Review Article

Pitfalls in molecular diagnosis of 21-hydroxylase deficiency in congenital adrenal hyperplasia

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Abstract

Congenital adrenal hyperplasia (CAH) is a putative error of metabolism with autosomal recessive heredity pattern. The main manifestations of classic form of CAH are salt-wasting, dehydration and simple virilization in both sexes and ambiguous genitalia in female gender. 21-hyroxylase (*CYP21A2*) impairment with prevalence value of 1 in 10,000–15,000 live births is the most common etiology of CAH. Because of consanguineous marriages, the frequency of the CAH in Iran is very high. A wide range of mutations diversity exists in *CYP21A2* gene and a large number of these mutations derived from a highly homologous pseudogene, *CYP21A1P*, through gene conversion. In addition, new mutations such as small and large deletion and point mutations can also result in enzyme deficiency. Various methods for mutation detection were performed. The main obstacle in molecular diagnosis of CAH is amplification of pseudogene during polymerase chain reaction of *CYP21A2*. All attempts focus on discrimination of pseudogene from gene; that is why, there is the majority of mutations on pseudogene, and if we have contamination with the pseudogene, the result will be unreliable. Here, we discuss this methods and advantage and disadvantage of those.

Key Words: 21-hydroxylase deficiency, congenital adrenal hyperplasia, molecular methods

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Received: 02.12.2014, **Accepted:** 17.02.2015

INTRODUCTION

Congenital adrenal hyperplasia (CAH) is one of the most common disorders with autosomal recessive hereditary pattern. This disorder is caused by 21-hydroxylase deficiency (21-OHD) in approximately 95% of cases. Frequency of the classic form of CAH is 1 in 14,000 newborns. A milder or nonclassic form has seen approximately 1 in 1000 female newborns with clinical evidence.[1] In newborn screening program, the serum level of 17-hydroxyprogesterone is measured which is elevated in patients with 21-OHD. Although biochemical testing is normally used in the diagnosis, molecular methods play an essential role in mutation detection of at-risk pregnancies.[2] In this article, a summary of molecular diagnostic methods used for diagnosis of 21-OH are reviewed, and the pros and cons of each method are discussed.

Biochemistry

21-hydroxylase is one of the cytochrome p450 enzymes, which converts 17-hydroxyprogesterone

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.164009

How to cite this article: Kolahdouz M, Mohammadi Z, Kolahdouz P, Tajamolian M, Khanahmad H. Pitfalls in molecular diagnosis of 21-hydroxylase deficiency in congenital adrenal hyperplasia. Adv Biomed Res 2015;4:189.

to 11-deoxycortisol and progesterone to deoxycorticosterone. These resulting metabolites represent the precursor of cortisol and aldosterone respectively. 21-OHD leads to reduce the amount of cortisol and aldosterone synthesis. Salt-wasting type of CAH arises from insufficient mineralocorticoid which could be life-threatening through dehydration. In addition, deficiency in cortisol synthesis induces overproducing of cortisol precursors by the adrenal cortex. Some of these precursors result in the biosynthesis of androgen. This may cause variable degrees of virilization in the external genitalia of affected female fetuses as well as rapid postnatal growth in male and female newborns.[3]

MOLECULAR GENETICS OF CONGENITAL ADRENAL HYPERPLASIA

21-hydroxylase is encoded by the *CYP21A2* gene and located on chromosome 6p21.3 within the human leukocyte antigen (HLA) Class III region. *CYP21A2* has a pseudogene called *CYP21A1P*. *CYP21A2* and *CYP21A1P* have 98% homology in exons and 96% in introns.[4,5] Both the gene and pseudogene are normally in tandem arrangement with the genes 4A and 4B gene. Multiple deleterious mutations in *CYP21A1P* render it inactive or nonfunctional gene. Common mutations include an 8-bp deletion in exon 3, a splice mutation in intron 2, a cluster of mutations in exon 6 and a nonsense mutation in exon 8 [Figure 1].[2] Multiple mutations have been known to be the cause of a wide range of CAH phenotypes.[6] The most frequent *CYP21* mutations are due to gene conversion from the pseudogene to the active gene.[7,8] However, about 15% of mutations are the deletion of the entire *CYP21* gene. The severity of the phenotype depends on the type of mutations. In general, gene deletions, the 8-bp deletion in exon 3, the exon 6 cluster of mutations and Arg356Trp all result in salt-wasting. The intron 2 mutation can indicate either salt-wasting or simple virilizing phenotype. The Ile172Asn mutation is associated with simple virilization, and Pro 30 Leu and Val 281 Leu are usually seen in patients with nonclassic form of the disease.[9]

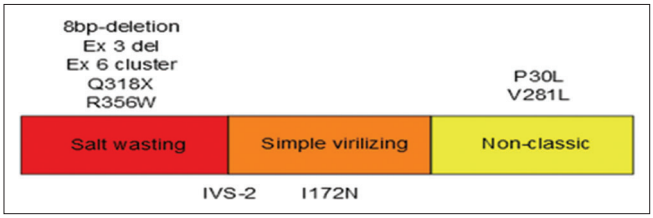

Figure 1: Eight common point mutations and deletion in *CYP21A2* and its relation with type of congenital adrenal hyperplasia

MOLECULAR DIAGNOSIS OF CONGENITAL ADRENAL HYPERPLASIA

Different methods have been used for molecular diagnosis of CAH disease. The presence of the highly homologous *CYP21P* gene poses a challenge to the study of the *CYP21*; for this reason, *CYP21* mutation detection requires selective *CYP21* gene amplification developed in the last few years. Some of the methods are robust and have been widely adopted. These methods are discussed below.

Targeted mutation analysis for common mutations should be performed first to confirm the diagnosis of 21-OHD CAH. If no mutation is identified or if only one mutation is identified, deletion/duplication testing should be performed next.

LINKAGE ANALYSIS

The first report on linkage of HLA serotypes with 21-OHD indicate the approximate position of the locus.[10]

In one study, through the use of indirect linkage analysis, ten highly polymorphic CA repeat microsatellite markers in 21-OHD have been identified in the Class III HLA region in families with affected children. The validity of the linkage analysis depends on several factors including: The proximity of the linked markers, their informativeness in the family, the use of several internal or flanking markers.[11]

In another study, six polymorphic microsatellite loci in the neighborhood of the *CYP21* gene were amplified by multiplex polymerase chain reaction (PCR) and then sequenced. Therefore, the number of repeat elements in the PCR products was found to indicate microsatellite type.[12]

Linkage analysis resulted in many diagnostic mistakes due to recombination or haplotype sharing. This approach is indirect and does not identify the causative mutation and would only be used to pinpoint the gene for further molecular techniques. However, for prenatal diagnosis, we recommend that microsatellite typing be used as a supplement to actual genotyping of *CYP21* in order to resolve ambiguities due to this phenomenon.

POLYMERASE CHAIN REACTION BASED METHODS FOR DETECTION OF *CYP21* MUTATIONS

In many genetic diseases, PCR is used for detection of mutations. However, in CAH disease, the PCR

method is not straightforward due to the existence of highly homologous pseudogene, *CYP21A1P*. Hence, primers must be specific to the sequence of *CYP21* and not amplify *CYP21P*. This problem severely limits the number of specific primers for *CYP21A2*.[13] Most authors have used two fractions of *CYP21A2,* which differ from *CYP21P*. One of them is 8-bp sequence in exon 3 of *CYP21* and deleted in the pseudogene while another one in exon 6 has four different nucleotide between gene and pseudogene.[2] At first, the *CYP21A2* gene should be amplified by specific primers that match the gene and not the pseudogene. Then mutation detection will be performed on the PCR product by allele-specific PCR, sequencing or other methods. The main point in CAH mutation detection is discrimination of the gene from the pseudogene, and if this process does not happen properly, there will be a lot of heterozygosity in the results.

ALLELE SPECIFIC POLYMERASE CHAIN REACTION

The use of allele-specific PCR to detect *CYP21* mutations has been reported in the majority of surveys.[14-16] In this method, allele-specific primer for each point mutation was used in order to detect mutations. For each mutation two PCRs were performed, one for detection of normal allele with normal primer and the other contains mutant primer for detection of mutant allele conjugation with common primer. The latter targets only *CYP21* without any contamination with *CYP21P*. Two primers for unrelated targets are used for confirmation of PCR performance as an internal control.

Individually for each exon, PCR products are run on 8% polyacrylamide gel electrophoresis. In normal sample normal allele is amplified and in homozygote sample, mutant allele is amplified. In heterozygote samples, both normal and mutant alleles generate electrophoresis gel band.[15] Allele-specific PCR is very sensitive and not authentic method.

ALLELE-SPECIFIC OLIGONUCLEOTIDE HYBRIDIZATION

Allele-specific oligonucleotides have been employed to detect mutations with probe PCR-amplified DNA in a dot blot format.[17-22] Initially, *CYP21* was divided into two segments with polymerase chain reaction using special primers for gene not pseudogene. PCR products were transferred onto a nylon membrane and dot blot hybridizations are performed with end-labeled, allele-specific normal and mutant oligonucleotide probes[23] for detection of point mutations. Analysis by PCR-allele specific oligonucleotide hybridization at the position 656 requires three oligonucleotide including the mutant and wild-type A and C oligonucleotides hybridize with DNA target to prevent incorrect genotyping.[20]

POLYMERASE CHAIN REACTION WITH RESTRICTION ENZYME DIGESTION

This method is based on performing two tandem PCR and then digesting with the restriction enzyme.[6,24,25] The primary PCR amplification of the active *CYP21* gene, without contamination of *CYP21P* sequence, was performed through *CYP21* specific primers.[25] Digestion with EcoRI enzyme was applied to confirm only the amplification of the active gene because this enzyme has one restriction site in the active gene and two restriction sites in the psoudegene.[6] The secondary PCR was performed on primary PCR product as template with primers specific to each common mutation[25] and each product incubated with a specific restriction enzyme. Noncommon mutation should be screened with direct sequencing of *CYP21* gene.[24] This method is fast and nonradioactive and suitable for prenatal diagnosis using amniotic cells or chorionic villi since small amount of material is required for PCR.[6]

LIGATION DETECTION REACTION

In few studies, ligation detection reaction (LDR) was used for detection of *CYP21* mutations.[12,26,27] DNA ligase is appropriate for mutation detection in many assays. In this method, the *CYP21* gene was amplified as two overlapping fragments. LDR oligonucleotides were synthesized by FAM labeled and adding poly (A) tail to the end of oligonucleotides with unique length per mutations. Therefore, this causes the generation of LDR products with different lengths. Subsequently LDR products are analyzed by polyacrylamide gel electrophoresis and florescence detection. LDR assay is fast, accurate and nonradioactive and can also detect all gene conversion of *CYP21A2* in a single tube reaction. In contrast to allele-specific PCR, LDR is able to detect single base insertion.[26]

MULTIPLEX MINISEQUENCING

Multiplex minisequencing has been used in a study to confirm mutation detection,[28] while DNA samples from CAH patients have been previously genotyped by direct DNA sequencing. One and half working days is needed for the procedure to finish. Unlike most methods which ordinary require two primers, this method only employs one primer.

In the first step, PCR was carried out by specific primer of the active gene. Then, single nucleotide extension of primers directly flanking the nucleotide of interest is used for the detection of common point mutations.

The primers are labeled with a fluorescently dideoxynucleotide triphosphate mixture. The 5′ end of primers was elongated with a poly (T) track, which is different in size to facilitate electrophoretic separation of diagnostic products.[28]

This method cannot be used solely for mutation detection in CAH patients and can only confirm the accuracy of DNA sequencing.

SINGLE STRAND CONFORMATIONAL POLYMORPHISM

In some research, single strand conformational polymorphism (SSCP) is performed for analyzing of *CYP21* gene.[29-33] In this method, primary separation of gene from the pseudogene by three overlap PCR was conducted. Then, specific primers were used to amplify each exon for SSCP analysis. For each one of the amplified DNA fragments, different electrophoretic conditions are needed,[33] and running SSCP gel has high sensitivity to temperature. This method did not identify the type of mutation and required sequencing for accurate detection. Therefore, this technique has not been widely accepted.[2]

REVERSE DOT BLOT

Normal and mutant oligonucleotides have been used for the common mutation sites and spotted onto a nylon membrane.[34] Exons containing mutations from samples are amplified and label with biotin-dUTP by PCR. These exons then are hybridizing to membrane strips. By chemiluminescence, signal detection is achieved. Reverse dot blot (RDB) method is accurate and cost-effective for the molecular diagnosis of *CYP21* point mutations in CAH, but requires DNA sequencing for confirmation.

MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION AND SOUTHERN BLOT

A great number of studies have made use of multiplex ligation-dependent probe amplification (MLPA)[35] or southern blot[36] for detection of large deletion or duplication in *CYP21A2* gene. Southern blot is a trusted method allowing for a secure diagnosis of the heterozygous arrangement.[36] However, compared to the Southern blot, MLPA could be appraised a high throughput analysis, allowing for the study of several samples in the same experiment at the same time and the analysis of both gene (*CYP21A2*) and pseudogene (*CYP21A1P*) in each patient.[37]

PRENATAL SCREENING

Prenatal hormonal therapy can alleviate the severity of virilization in affected newborns,[38] but the virilization begins in the middle of the first trimester of pregnancy and should not await for genetic testing.[14] Therefore, the treatment with dexamethasone to the mother begin concurrent with the result of analysis of 17-hydroxyprogesterone in amniotic fluid in pregnant women. Genetic testing with DNA analysis has been performed. Therapy can be stopped if the fetus is a male or an unaffected female as a result of genetic testing.[39]

CONCLUSION

Study of *CYP21A2* gene because of highly homologous pseudogene is not easy, but in the various methods for mutation detection of *CYP21A2* gene defect, it seems RDB method for point mutations detection and MLPA for deletion/duplication mutations are the best and reliable technique.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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