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Clinical, cytogenetic and molecular analysis of androgen insensitivity syndromes from south Indian cohort and detection and *in-silico* characterization of *androgen receptor* gene mutations

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ABSTRACT

Rare cases of 9 complete androgen insensitivity syndromes, 9 cases of partial androgen insensitivity syndromes and equal number of male control samples were selected for this study. Few strong variations in clinical features were noticed; Giemsa banded metaphase revealed a 46,XY karyotype and the frequency of chromosome aberrations were significantly higher when compared with control samples. DNA sequence analysis of the androgen receptor gene of androgen insensitivity syndromes revealed three missense mutations — c.C1713>G resulting in the replacement of a highly conserved histidine residue with glutamine p.(His571Glu) in DNA-binding domain, c.A1715>G resulting in the replacement of a highly conserved tyrosine residue with cysteine p.(Tyr572Cys) in DNA-binding domain and c.G2599>A resulting in the replacement of a highly conserved valine residue with methionine p.(Val867Met) in ligand-binding domain of androgen receptor gene respectively. The heterozygous type of mutations c.C1713>G and c.G2599>A observed in mothers of the patients for familial cases concluding that the mutation was inherited from the mother. The novel mutation c.C1713>G is reported first time in androgen insensitivity syndrome. *In-silico* analysis of mutations observed in androgen receptor gene of androgen insensitivity syndrome predicted that the substitution at Y572C and V867M could probably disrupt the protein structure and function.

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1. Introduction

Androgen insensitivity syndrome (AIS; testicular feminization; OMIM# 300068) is an X-linked recessive disorder characterized by variable defects in the virilization of *46,XY* individuals. This is due to the loss of function of gene because of *androgen receptor* gene mutations (*AR*; OMIM# 313700), which results in peripheral androgen resistance.

The clinical phenotypes of AIS (androgen insensitivity syndrome) are variable and are classified into three main categories: Complete androgen insensitivity syndrome (CAIS), partial androgen insensitivity syndrome (PAIS) and mild androgen insensitivity syndrome (MAIS), the designations reflecting the severity of androgen resistance.

Reports on the comparative analysis of the phenotypic relationship among the complete and partial AIS with a correlation of the varying degree of androgen defects are inadequate and inconclusive. However few attempts were made in that direction but ended in more complicated and conflicting results [1]. It was observed that the same mutation of the *AR* gene in different affected AIS can cause variable clinical phenotypes [2]. Though the mutations in the *AR* gene are a frequent cause of AIS, the associations of certain other factors are yet to be proved. *G2445A* substitution is known to cause both PAIS [3] and CAIS [4]. *C2296A* substitution, with proven pathogenicity in PAIS [5] and CAIS [6] patients, was also observed in an absolutely normal individual [7]. Several of those reports deal only with the clinical and hormonal features and did not include an analysis of underlying mutations in the subjects. Hence it requires further attempt to understand important biological phenomena to correlate the androgen activity with the clinical features of all the three types of androgen insensitivity syndromes. In this study moderately fair sample size was selected and a thorough analysis of all the clinical features in complete and partial AIS were recorded and a complete analysis of *AR* gene sequencing in all those syndromes were made along with pedigree analysis in all the cases.

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Sex assignment at birth of patients with incomplete AIS is based upon the virilization of the external genitalia at birth. Major clinical problems of PAIS are the acceptability and advisability of male *vs.* female sex assignment. The genetic makeup and phenotype relationship in AIS became relevant when the genetic diagnosis became available. A more precise prognosis was expected from the knowledge of a specific AR mutation and its residual androgen action, which might facilitate the clinician for sex assignment of a 46,XY subject with AIS and help genetic counseling of carrier females.

It is known that the structure of human populations is relevant in various epidemiological contexts. As a result of variation in frequencies of both genetic and non genetic risk factors, rates of disease and of such phenotypes as adverse drug response vary across populations [8]. It was thought that it is reasonable to check the type and nature of AIS mutations associated with AIS that may provide different types of useful information in a new rare geographical population such as one of South East seashore Indian population, Chennai and its surrounding area. We studied the genetic makeup and phenotype relationship in families with multiple affected subjects with clinical as well as molecular level of complete and partial AIS and the occurrence and possible causes of phenotypic variation.

2. Materials and methods

2.1. Participants and blood sampling

9 cases of complete androgen insensitivity syndrome (CAIS), 9 cases of partial androgen insensitivity syndrome (PAIS) and 6 cases of their relatives—3 females and 3 cases of males and equal number of age matched controls were selected with the help of qualified Gynecologist from the Institute of Obstetrics and Gynaecology (IOG), Madras Medical College (MMC), Government Hospital for Women and Children, Egmore, Chennai, Tamil Nadu, India.

3 ml of blood sample in an EDTA vaccuntainer for DNA analysis and 2 ml of blood sample in a heparinized vaccuntainer for cytogenetic analysis were collected from all the patients, relatives and control subjects for this study.

Relatives at risk of being affected with AIS or at risk of being carriers of AIS were offered diagnosis and counseling. A written informed consent was obtained from either the patients or their parents. The study was approved by the University Human Ethical Committee of the VIT University.

2.2. Clinical analysis

A total number of 35 cases of suspected AIS cases were referred to us for karyotyping analysis through the Institute of Obstetrics and Gynaecology, Egmore, Chennai, India. Out of 35 cases 18 cases were confirmed with 46,XY confirming their AIS nature. Informed consent was obtained from all the patients that were subjected to physical and clinical evaluations and family histories of all the patients were recorded (Tables 1 & 2). Upon complete clinical examination, phenotypes were diagnosed. With the help of the physicians all the clinical features that are needed to classify the androgen insensitivity syndrome (AIS) were recorded. On the basis of the clinical features they were assigned to CAIS and PAIS. In all the cases pedigree analysis was carried out and mutation carriers were recorded.

2.3. Cytogenetic analysis

The cytogenetic studies were carried out in all the cases using heparinized blood samples to find out the karyotype and chromosomal aberrations. Chromosome preparations were obtained from PHA-stimulated peripheral blood lymphocytes by using the modified method of Hungerford [9]. At least fifty well spread metaphase plates were scored by direct microscopic analysis. Well spread metaphases were photographed under oil immersion objective lens (100×) of Leica DM2000 microscope with Metasystems camera and the photomicrographs of banded spreads were karyotyped using automatic IKaros software (Metasystems). The karyotype was described according to the International System for Human Cytogenetic Nomenclature [10].

2.4. Molecular analysis

The molecular study was carried out in all the cases to make a thorough analysis of the mutations in AR gene to correlate with the clinical features.

2.4.1. DNA extraction

DNA was isolated from the blood samples using the following procedure: 2–3 ml from each sample was placed in a 15-ml sterile polypropylene centrifugation tube with 6 ml of RBC lysis buffer (0.01 M Tris–HCL, 320 mM sucrose, 5 mM MgCl2, 1% Triton X-100 and adjust pH to 8.0), mixed it in a roller mixer at room temperature and centrifuged at 10,000 rpm for 10 min at 4 °C. Discard supernatant without disturbing the cell pellet, remove remaining moisture by inverting the tube and blotting onto tissue paper. Re-suspend the pellet in 1 ml of WBC lysis buffer (0.4 M Tris–HCl, 0.06 M EDTA, 150 mM NaCl, 1% SDS and adjust pH to 8.0) and 250 μl of 5 M sodium per chlorate monohydrate and mix it by inverting the tubes several times. Incubate the content at 65 °C for 20 min in water bath and allow it to cool to room temperature. Add 2 ml of ice-cold chloroform and mix it for 20 min using a roller mixer and centrifuge at 10,000 rpm for 10 min at 4 °C. Transfer the aqueous phase into a new sterile polypropylene centrifugation tube and add twice the volume of ice-cold ethanol. Invert the tube several times gently, until the DNA precipitate is visible. Spool the DNA using a wide bore pipette tip and transfer it to a new sterile Eppendorf tube. DNA was washed twice with ethanol and kept the DNA to dry for 20 min at 37 °C. Finally dissolved the DNA in an appropriate volume of TE buffer (1 M Tris–HCl, pH 8.0; 0.5 M EDTA) typically about 100–200 μl according to the volume of pellet, and place the DNA sample in a 55 °C water bath for 10–15 min to dissolve DNA in TE buffer.

2.4.2. Quantification and PCR analysis

Qualitative analysis of DNA was carried out by 0.8% agarose gel electrophoresis and quantification of DNA by using a Biophotometer (Eppendorf). Dilutions of DNA were made up to 10 ng/μl concentration by using TE buffer, pH-8.0. The 10 ng/μl of concentrated DNA solution was checked on 0.8% agarose gel electrophoresis.

2.4.3. Molecular studies in AR gene

The DNA sequence of AR gene (accession number: ENSG00000169083) was downloaded from Ensembl database (www.ensembl.org) and 12 pairs of primers covering the entire coding region and primer sequence were designed using Gene Tool software. Polymerase chain reaction analysis of AR gene was carried out in an Eppendorf thermocycler. PCR consisted of 10 μl PCR reaction mixture and included 1.0 μl PCR buffer (10×), 1.0 μl MgCl2 (25 mM), 0.8 μl deoxynucleotide tri-phosphates (10 mM), 0.5 pM of each primer, 1 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, California) and 20 ng of genomic DNA. Polymerase chain reaction conditions for AR gene consisted of initial denaturation at 94 °C for 12 min followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. PCR products were checked on 2% agarose gel, stained with ethidium bromide and visualized under a UV transilluminator. The amplicons were further subjected to automated DNA sequencing [11], using Big Dye Terminator cycle sequencing kit (v 3.1, Applied Biosystems, USA) and 3730 DNA analyzer (Applied Biosystems, USA).

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