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MUTATIONS IN THE *CYP21A2* GENE ANALYZED BY THE POLYMERASE CHAIN REACTION

MUTAÇÕES NO GENE *CYP21A2* ANALISADAS ATRAVÉS DA REAÇÃO DA POLIMERASE EM CADEIA

MUTACIONES EN EL GEN *CYP21A2* ANALIZADAS A TRAVÉS DE LA REACCIÓN DE LA POLIMERASA EN CADENA

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RESUMO

Objetivo: padronizar a Reação em Cadeia da Polimerase Alelo Específico (PCR-AS), no Laboratório de Genética Molecular Humana do Hospital Universitário Professor Alberto Antunes (HUPAA), para a investigação das mutações p.Pro30Leu, c.290-13A/C>G, p.Gly110fs, p.Ile172Asn, Cluster6 (p.Ile236Asn+p.Val237Glu+p.Met239Lys), p.Val281Leu, p.Gln318* e p.Arg356Trp no gene *CYP21A2*, todas de reconhecida frequência e provenientes do pseudogene *CYP21A1P*. **Método:** as PCR-AS foram realizadas utilizando amostras-controle para cada mutação. Oligonucleotídeos para a seleção do alelo, com a alteração (mutante) e sem a alteração (normal), foram utilizados para cada mutação analisada. Os produtos das PCR-AS foram testados por meio de eletroforese em gel de agarose. A análise dos resultados das reações foi realizada por meio da presença ou ausência do fragmento de interesse. **Resultados:** os alelos normal e mutante foram amplificados para as mutações p.Pro30Leu, c.290-13A/C>G, p.Gly110fs, p.Ile172Asn, Cluster6 (p.Ile236Asn+p.Val237Glu+p.Met239Lys), p.Val281Leu, p.Gln318* e p.Arg356Trp. **Conclusão:** a padronização PCR-AS para o diagnóstico molecular de HAC devido às mutações no gene *CYP21A2,* no Laboratório de Genética Molecular Humana, proporcionará a elucidação diagnóstica de casos triados pelo Programa Nacional de Triagem Neonatal e/ou atendidos no Serviço de Genética Clínica do HUPAA/UFAL. Além disso, proporcionará o reconhecimento das mutações mais frequentes no gene *CYP21A2* na população de Alagoas, assim como a ampliação do conhecimento sobre as relações genótipo-fenótipo para os casos de HAC. Este conhecimento é de fundamental importância para incrementar a abordagem diagnóstica, o tratamento, o aconselhamento genético e a prevenção.

Descritores: Reação em Cadeia da Polimerase; Genética Médica; Hiperplasia.

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ABSTRACT

Objective: to standardize the Allele-Specific Polymerase Chain Reaction (PCR-AS) in the Laboratory of Human Molecular Genetics of the Professor Alberto Antunes University Hospital (HUPAA) to investigate the mutations p.Pro30Leu, c.290-13A / C> G, p .Gly110fs, p.Il172Asn, Cluster6 (p.Ile236Asn + p.Val237Glu + p.Met239Lys), p.Val281Leu, p.Gln318 * and p.Arg356Trp in the CYP21A2 gene, all of recognized frequency and from the pseudogene CYP21A1P. **Method:** PCR-AS were performed using control samples for each mutation. Oligonucleotides for allele selection, with alteration (mutant) and without alteration (normal), were used for each mutation analyzed. PCR-AS products were tested using agarose gel electrophoresis. The analysis of reaction results was performed through the presence or absence of the fragment of interest. **Results:** the normal and mutant alleles were amplified for the mutations p.Pro30Leu, c.290-13A / C> G, p.Gly110fs, pIl172Asn, Cluster6 (p.Ile236Asn + p.Val237Glu + p.Met239Lys), p .Val281Leu, p.Gln318 * and p.Arg356Trp. **Conclusion:** the PCR-AS standardization for the molecular diagnosis of CAH due to mutations in the CYP21A2 gene in the Laboratory of Human Molecular Genetics will provide the diagnostic elucidation of cases screened by the National Neonatal Screening Program and / or attended at the Clinical Genetic Service of HUPAA / UFAL. In addition, it will provide recognition of the most frequent mutations in the CYP21A2 gene in the population of Alagoas, as well as the increase of knowledge about genotype-phenotype relationships for cases of CAH. This knowledge is of fundamental importance to increase the diagnostic approach, treatment, genetic counseling and prevention.

Descriptors: Polymerase Chain Reaction; Genetics, Medical; Hyperplasia.

RESUMEN

Objetivo: estandarizar la Reacción en Cadena de la Polimerasa Alelo Específico (PCR-AS), en el laboratorio de Genética Molecular Humana del Hospital Universitario Profesor Alberto Antunes (HUPAA), para la investigación de las mutaciones p.Pro30Leu, c.290-13ª /C>G, p.Gly110fs, p.Ile172Asn, Cluster6 (p.Ile236Asn+p.Val237Glu+p.Met239Lys), p.Val281Leu, p.Gln318* y p.Arg356Trp en el gen *CYP21A2*, todas de reconocida frecuencia y provenientes del pseudogén *CYP21A1P*. **Método:** las PCR-AS se realizaron utilizando muestras de control para cada mutación. Se utilizaron los Oligonucleótidos para la selección del alelo, con la alteración (mutante) y sin la alteración (normal), para cada mutación analizada. Los productos de las PCR-AS fueron probados a través de electroforesis en gel de agarosa. El análisis de los resultados de las reacciones se realizó a través de la presencia o ausencia del fragmento de interés. **Resultados**: Los alelos normal y mutante se han amplificado para las mutaciones p.Pro30Leu, c.290-13A / C> G, p.Gly110fs, p.Ile172Asn, Cluster6 (p.Ile236Asn + p.Val237Glu + p.Met239Lys), p .Val281Leu, p.Gln318 * y p.Arg356Trp. **Conclusión:** la estandarización PCR-AS para el diagnóstico molecular de HAC debido a las mutaciones en el gen CYP21A2, en el Laboratorio de Genética Molecular Humana, proporcionará la elucidación diagnóstica de casos triados por el Programa Nacional de Triaje Neonatal y / o atendidos en el Servicio de Genética Clínica del HUPAA / UFAL. Además, proporcionará el reconocimiento de las mutaciones más frecuentes en el gen CYP21A2 en la población de Alagoas, así como la ampliación del conocimiento sobre las relaciones genotipo-fenotipo para los casos de HAC. Este conocimiento es de fundamental importancia para incrementar el abordaje diagnóstico, el tratamiento, el asesoramiento genético y la prevención.

Descriptores: Reacción en Cadena de la Polimerasa; Genética Médica; Hiperplasia.

INTRODUCTION

Sex Differentiation Disorders (SDD) are congenital conditions where genital or gonadal development is incomplete or disorderly, leading to a disagreement between the genetic, gonadal and phenotypic gender of the affected individual.[1] Among the many cases of these disorders, we found Congenital Adrenal Hyperplasia (CAH), an inborn error of metabolism characterized by the deficiency of one of the five enzymes involved in adrenal steroidogenesis, from cholesterol. About 90-95% of cases occur due to deficiency of the CYP21A2 enzyme, encoded by the CYP21A2 gene (ENSG00000231852).[2-3]

The clinical manifestations associated with CAH are divided into two forms: the classical form, with the salt loser phenotype (SL) and simple virilizer (SV) phenotypes; and the non-classical or late onset form, which includes the symptomatic and asymptomatic subgroups.[1-3]

The clinical spectrum of CAH is broad, and may vary according to residual activity of the altered CYP21A2 enzyme. The SV form occurs because the enzymatic residual activity is between 1% and 5%, resulting in only the deficiency of cortisol production alone, with no damage to aldosterone production. On the other hand, the SL form occurs when the enzymatic residual activity is zero or less than 1%, resulting in the deficiency of cortisol and aldosterone biosynthesis, with route deviation for the production of androgens.[3]

When untreated, clinical signs of hyperandrogenism can be observed in all age groups, from birth to adulthood. These signs include genital ambiguity of different degrees in subjects 46, XX, precocious puberty, hirsutism, amenorrhea / oligomenorrhea and infertility. In the salt-losing form, corresponding to 75% of cases, cortisol and mineralocorticoid deficiency may be associated with hydro-electrolytic changes such as dehydration, hyponatraemia and hyperkalemia, vomiting, metabolic acidosis, hypovolemic shock, with a significant increase in mortality, especially in the period neonatal.[4-5]

The non-classical symptomatic form is characterized because it does not present prenatal virilization and the symptoms develop at variable times. Young women may have acne, hirsutism and menstrual disorders; affected children are characterized by precocious puberty, accelerated growth velocity and advanced skeletal maturation.[2-3,6-7] CAH in non-classic asymptomatic form presents a

hormonal profile similar to the symptomatic form, but does not develop clinical manifestations.[1,8-9]

CAH has an overall prevalence of 1: 10,000 to 1: 15,000 live births.[4-5] Neonatal screening data in some Brazilian states indicates an incidence of 1: 10,325 in Goiás,[10] 1: 19,927 in Minas Gerais,[11] and 1: 11,655 in Santa Catarina,[12] but there is still no single precise data that can inform the incidence of CAH in Brazil.

According to "The Human Gene Mutation Database" (2018), a total of 302 mutations have been described in CAH-related CYP21A2.14 Of these, eight mutations derived from the CYP21A1P pseudogene are found more frequently in the affected individuals, they are: p.Pro30Leu, c.290-13A/C>G, p.Gly110*fs*, p.Ile172Asn, Cluster6 (p.Ile236Asn+p.Val237Glu+p.Met239Lys), p.Val281Leu, p.Gln318* and p.Arg356Trp.

Screening for frequent mutations in CYP21A2 can be performed by the allele-specific polymerase chain reaction (PCR-AS), whose molecular data are of great importance for the definition of the diagnosis. Based on the above, the objective of this work was to standardize the PCR-AS in the Laboratory of Human Molecular Genetics of the University Hospital Professor Alberto Antunes of the Federal University of Alagoas (LGMH / HUPAA / UFAL) to investigate mutations p.Pro30Leu, c.290-13A/C>G, p.Gly110fs, p.Ile172Asn, Cluster6 (p.Ile236Asn+p.Val237Glu+p.Met239Lys), p.Val281Leu, p.Gln318* and p.Arg356Trp, all of recognized frequency in subjects with CAH.

METHOD

The experiments were carried out at LGMH / HUPAA / UFAL. The funds for the implementation of the project came from the Universal - FAPEAL project entitled: Molecular Characterization of Families with Congenital Adrenal Hyperplasia in Alagoas: Pilot Study (UNIVERSAL FAPEAL NO. 04/2016 - PROCEDURE N. 60030001071/2016).

This work was conducted under the approval of the Research Ethics Committee of the Federal University of Alagoas (CAAE: 59931616.6.0000.5013 - Opinion 1,752,433, 9/29/2016).

The genomic DNA of previously studied patients served as controls of the reactions. The controls were kindly provided by Dr. Maricilda Palandi de Mello of

the Center for Molecular Biology and Genetic Engineering of the Statistical University of Campinas. The mutations analyzed were: p.Pro30Leu, c.290-13A/C>G, p.Gly110fs, p.Ile172Asn, *Cluster* 6 (p.Ile236Asn+p.Val237Glu+p.Met239Lys), p.Val281Leu, p.Gln318* and p.Arg356Trp.

For the identification of normal and mutant alleles, two PCRs were performed. In one reaction the normal oligonucleotide and the anchor oligonucleotide were used and in the other reaction, mutant oligonucleotide and anchor performed in different tubes. Due to the great homology between gene and pseudogene, the anchor oligonucleotide is fundamental for the exclusive selection and amplification of the CYP21A2 gene.

The amplified fragments were analyzed by means of 1% agarose gel electrophoresis, with the aid of a molecular weight ruler 1kb plus DNA Ladder.

The gels were stained in Ethidium Bromide solution (0.5µg / mL) and the results were analyzed in transliner, where the photos of the gels were captured. Analysis of the results was performed by the presence or absence of the fragment of interest.

RESULTS

Amplification of the fragments of interest through the AS-PCR was achieved for the mutations: p.Pro30Leu, c.290-13A/C>G, p.Gly110fs, p.Ile172Asn, Cluster6 (p.Ile236Asn+p.Val237Glu+p.Met239Lys), p.Val281Leu, p.Gln318* and p.Arg356Trp.

Table 01 - Reagents used in each reaction.

Reagents (microliters)	p.Pro30Leu***	SPLC C e G*	SPLC A	p.Gly110fs*	p.Il172Asn **	Cluster6 *	p.Val281Leu ***	p.Gln318*	p.Arg356Trp*
Water	17	20	19.9	20.1	20.3	20.1	19.5	20	20.3
Plug	6	3	3	3	3	3	3	3	3
$MgCl_2$	1	0.7	0.8	0.9	0.7	0.9	1	1	0.7
DNTP	2.5	2.5	2.5	2.5	2.5	2.5	3	2.5	2.5
Primer 1	1	1	1	1	1	1	1	1	1
Primer 2	1	1	1	1	1	1	1	1	1
BSA 1%	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Taq	0.2	0.3	0.5	0.2	0.2	0.2	0.2	0.2	0.2

Form: *Salt looser; **Simple viralizer; ***Not classic

Table 02 – Standard cycles.

	p.Pro30Leu***	SPLC Ae G	SPLC C	p.Gly110fs*	p.Il172Asn **	Cluster6 *	p.Val281Leu***	p.Gln318*	p.Arg356Trp*
Cicles	94 ºC - 5 min	94 ºC - 5 min	94 ºC - 5 min	94 ºC - 5 min	94 ºC - 5 min	94 ºC - 5 min	95 ºC - 5 min	94 ºC - 5 min	94 ºC - 5 min
	94 ºC - 1 min	94 ºC - 1 min	94 ºC - 1 min	94 ºC - 1 min	94 ºC - 1 min	94 ºC - 1 min	95 ºC - 1 min	94 ºC - 1 min	94 ºC - 1 min
	67 ºC - 1 min	67,5 ºC - 1 min	67 ºC - 1 min	67,5 ºC - 1 min	67 ºC - 1 min	67,5 ºC - 1 min	61 ºC - 1 min	58 ºC - 1 min	62 ºC - 1 min
	72ºC - 2min 30 seg	72ºC - 1 min	72ºC - 2 min 30 seg	72ºC - 1 min	72ºC - 10 min	72ºC - 1 min	72ºC - 2 min	72ºC - 1 min	72ºC - 1 min
	72 ºC - 5 min	94ºC - 1 min	72 ºC - 5 min	72 ºC - 5 min	94ºC - 1 min	72 ºC - 5 min	72 ºC - 10 min	72 ºC - 10 min	72 ºC - 10 min
	15 ºC - ∞	66ºC - 1 min	15 ºC - ∞	15 ºC - ∞	62ºC - 1 min	15 ºC - ∞	15 ºC - ∞	15 ºC - ∞	15 ºC - ∞
		72ºC - 1 min 30 seg			72ºC - 1 min 30 seg				
		72 ºC - 5 min 15 ºC - ∞			72 ºC - 5 min 15 ºC - ∞				

Legenda: Repete-se 30x; Repete-se 35x; Repete-se 5x.

DISCUSSION

CAH is the most frequent form of SDD cases and the main cause of genital ambiguity. Investigation of mutations derived from the CY21A1P pseudogene by AS-PCR can define the diagnosis in about 90% of the cases.[6]

The mutations investigated herein are related to SL, SV and non-classical CAH.

The p.Pro30Leu mutation (c.89C> T) is observed in 17% of the non-classical CAH alleles.[15]

The mutation c.290-13A / C> G is characterized by the transition from the A / C alleles to G in the splicing donor region of intron 2, the most frequent mutation being described in the literature.[16] This change is responsible for the formation of an anomalous splicing donor region that is associated with a severe CYP21A2 deficiency and is related to the OS phenotype.[16-17]

The frameshift mutation p.Gly110fs (c.329-336delGAGACTAC) is characterized by the deletion of eight nucleotides at position 707-714 in exon 3 of the CYP21A2 gene, generating an enzyme with no functional activity. This

alteration is found in 3 to 10% of alleles of the SL form (HIGASHI et al., 1988; MELLO et al., 2002).[15]

The missense p.Ile172Asn mutation is characterized by the exchange of a thymine by an adenine (c.515T> A) at amino acid 172 of CYP21A2. This mutation is associated with the SV phenotype, allowing a small production of aldosterone, being enough to avoid crises of salt loss. This mutation has a frequency of 5% to 10% in the affected alleles.[15]

The change called Cluster 6 is characterized by a group of three missense mutations that occur simultaneously in codons 236, 237 and 239 of exon 6 of CYP21A2, causing the substitution of three amino acids: Isoleucine, Valine and Methionine for Asparagine, Glutamic Acid and Lysine, respectively. These changes cause the mutated gene to encode a protein without activity, thus, this set of mutations is associated with SL form and has a worldwide frequency of 3-17% among all affected alleles.[6,15]

The p.Val281Leu mutation (c.841G> T) reduces CYP21A2 activity to 20% and is related to the non-classical form of CAH.[18]

The nonsene p.Gln318 * mutation (c. 952C> T) generates a truncated protein with zero activity. This change abolishes the function of CYP21A2 and is associated with SL, with a frequency of 4 to 7% in SL cases.[15-19]

The p.Arg356Trp mutation (c.1066C> T) generates an enzyme with approximately 2% activity, which explains the association with the classical SL form. This mutation occurs in 14% of the alleles. In Brazil, its frequency is 8.2-8.6%, and in the state of Sergipe the frequency of this change is 14.3%.[15,19-20]

The molecular approach is fundamental to complement the clinical diagnosis of CAH to thus provide the most appropriate and individualized treatment. The PCR-AS technique has the limitation of only indicating selected mutations. When clinical and laboratory characteristics indicate CAH, but genetic alteration is not found through AS-PCR, other techniques are used for diagnostic elucidation, such as the sequencing of the CYP21A2 gene.

CONCLUSION

PCR-AS standardization for molecular diagnosis of CAH due to mutations in the CYP21A2 gene, in the Laboratory of Human Molecular Genetics, will provide the diagnostic elucidation of cases screened by the National Neonatal Screening Program and / or attended at the HUPAA / UFAL Clinical Genetic Service. In

addition, it will provide recognition of the most frequent mutations in the CYP21A2 gene in the population of Alagoas, as well as the increase of knowledge about genotype-phenotype relationships for cases of CAH. This knowledge is of fundamental importance to increase the diagnostic approach, treatment, genetic counseling and prevention.

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