CAH-05	None	N / A	Deletion	Homozygous	Involving exons 6 and 8 / reported mutation
CAH-06	None	N / A	Deletion	Homozygous	Involving exons 6 and 8 / Reported mutation
CAH-07	None	N / A	Deletion	Homozygous	Involving exons 6 and 8 / Reported mutation
CAH-08	c.290 -13 C>G (IVS2-13C>G)	Homozygous	None	N / A	Intron 2 / reported mutation
CAH-09	c.952 C>T (p.Gln318X)	Homozygous	None	N / A	Involving exon 8 / reported mutation
CAH-10	c.290 -13 C>G (IVS2-13C>G)	Homozygous	None	N / A	Intron 2 / reported mutation
CAH-11	c.290 -13 C>G (IVS2-13C>G)	Homozygous	None	N / A	Intron 2 / reported mutation
CAH-12	None	N / A	Deletion	Heterozygous	Involving exons 6 and 8 / reported mutation
CAH-13	c.1436 G>T (p.Arg479Leu)	Heterozygous	None	N / A	Involving exon 10 / reported mutation

MLPA - multiple ligation dependent probe amplification

with congenital adrenal hyperplasia and 2 subjects with history of affected siblings. Utilizing Sanger sequencing, we managed to detect 11 homozygous and 2 heterozygous mutations in 13 subjects (Table 2). We found p.Gln318X mutation in 4 patients and c.290 -13 C>G (IVS2-13C>G) in another 4. Four subjects had, what seems to be, a common deletion in our cohort detected by MLPA (a technique designed to detect alterations (deletion/duplication). This deletion involves exons 6 and 8 of the CYP21A2 gene. This deletion was detected in a homozygous status in 3 patients and in a heterozygous status in one patient (Table 2).

Discussion. Classic CAH is an inherited disorder characterized by profound deficiency of the enzyme 21-hydroxylase. This leads to acute presentation with salt losing crisis in the first few weeks of life. If unrecognized and not managed effectively, this will lead to shock, hypoglycemia, and death.^{1,2} Patients who survive this adrenal crisis may suffer from developmental delay secondary to brain insult. Females with classic CAH are likely to be diagnosed early as they usually present with ambiguous genitalia at birth.⁸ This results from the overproduction of androgen in utero as a result of the block in the synthesis of cortisol and aldosterone that leads to diversion of the precursors towards the synthesis of androgen.^{1,2} Nine of our patients in this series had classic CAH and presented with ambiguous genitalia and or salt losing crisis. Similarly, 72% of a Tunisian cohort with CAH presented with ambiguous genitalia and or salt losing crisis.⁶ This can be explained by the lack of newborn screening for CAH in this population. Universal neonatal screening for CAH aims at identification of affected infants and starting hormonal replacement therapy, thus preventing salt losing crisis and its consequences, including mental retardation, and death.² Newborn screening has been recently implemented in Saudi Arabia.³ The first report of this program showed that the incidence of CAH is one in 6400, which is high compared with reports from other countries.^{2,3}

Non-classic CAH results from residual enzyme activity of 21-hydroxylase. This leads to late presentation with virilization and early puberty.^{8,10} Two males in our study had non-classic CAH and presented with precocious puberty. Non-classic CAH is an uncommon but treatable cause of precocious puberty. Therefore, physicians should consider this diagnosis in any child presenting with signs of early puberty. Similarly, Bizzarri et al¹⁰ investigated clinical features suggestive

of non-classical 21-hydroxylase deficiency in children presenting with precocious pubarche and found that earlier onset of pubarche and advanced bone age suggest non-classic CAH compared to the other causes.

According to the human genome mutation database (HGMD there are currently 243 various types of mutations detected in the CYP21A2 gene.^{11,12} The majority of which (64.2%; 156/243) are the miss-sense/non-sense type of mutations. Most mutations (~80-98%) can be detected by either targeted mutation or by deletion/duplication analysis. Using the strategy of screening the CYP21A2 gene first by Sanger sequencing and second, carrying out MLPA on negative individuals, we observed few mutations including p.Gln318X in 4 patients. The worldwide frequency of this mutation ranges from 0-14%.¹³ It presented with classic salt losing CAH in all our 4 patients. We also detected c.290 -13 C>G (IVS2-13C>G) mutation in another 4 patients, 2 presented with salt losing classic CAH, and the other 2 with non-classic CAH. This demonstrates the variability in expression of the CYP21A2 gene leading to different phenotype within the same population. Furthermore, 4 subjects had, what seems to be, a common deletion involving exons 6 and 8 of the CYP21A2 gene. This deletion was observed in a homozygous status in 3 patients presenting with salt losing classic CAH, and in a heterozygous status in one asymptomatic patient. Interestingly, all our patients with both classic and non classic CAH were homozygous for various mutations, and this may not be surprising as consanguinity reaches up to 56% in certain parts of Saudi Arabia.¹³ This is in contrast to other populations, where the majority of individuals with 21-hydroxylase deficiency CAH are compound heterozygote.^{1,11,12} We searched the published database of CYP21A2 gene mutation in our neighboring countries such Iran, Turkey, and other Arabic countries, and we did not find any of the mutations we detected in our cohort observed in these populations.⁴⁻⁷ This could be explained by the small size of our cohort and by the fact that the CYP21A2 gene has a large number of known mutations. We cannot make strong statements of the mutation hot spot areas as our cohort is relatively small. Another limitation of this study is the fact that all patients were seen in a single institute in the Eastern province of Saudi Arabia, which may not be representative of the whole country.

In conclusion, detecting mutations in all our patients indicates that our study was successful, which constitutes screening the gene first by Sanger sequencing and second, carrying out MLPA on negative individuals.

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