Molecular genetic analysis of CYP21A2 gene in patients with congenital adrenal hyperplasia

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ABSTRACT

Context: Congenital adrenal hyperplasia (CAH) is one of the inborn errors of metabolic disorder inherited in an autosomal recessive manner caused by the defects in the steroid 21 hydroxylase CYP21A2 gene. We analyzed the genotype of 62 patients with classic CAH.

Aims: To find out the underlying mutations of CYP21A2 gene.

Settings and Design: Cohort of CAH patients.

Materials and Methods: Sixty-two patients with CAH were recruited from the endocrine clinic at AIIMS. Electrochemiluminiscence method was used for estimating the levels of cortisol. Radioimmunoassay kit-based method was used for estimating the 17 OHP levels. Polymerase chain reaction amplification was done using specific primers to amplify the CYP21A2 gene.

Statistical Analysis Used: Statistical analysis was done by using Epi Info Version 3.5.1.2008.

Results: Out of 62 patients, 50 were simple virilizers (SV) and 12 were salt wasters (SW). Fifty-six were females and six were males. Five 46, XX children were reared as males. Age at presentation varied from 8 months to 38 years. Molecular genetic analysis revealed that the highest number of patients harboured (In 2) IVS2-13 A/C > G (48%), followed by p.P30L (46%), p.Q318X (35%), (Δ 8 bp) deletion 8 bp (26%), p.I172N (26%), and p. R356W (20%) mutations.

Conclusion: This is among the few studies to analyze the mutational spectrum of CYP21A2 gene in a large CAH cohort from India. Molecular diagnosis of CYP21A2 gene should be considered as part of the CAH evaluation to assess the risk of the patients/parents/siblings and to offer genetic counseling.

Key words: Ambiguous genitalia, CYP21A2 gene, phenotype, salt wasting, simple virilizing

INTRODUCTION

Congenital adrenal hyperplasia (CAH) is an autosomal recessive inherited disorder caused by steroid 21-hydroxylase deficiency. The deficiency of this enzyme leads to impaired cortisol production and increased 17-OH-progesterone (17 OHP) biosynthesis. The increased androgen levels during embryonic development cause virilization of the external genitalia in females. Depending on the enzyme activity, patients with CAH are categorized into classic and nonclassic type.

The gene encoding the 21-hydroxylase enzyme (CYP21A2) and a nonfunctional pseudogene (CYP21A1P) are located within the human leukocyte antigen class III gene region on the short arm of chromosome 6 (6p21.3), closely adjacent in tandem arrangement with the C4A and C4B genes encoding for the fourth component of the serum complement.[1-3] These two genes consist of 10 exons and show a high homology with a nucleotide identity of 98% in their exon and 96% in their intron sequences.[4,5] Most of the inactivating mutations are generated by unequal crossing over or gene conversion between the functional (CYP21A2) gene and a nonfunctional (CYP21A1P) pseudogene.[6,7] As a result, complete gene deletions/large gene conversions/8-bp/single point mutations are manifested with severe phenotypic anomalies in patients with CAH.[2,8]

To date, many studies from different ethnic groups around the world have reported the ethnic specific frequency of
CYP21A2 gene mutations and their impact on the clinical features of CAH patients. The classic CAH disease has a worldwide incidence of about 1:10,000 to 1:16,000 live births\(^9\,^10\) and a carrier frequency of 1:60.\(^11\) Only two studies are available on the frequency of CYP21A2 gene mutations from India.\(^12\,\,^13\) Therefore, in this study we analyzed the genotype of patients with classic CAH from the Indian subcontinent.

**Materials and Methods**

**Patients**

This study was approved by AIIMS Ethics Committee. Patients with CAH were recruited from the endocrine clinic at AIIMS. These patients were classified into the salt wasting, and simple virilizing as per their phenotype, clinical history, and hormonal profile. Detailed family history of the patient was taken. Physical examination was done including prader staging for genital appearance, sex of rearing, and hirsutism status.

**Methods**

**Hormonal analysis**

Electrochemiluminiscence method was used for estimating the levels of cortisol, adrenocorticotropic hormone, testosterone, and dehydroepiandrosterone sulfate. Radioimmunoassay kit-based method was used for estimating the 17 OHP levels (Diagnostic Systems Laboratories, Inc., Webster, TX, supplied by Immunotech Marcelle, France).

**Cytogenetic analysis**

Conventional cytogenetic analysis was carried out on peripheral blood using standard techniques. Karyotyping was carried out on G-banded metaphases obtained from 72-h cultures.

**Molecular analysis**

Informed consent to carry out molecular genetic studies was obtained from the patient/parents. DNA was isolated from blood samples by using the Qiagen DNA isolation kit as per the manufacturer’s instructions. DNA was quantified and subjected to polymerase chain reaction (PCR) amplification using specific primers to amplify the CYP21A2 gene.\(^14\) This gene was amplified in two segments using specific primers as shown in [Figure 1]. Using fragment 1 as template, we performed a secondary PCR with the appropriate primers [Figure 1, upper left] to detect p.P30L, In2, and Δ 8 bp mutations. Fragment 2 was digested with Apal restriction enzyme [Table 1] to detect both p.V281L and p.R339H mutations at the same time. Fragment 2 was also used as template to perform a secondary PCR with the appropriate primers [Figure 1, lower right] to detect p.I172N, p.Q318X, p.R356W, and p.P453S mutations.

Secondary PCR products were digested with the appropriate restriction enzymes [Table 1] to identify the common mutations using restriction fragment length polymorphism method\(^15\,\,^21\) followed by 1% agarose gel electrophoresis/10% polyacrylamide gel electrophoresis to separate the restriction fragments.

**Genotype classification**

These patients were divided into three groups as described previously.\(^22\) Group 0/Null consisted of mutations [Figures 2 and 3] with complete loss of enzyme activity (deletions, conversions, Δ 8 bp deletion, p.Q318X, and p.R356W). Group A consisted of In2 mutation with low enzyme activity and group B consisted of p.I172N with relatively low normal enzyme activity.

**Results**

Out of 62 patients, 56 were females and 6 were males. Five 46, XX children were reared as males. Seventeen were siblings. Five patients were from consanguineous marriage. Age at presentation varied from 8 months to 38 years. The mean age was 15.39 ± 10.44 years. The mean 17 OHP and cortisol levels were 17.1 ± 32.93 ng/ml and 8.59 ± 7.63 µg/dl, respectively. Except nine patients, all were on treatment at the time of sample collection for hormonal and molecular analysis. Among these 62 CAH patients, 56 (90%) have ambiguous genitalia. Fifty patients (81.0%) were simple virilizers (SV) and 12 patients (19.0%) were salt wasters (SW). The highest number...
Table 1: Primers and Restriction Enzymes used for the detection of CYP21A2 gene mutations

| Mutation Type | Primers | PCR product, bp | Restriction Enzymes | Fragment sizes, bp |
|---------------|---------|----------------|---------------------|-------------------|
| Pro 30 Leu    | P5 P6   | 249            | HhaI                | 21,228            |
| Intron(2,C-G) | P7 P8   | 378            | HhaI                | 378               |
| Exon 3 (8bp deletion) | P9 P10 | 89 | NA | 89 |
| Ile 172 Asn   | P11 P12 | 416            | TaqI                | 416               |
| Val 281Leu+Arg 339 His | P3 P4 | 2219 | ApaLI | 376,853,990 |
| Gln 318 stop  | P12 P13 | 136            | Pst I               | 25,111            |
| Arg 356 Trp   | P14 P15 | 213            | Fnu4 H1             | 9,33,81,90        |
| Pro 453 Ser   | P16 P17 | 223            | HhaI                | 2,23,51,147       |

Figure 2: Approximate location of the CYP21A2 gene mutations

Table 2: Distribution of common mutations in classic CAH patients

| Mutation type | No | % |
|---------------|----|---|
| Pro 30 Leu    | 21 | 46 |
| Intron 2      | 22 | 48 |
| Exon 3 (Del 8bp) | 12 | 26 |
| Ile 172 Asn   | 12 | 26 |
| Val 281 Leu+Arg 339 His | 0 | 0 |
| Gln 318 Stop  | 16 | 35 |
| Arg 356 Trp   | 09 | 20 |
| Pro 453 Ser   | 0  | 0 |

Figure 3: Residual enzymatic activity according to in vitro CYP21A2 functional studies. The mutation groups refer to the Krone et al. (2000) classification and correspond to the genotypes clustered in the underscored line

Table 3: Allele frequency of the common mutations in CYP21A2 gene in Different Populations

| Mutations type | No. of alleles | 1n2 | Intron 2 | Exon 3 | Ile 172 | Arg 356 | Pro 30 Leu | Gln 318 Stop | Reference |
|----------------|----------------|-----|----------|--------|---------|---------|------------|-------------|-----------|
| Pakistan       | 58             | 27  | 26       | 19     | 9       | 16      | 0          | 0           | [25]      |
| Iran           | 100            | 28  | 9        | 0      | 0       | 0       | 0          | 0           | [25]      |
| Turkey         | 31             | 22  | 11.4     | 0      | 0       | 0       | 0          | 0           | [25]      |
| India          | 46             | 27.2| 31.8     | 0      | 2.2     | 22.7    | 0          | 0           | [12]      |
| Present study  | 124            | 48  | 26       | 20     | 46      | 35      | 0          | 0           | Present study |

DISCUSSION

Most of our patients sought medical consultation during puberty due to ambiguous genitalia and primary amenorrhea. SV type is more prevalent than SW type. This may be due to the salt wasting crisis leading to infant mortality before the diagnosis. Moreover, females were higher than males as evident from the literature that most of the male children miss the correct diagnosis during infancy until and unless they are tested for precocious puberty.

We observed an interesting pattern of mutational spectrum in CYP21A2 gene in our patients. Out of 124 alleles, we identified mutations in 92 alleles (74.2%) in our study.
population which is almost similar to the previous study.\textsuperscript{[12]} Among the nine common mutations analyzed, In2 was the most prevalent mutation and the similar findings were reported in East Indians, Hong Kong Chinese, Singapore Chinese, Indians and Malays, Japanese, Iranian, French, Pakistani Taiwanese, and in American populations.\textsuperscript{[12,23,30]}

This single-nucleotide point mutation In2 associated with the classic 21-OHD by the aberrant splicing of In2 leading to a null activity of CYP21A2. Other disease causing severe mutations like, p.Q318X, Δ 8 bp, and p.R356W were also identified in an increasing frequency, and the similar pattern was reported in East India, France, Mexico, and Spain.\textsuperscript{[12,13,31,32]} In SV type, p.I172N mutation was found to be high and this trend was reported in many studies.\textsuperscript{[22,23,33,34]} I172N is the only one mutation specifically associated with the SV form of the disease, and mutation of this hydrophobic residue to a polar residue results in an enzyme with approximately 1% or normal activity.\textsuperscript{[21,33]} The frequency of p.P30L mutation was relatively high in our study, when compared with other studies.\textsuperscript{[12,13,36,37]} This mutation was first described in a nonclassic CAH patient and considered as a part of larger gene conversion or a chimeric CYP21A1P/CYP21A2 gene. Subsequently, this mutation has been described in SV, NC, and also in SW CAH patients.\textsuperscript{[17,38]}

This study is among the few studies to analyze the mutational spectrum of CYP21A2 gene in a large CAH cohort from India. Direct sequencing is required to characterize the complete gene. Molecular diagnosis of CYP21A2 gene should be considered as part of the CAH evaluation in order to assess the risk of the patients/parents/siblings and to offer genetic counseling.

**Acknowledgement**

This study is being supported by the Department of Biotechnology (Ref.N0.BT/PR7205/Med/12/277/06), Government of India.

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Cite this article as: Marumudi E, Sharma A, Kulshreshtha B, Khadgawat R, Khurana ML, Ammini AC. Molecular genetic analysis of CYP21A2 gene in patients with congenital adrenal hyperplasia. Indian J Endocrinol Metab 2012;16:384-8.

Source of Support: Department of Biotechnology (Ref.NO.BT/PR7205/Med/12/277/06), Government of India. Conflict of Interest: None declared.